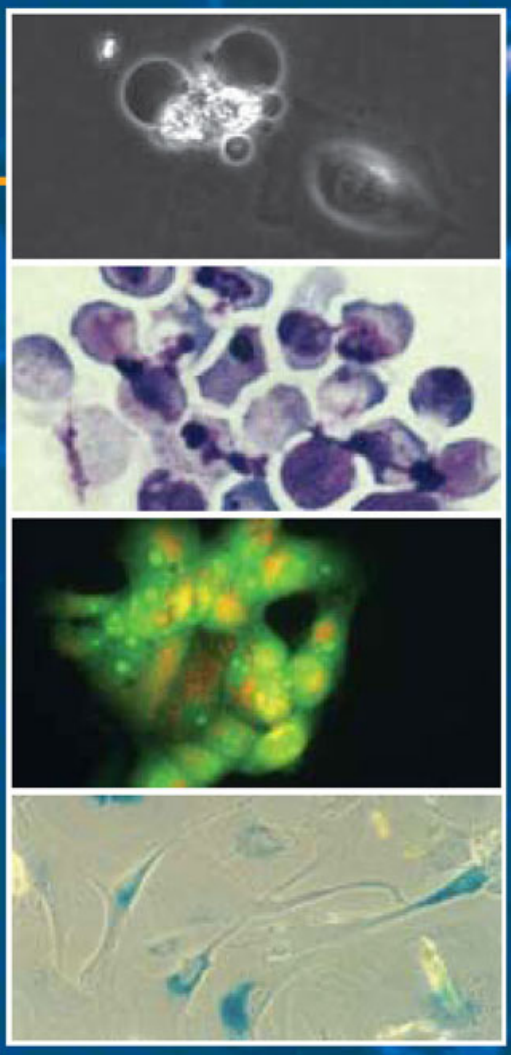
CANCER DRUG DISCOVERY AND DEVELOPMENT

Apoptosis, Senescence, and Cancer

Second Edition



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Apoptosis, Senescence, and Cancer

Cancer Drug Discovery and Development

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Cover Illustration: The images on the cover represent four different modes of “cell death” that are discussed extensively in the text. These include (top to bottom): mitotic catastrophe, apoptosis, autophagy and senescence.

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Preface

The goals of chemotherapy (and radiotherapy) are to eliminate tumor cell targets by promoting cell death. In recent years, a major focus has been placed on programmed cell death or apoptosis as the primary mechanism of cell killing. However, tumor cells may respond to various forms of treatment in diverse ways, only some of which culminate in cell death and loss of clonogenic survival. In addition to apoptosis, cell death may occur through mitotic catastrophe, autophagy (a subtype of apoptosis), or anoikis. Alternatively, cells may undergo either transient or prolonged growth arrest; in addition, senescence arrest or accelerated senescence is now recognized as a response to various treatments, which may also play a role in preventing cell transformation. Consequently, "permanent" growth arrest, possibly mediated through senescence, could contribute to loss of self-renewal capacity, particularly in solid tumors. The question of why some tumor cells within a population ultimately recover proliferative capacity (whether in cell culture, in xenograft models as a component of tumor growth delay, or in patients in relation to disease recurrence) remains an unresolved question in the fields of experimental chemotherapy and radiotherapy and a critical problem in the clinical treatment of malignancies. The possibility that surviving and recovering cells represent a resistant stem cell population has recently gained credence, although evidence in support of this hypothesis is far from conclusive.

The purpose of this book is to contribute to an understanding of the growth arrest and cell death pathways mediating the response to chemotherapy in tumor cells. The book is divided into six sections. The first reviews the major cell death pathways. The second develops the themes of telomeres, telomerase, and senescence in genetic stability and tumorigenesis. The third provides an in-depth dissection of the critical DNA damage and response signaling pathways. The fourth deals with the fundamental limitations on therapy conferred by drug resistance, as well as current approaches to circumvent or attenuate drug resistance. The fifth and sixth sections provide an analysis of our understanding of the responses to both conventional strategies and newly developed therapies against cancer.

It is our hope that this book will provide basic scientists and clinicians with a deeper and more thorough understanding of the cellular responses of malignant cells to common therapeutic modalities, which may determine the effectiveness of treatment, both in the initial phase of the disease and the latter stages, including recurrence and metastatic disease.

David A. Gewirtz

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Introduction and Overview

The title of this book might suggest that one mode of cell death (*apoptosis*) and one mode of growth arrest (*senescence*) represent the critical elements of tumor cell responses to various forms of cancer therapy. However, a quick glance through the section and chapter headings will readily convey the range of possible responses to both conventional therapies, such as standard cytotoxic drugs and radiation, to more recent therapies, such as monoclonal antibodies and targeting of specific receptor and signaling pathways, to developing modalities, such as photodynamic therapy and approaches targeting the vascular system. With regard to senescence, in addition to the relatively recent realization that senescence is likely to mediate the growth arrest response to many therapeutic treatments and could potentially act as a rational drug response, a considerable section of this book has been devoted to the role of senescence in genomic instability and tumor development. In addition, the reader will find that the theme of genomic instability runs through other sections, such as the consideration of mitotic catastrophe, as well as regulatory functions of ataxia telangiectasia mutated (ATM)/ATM-related (ATR) and DNA-dependent protein kinase. Likewise, the relationship between c-myc and cyclin-dependent kinase inhibitors is considered in terms of tumor progression, as well as their relevance for cancer therapy and the promotion of apoptosis.

CELL DEATH PATHWAYS

For almost two decades, the cell death response to chemotherapy and radiation has focused almost exclusively on *apoptosis*. In view of the importance of apoptotic cell death, two introductory chapters in this section provide a detailed and learned overview of both the intrinsic and the extrinsic pathways of apoptosis. However, there is extensive evidence that apoptosis is not the only mode of cell death or possibly the primary mode of cell death in solid tumors. It is therefore of particular importance to review the approaches utilized to conclude that a particular mode of cell death is relevant to drug or radiation treatment.

It has been well established for many years that at least one other mode of cell death, that of *mitotic catastrophe*, is quite common, particularly in tumors that have been exposed to ionizing radiation. However, the basis for the cell "choosing" or preferring a particular mode of cell death is not understood, even at the most fundamental level. Relatively recently, at least one additional mode of cell death has been recognized, known as *autophagy*. Autophagy is a complex response because, unlike apoptosis or mitotic catastrophe, autophagy can function as a cytoprotective mechanism when initiated under certain modes of cell stress, such as nutrient deprivation. Finally, an area that is appropriately receiving renewed attention is a subspecies of apoptosis that is termed "anoikis," or cell death subsequent to loss of adhesion to substratum. The ability of tumor cells to resist anoikis may provide the necessary survival advantage that permits a tumor cell to metastasize.

SENESCENCE GROWTH ARREST

Within the same time frame that autophagy has been recognized as a mode of cell death, *stress-induced senescence* (also known as premature or accelerated senescence) has been recognized as a unique mode of growth arrest. The uniqueness and signaling elements of this pathway has resisted facile dissection as the genetic elements that appear to be involved overlap quite closely with those involved in the G1 arrest pathway, specifically induction of p53 (although it is clear that p53-independent senescence also exists), $p21^{waf1/cip1}$, dephosphorylation of pRb- or Rb-related proteins such as p130 and p107, and suppression of E2F-mediated transcription. Analysis of the molecular elements involved in stress-induced senescence has also been hampered by reliance on a limited number of senescence markers, specifically senescence-associated β-galactosidase staining, cell morphology (flattening, enlargement, and granulation), and *telomere dysfunction*, which is in dramatic contrast to the multiple signaling and regulatory elements events that have been identified for death-related pathways. Of particular interest, the specific therapeutic targeting of telomeres to induce senescence may provide an under-explored, if not novel, targeted approach for cancer therapy.

DRUGS, MECHANISMS, AND RESISTANCE

Although a great deal of information has been published on the mechanism of action of different classes of antitumor drugs, the chapters in this book attempt to focus, in part, on the importance of different modes of cell death. We have attempted to cover major classical chemotherapeutic agents including the *antimetabolites, platinum*-based compounds and *alkylating* agents, *topoisomerase I* and *topoisomerase II* inhibitors, and *microtubule* poisons. In addition to these classical and conventional therapies, relatively new approaches such as *antibody* therapy, *tyrosine kinase*, and *epithelial growth factor receptor* (EGFR) inhibitors are described in detail in this book. Many "cutting edge" therapies are at various stages of development, including *antiangiogenic* agents and *photodynamic* therapy. Finally, the possibility of *TRAIL* as a therapeutic target has been considered, in particular because of its potential to be a highly selective target that is limited to tumor cells.

It is likely that any reader of this book will already be familiar with the difficulties encountered in cancer therapy, many of which are associated with various forms of resistance, including intrinsic mechanisms or those that develop in response to the treatment challenge. To address this issue, specific signaling pathways are considered both in the context of conferring resistance and for converting these pathways into potential targets for drug development and sensitization to existing therapies.

DNA DAMAGE RESPONSE

Although not all cancer therapies involve DNA damage, many of the traditional and conventional treatments do promote cellular stress, either directly through damage to DNA or indirectly through interference with the function of alternative targets. It was therefore considered to be of particular relevance to address specific components of the DNA damage response and signaling pathways, focusing on *ATM/ATR, H2AX/53BP1*, and *DNA PK*.

UNANSWERED QUESTIONS

What we still do not understand, despite our best efforts, is precisely how tumor cells "decide" on the nature of their response to these treatment modalities, especially related to *recovery* and/or *resistance*. It is generally thought that less severe "lesions" result in a transient growth arrest, and once such lesions are repaired (or the cell determines that the lesion is not "life-threatening"), growth will resume. Although without solid experimental evidence, it is possible to speculate that disease recurrence at the site of the primary tumor could be related to recovery after transient growth arrest; that is, one can consider this transient growth arrest to be analogous to tumor cell dormancy. A closely related question, one which we have not attempted to address in this book, is the nature of the signaling response that is required for proliferative recovery in "dormant" tumors. Finally, the basis for the therapeutic selectivity of many if not most of current conventional or more novel treatment modalities is still far from being fully elucidated.

The editors express their deep appreciation to all the contributors to this text, scientists, researchers, and clinicians who somehow managed to take the time and effort to provide the benefits of their expertise in specific fields of research to contribute chapters to this book. We also credit the editors at Humana Press for their endless patience with the process of developing this book.

We would like to dedicate this book to all those who have suffered and continue to suffer from the ravages of cancer in the hope that the information gathered in this text might provide some small element of guidance in the efforts of our scientific colleagues to defeat this disease.

David A. Gewirtz
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I Apoptosis and Alternative Modes of Cell Death

1 The Intrinsic Pathway of Apoptosis

Scott H. Kaufmann, MD, PhD

Summary

Virtually all anticancer drugs induce apoptosis in susceptible cell types. This biochemically distinct form of cell death reflects, in large part, activation of caspases, a distinct family of intracellular cysteine proteases. At least two separable processes, one starting with ligation of specific cell surface receptors (so-called death receptors) and the other involving release of cytochrome c from mitochondria, result in transduction of various signals into caspase activity. The mitochondrial or intrinsic pathway appears to play a predominant role in the apoptotic response to anticancer drugs. Bcl-2 family members regulate this pathway by modulating the release of key proapoptotic polypeptides, including cytochrome c and second mitochondrial activator of caspases (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO), from mitochondria. Several Bcl-2 family members that facilitate mitochondrial permeabilization are transcriptional targets of the p53 tumor suppressor gene, providing a partial explanation for the ability of DNA-damaging agents to induce apoptosis. Other proapoptotic Bcl-2 family members are released from cytoskeletal sites upon treatment with paclitaxel or loss of adherence. The antiapoptotic protein XIAP (X chromosome-linked IAP) binds procaspase 9 and prevents its activation. The gene encoding XIAP is activated by nuclear factor-κB, contributing to the antiapoptotic effects of this transcription factor. In this chapter, the various components of the intrinsic pathway are reviewed, alterations in this pathway in various cancers are described, and evidence that some of these same antiapoptotic alterations might contribute to anticancer drug resistance under certain circumstances is discussed.

Key Words: Apoptosis; caspases; carcinogenesis; Bcl-2; IAP; drug resistance.

1. ANTICANCER DRUGS CAN INDUCE APOPTOSIS

Studies performed over the past 15 years have demonstrated that virtually all the agents currently utilized to treat cancer can induce apoptosis *(1)*, a morphologically and biochemically distinct cell death process *(2)*, in susceptible cells. Morphologically, this process is characterized by plasma membrane blebbing, cell shrinkage, and chromatin condensation followed by disassembly of the cell into multiple membrane-enclosed fragments, which are then engulfed by neighboring cells or professional phagocytes *(2)*.

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These morphological changes are thought to reflect, at least in part, the action of caspases, unique intracellular proteases that digest polypeptides required for cellular integrity and survival *(3–5)*.

Additional analyses have suggested that apoptosis also occurs after administration of antileukemic therapy in the clinical setting *(6–10)* and after treatment of solid tumors in certain animal models *(2,11,12)*. These observations, which suggest that proximal effects of divers anticancer drugs can lead to activation of a common final death pathway, have prompted many investigators to speculate that alterations in the apoptotic machinery or its regulation might lead to a form of broad-spectrum drug resistance *(13–17)*. It is important to emphasize, however, that cells can also suffer other fates, including senescence (see Chaps. 11 and 19) and so-called mitotic catastrophe (see Chap. 4), after exposure to chemotherapeutic agents *(18–20)*. These alternative outcomes need to be considered in determining the overall response of tumor cells to antineoplastic agents *(20)*.

This chapter describes apoptosis as an endpoint of anticancer drug exposure. In the sections that follow, current understanding of the apoptotic machinery, particularly the intrinsic pathway of apoptosis, is summarized. In addition, cancer-associated alterations of this pathway are described, and evidence suggesting that these alterations might contribute to drug resistance is discussed.

2. CASPASE ACTIVATION: SHARPENING THE SCALPEL

Although early studies suggested that the vast majority of cellular polypeptides remain intact as cells undergo apoptosis, more than 500 polypeptides are now known to be selectively degraded during this death process *(4,5)*. Many proteases, including calpains, cathepsins, and the proteasome, have been implicated in these proteolytic events *(21,22)*, but the vast majority of cleavages are thought to be mediated by caspases *(4,5,23)*, a family of intracellular cysteine proteases that cleave next to aspartate residues.

Of the 12 known human caspases, seven have established (caspases 3, 6, 8, 9, and 10) or postulated (caspases 2 and 7) roles in apoptosis. Each of these is synthesized as an inactive zymogen that contains a prodomain as well as sequences that give rise to a large subunit and a small subunit.

Recent studies have suggested that the process of caspase activation differs for initiator caspases, which transduce various signals into protease activity, and effector caspases, which are responsible for the bulk of proteolytic cleavages within apoptotic cells. In particular, the initiator caspase of the intrinsic pathway, procaspase 9, appears to be a monomer *(24)* with misaligned catalytic cysteine and histidine residues and a catalytic pocket that cannot bind substrates *(25)*. Procaspase 9 dimerization *in vitro* results in conformational changes that open the substrate-binding pocket and align the catalytic residues at one of the two active sites in the dimer *(25)*. Notably, these changes can occur without any proteolytic cleavage of procaspase 9 *(25–27)*. Other initiator caspases such as procaspases 8 and 10 also appear to exist as monomers that can be activated by dimerization *in vitro (24,28)*.

In contrast to initiator caspases, the zymogens of effector caspases are dimers under physiological conditions *(24,29)*. Activation of these caspases involves proteolytic cleavage at several aspartate residues, including one between the large and

small subunits and another between the prodomain and the large subunit *(4,23,30)*. X-ray crystallography of procaspase 7 and the mature enzyme has revealed that these proteolytic cleavages are accompanied by release of the two prodomains and by conformational changes that produce an enzyme with two catalytically competent active sites, each containing residues from one large and one small subunit *(29,30)*. Caspases 3 and 6 are likely activated in a similar fashion.

3. CLEAVAGE BY EFFECTOR PROTEASES: SURGICAL EXCISION OF CRITICAL CELLULAR COMPONENTS

Once activated, caspase 3 selectively cleaves several hundred substrates *(4,5)*, and caspase 6, which is activated by caspase 3 *(31)*, cleaves a smaller group of polypeptides *(4,32)*. These cleavages contribute to many of the changes observed during apoptosis. For example, caspase 3 cleaves the actin filament severing enzyme gelsolin to a constitutively active fragment that has been implicated in cytoplasmic blebbing, one of the morphological hallmarks of apoptosis *(33,34)*. Caspase 3 also cleaves intermediate filament proteins, including lamin B, the nuclear/mitotic apparatus protein NuMA, and cytokeratins *(4,5)*, whereas caspase 6 appears to be uniquely capable of cleaving lamin A in nuclei that contain this particular intermediate filament protein *(32,35)*. To the extent that these polypeptides are normally responsible for the maintenance of cell shape, the destabilizing caspase-mediated cleavages of these intermediate filaments likewise contribute to apoptotic morphological changes.

Caspase-induced cleavages also participate in the biochemical changes observed in apoptotic cells. For example, caspase 3 cleaves the nuclease inhibitor ICAD (inhibitor of caspase-activated deoxyribonuclease), allowing subsequent internucleosomal cleavage of DNA *(36)* by the constitutively expressed nuclear enzyme CAD *(37–39)*. In addition, caspase 3 cleaves many protein kinases, removing their inhibitory domains and increasing their activity *(4)*. Although the manner in which this unregulated kinase activity contributes to the apoptotic phenotype remains largely unknown, the constitutively active fragment of protein kinase Cδ *(40)* activates a phospholipid scramblase that catalyzes transfer of phosphatidylserine from the inner leaflet of the plasma membrane, where it is normally located, to the outer leaflet *(41)*. This loss of phospholipid asymmetry, another hallmark of apoptotic cells *(42)*, provides a signal recognized by the adaptor molecule MFG-E8 *(43,44)* and/or a phosphatidylserine receptor present on the surfaces of macrophages *(45–47)*, thereby facilitating phagocytosis of the dying cell. Caspase-mediated degradation of additional polypeptides inhibits many processes that are required for continued cell survival, including DNA repair pathways and components of the protein translation machinery *(4,5)*. Collectively, these caspase-mediated cleavages interfere with cellular homeostasis and appear to reinforce the "decision to die."

4. CYTOCHROME C: A CRITICAL MOLECULE FOR LIFE AND DEATH

Within the cell, two distinct but interacting pathways can lead to the activation of caspases 3 and 6. One involves transduction of receptor-mediated events into proteolytic activity of caspase 8 and/or caspase 10. This pathway is reviewed in Chapter 2. The other involves transduction of various signals into a change in permeability of the

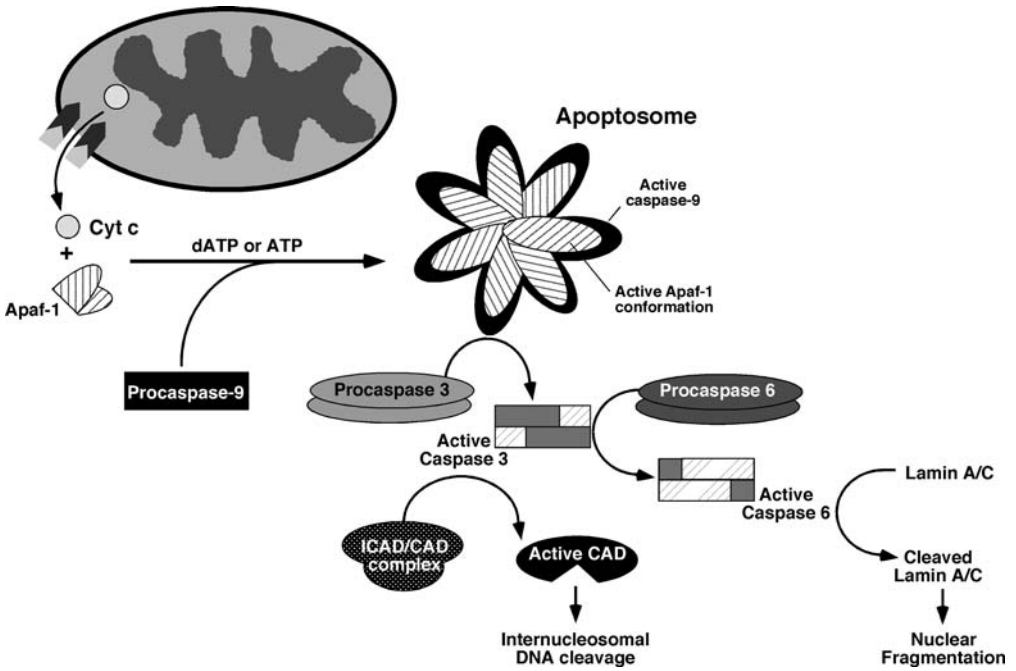

Fig. 1. The mitochondrial pathway. Upon release from the mitochondrial intermembrane space, cytochrome c binds to and induces an ATP dATP-dependent *(48)* conformational change in Apaf-1, thereby facilitating apoptosome formation. Activated caspase 9 then proteolytically activates caspase 3, which cleaves a myriad of substrates, including the nuclease inhibitor ICAD (inhibitor of caspase-activated deoxyribonuclease) and other procaspases, to produce apoptotic biochemical and morphological changes as described in Section 3.

outer mitochondrial membrane, which then leads to activation of caspase 9. This signal transduction cascade, which is termed the intrinsic (or mitochondrial) pathway (Fig. 1), is the subject of the remainder of this chapter.

A critical step in this pathway is the selective release of polypeptides from the mitochondrial intermembrane space into the cytoplasm *(49,50)*. The most widely studied of these released polypeptides is cytochrome c, a component of the electron transport chain that is loosely bound to the outer leaflet of the inner mitochondrial membrane. Release of the cytochrome c from mitochondria within any particular cell during apoptosis appears to be rapid and quantitative *(51)*. Upon its appearance in the cytoplasm, cytochrome c binds the scaffolding molecule Apaf-1 (apoptotic protease-activating factor-1) *(52)*, which then undergoes a nucleoside triphosphate-dependent conformational change and binds procaspase 9 *(53)*. The resulting $M_r \sim 700, 00$ complex termed the "apoptosome" contains multiple Apaf-1 and procaspase 9 molecules *(54–57)*. In a manner that is not completely understood at the molecular level *(58)*, binding to this complex activates procaspase 9, which then proteolytically activates caspase 3 *(31,53)* to initiate cellular disassembly as described in Section 3.

The role of cytochrome c in caspase activation has been extensively studied. To activate caspase 9 *in vitro*, cytochrome c must contain the heme prosthetic group *(59,60)*, although either oxidized or reduced cytochrome c will suffice *(61)*. Once

the apoptosome forms, cytochrome c appears to become dispensable. The absence of detectable cytochrome c in the active apoptosome *(26,56,57)* and the ability of Apaf-1 truncation mutants to activate procaspase 9 in the absence of cytochrome c *(31)* suggest that cytochrome c is only required to induce the Apaf-1 conformational change that leads to apoptosome formation.

The biochemical basis for the efflux of cytochrome c from mitochondria has been the subject of considerable speculation (reviewed in *49,62–65*). Some models have suggested that cytochrome c release reflects opening of a permeability transition pore composed of resident mitochondrial membrane proteins such as the voltage-dependent anion channel (VDAC) and adenine nucleoside translocator (ANT) acting in concert with the mitochondrial matrix protein cyclophilin D. Results of murine gene-targeting studies, however, have failed to confirm an essential role for VDAC *(66)*, ANT *(67,68)*, or cyclophilin D *(69–71)* in apoptosis triggered during development or by exogenous stimuli. Accordingly, more recent studies have focused on the role of Bcl-2 family members in the release of mitochondrial proteins to the cytoplasm.

5. BCL-2 FAMILY MEMBERS AND THE INTEGRATION OF CELLULAR STRESS

The biology and biochemistry of Bcl-2 family proteins have been extensively reviewed elsewhere *(72–74)* and are only briefly described here. *Bcl-2*, the founding gene in this family, was identified because it is juxtaposed to the immunoglobulin heavy chain promoter in indolent B-cell lymphomas containing the t(14;18) chromosomal translocation. Subsequent analysis demonstrated that Bcl-2 overexpression inhibits cell death *(75,76)*. Since these early observations, approximately 20 related mammalian polypeptides have been identified. On the basis of functional and structural criteria, these polypeptides can be divided into three groups (Fig. 2), the antiapoptotic group I family members and the proapoptotic group II and group III family members. Group I family members, which include Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1/Bfl1, Boo/Diva,

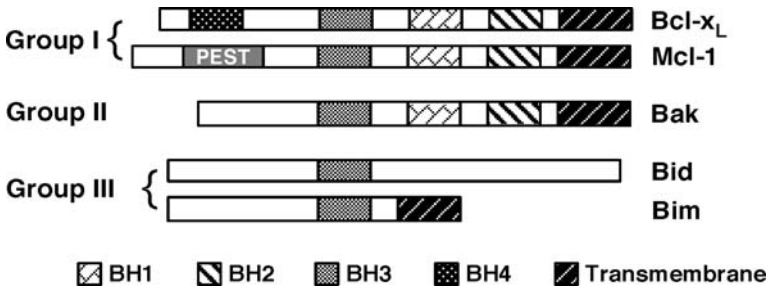

Fig. 2. Schematic representation of selected Bcl-2 family members. Group I polypeptides are antiapoptotic and include Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, Boo/Diva, Nrf3, and Bcl-B. Among these, Mcl-1 is unique in lacking a BH4 (Bcl-2 homology 4) domain and containing a proline/glutamate/serine/threonine-rich (PEST) sequence, which is often seen in short-lived polypeptides. Group II family members are proapoptotic and include Bax, Bak, and Bok/Mtd. Among this group, Bax and Bak are generally expressed in somatic cells. Group III family members, which share limited sequence homology only in their 15-amino acid BH3 domains, include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G.

Nrf3, and Bcl-B *(72–74)*, generally contain four short, conserved BH (Bcl-2 homology) domains, BH1–BH4, and most contain a C-terminal transmembrane domain that targets them to the cytoplasmic surfaces of various intracellular membranes, including the outer mitochondrial membrane and the endoplasmic reticulum. Group II family members, which include Bax, Bak, and Bok/Mtd *(72–74)*, lack the N-terminal BH4 domain but contain the other BH domains. Finally, group III family members, which include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G, are a more heterogeneous collection of polypeptides that share limited sequence homology only in their 15-amino acid BH3 domains *(73,77)*.

Current understanding, which is summarized in Fig. 3, suggests that group II Bcl-2 family members, especially Bax and Bak, directly mediate cytochrome c

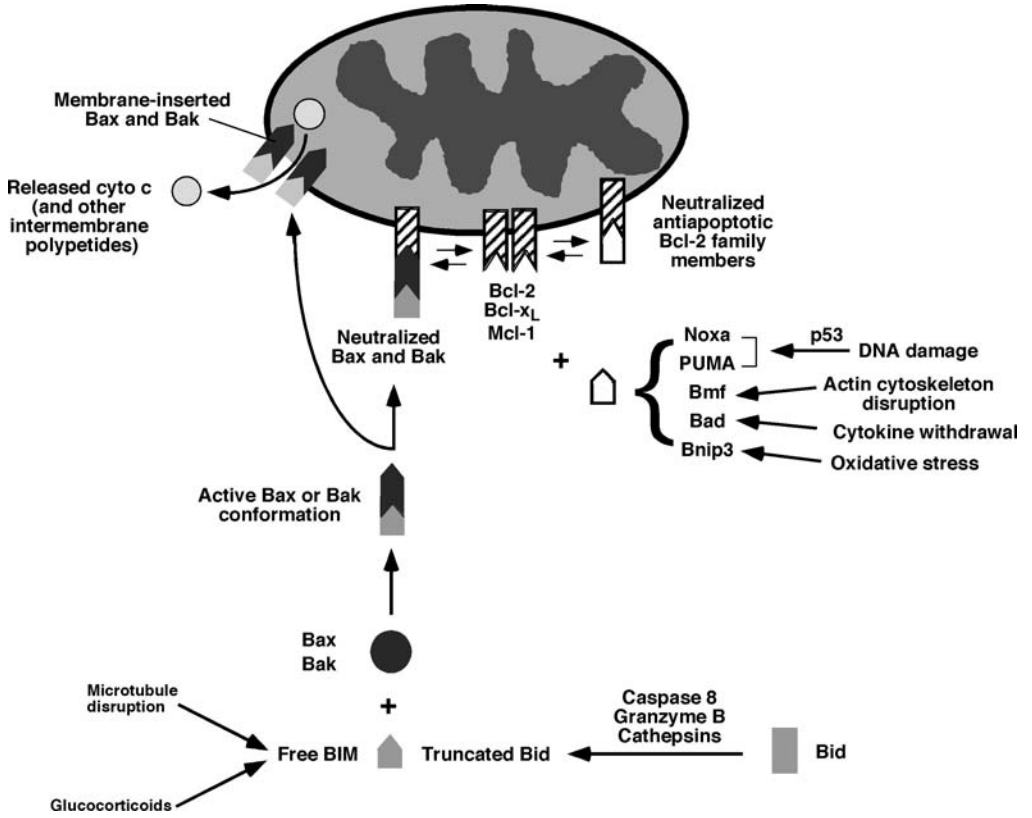

Fig. 3. Regulation of the mitochondrial pathway by Bcl-2 family members. As indicated in the text, insertion of Bax and/or Bak into the outer mitochondrial membrane appears to be directly responsible for the release of cytochrome c and other polypeptides of the mitochondrial intermembrane space. One group of BH3-only polypeptides, typified by Bim and truncated Bid, is currently thought to directly induce the conformational change implicated in Bax- and/or Bak-induced mitochondrial outer membrane permeabilization. Another group of BH3-only polypeptides, including Puma, Bmf, and Bad, is thought to influence Bax and/or Bak membrane insertion less directly, that is, by binding and neutralizing antiapoptotic Bcl-2 family members. Recent data also indicate that certain group III family members bind preferentially to different group I family members *(79)*, providing a potential explanation for the ability of various antiapoptotic Bcl-2 family members to protect differentially against different types of cellular stress.

release *(63,73,74)*. Bax is found in the cytoplasm or loosely bound to the mitochondrial surface in non-apoptotic cells *(78)* but is inserted in the outer mitochondrial membrane after exposure of cells to apoptotic stimuli *(80)*. This membrane insertion appears to involve changes in the conformation and oligomerization state of Bax *(81–83)*. During the process of apoptosis, Bak, which normally resides in the outer mitochondrial membrane, also undergoes a conformational change that results in its deeper insertion in the outer mitochondrial membrane *(84–86)*. Importantly, deletion of either *BAX* or *BAK* modestly diminishes the ability of various stimuli to induce mitochondrial cytochrome c release and subsequent development of apoptotic changes in fibroblasts, whereas deletion of both genes has a much more profound effect *(85,87)*.

Further support for a role of these polypeptides in outer mitochondrial membrane permeabilization comes from a series of observations made under cell-free conditions. After activation (see Section 6 below), Bax induces cytochrome c release from isolated mitochondria *(88,89)* or lipid vesicles prepared from mitochondrial membranes *(89)*. The latter observation again calls into question earlier claims that resident mitochondrial membrane proteins are required for cytochrome c release and instead suggests that group II Bcl-2 family members are sufficient by themselves to breach the outer mitochondrial membrane.

Although it is clear that Bax and Bak play important roles in cytochrome c release, several points still require further study. Although it is possible that Bax and/or Bak oligomerize to form a pore *(90)*, the details of this pore are unclear *(91)*. In addition, it is unclear whether other mitochondrial proteins are released by the same process as that of cytochrome c. It has been observed, for example, that cytochrome c release during apoptosis in fibroblasts requires expression of either Bax or Bak, whereas second mitochondrial activator of caspases (Smac) release (see Section 9 below) requires expression of both *(92)*. The mechanistic basis for this observation remains to be determined.

6. GROUP III FAMILY MEMBERS: ARMING THE ASSASSIN

Insertion of Bax and Bak into the mitochondrial outer membrane appears to be facilitated by group III Bcl-2 family members, the so-called BH3-only members of this family. As recently reported by Kuwana et al. *(93)* and summarized in Fig. 3, it currently appears that BH3-only polypeptides can do this in one of two ways.

Some of the BH3-only polypeptides are thought to interact directly with group II polypeptides to induce their insertion into the mitochondrial outer membrane. Evidence in support of this model includes the detection of a direct interaction of some of these polypeptides (notably Puma and a truncated form of Bid) with Bax *(94)*, the demonstration that truncated Bid is capable of inducing Bax-mediated permeabilization of the mitochondrial outer membrane or vesicles prepared from mitochondrial membrane lipids *(89)*, and the demonstration that the effects of truncated Bid on Bax-mediated permeabilization can be reproduced by 24- or 25-mer peptides containing the BH3 domains of Bid or Bim *(93)*.

By contrast, peptides corresponding to the BH3 domains of other group III Bcl-2 family members are unable to directly induce Bax-mediated permeabilization of lipid vesicles *in vitro (93)*. Nonetheless, all these BH3 domain peptides are able to facilitate membrane permeabilization in a cell-free system containing lipid vesicles, truncated Bid, Bax, and the antiapoptotic family member Bcl-x_L. These observations suggest that

the other BH3-only family members act by neutralizing group I Bcl-2 family members as described in greater detail in the next section.

Why are there so many different group III polypeptides? It appears as if these polypeptides monitor and respond to different types of cellular stress. Bid is a constitutively expressed cytoplasmic polypeptide that is cleaved by caspase-8 *(95,96)*, granzyme B *(97)*, or lysosomal cathepsins *(98)* to release a fragment with enhanced ability to activate Bax. Other constitutively expressed BH3-only polypeptides are activated in other ways: Bad by dephosphorylation and release from cytoplasmic 14-3-3 proteins upon cytokine withdrawal *(99)*; Bim by detachment from microtubules during paclitaxel treatment *(100,101)*; and Bmf by release from actin filaments when cell-substrate attachment is disrupted *(102)*. By contrast, Noxa and Puma are synthesized in a p53-dependent manner in response to DNA damage *(103,104)*, providing a potential explanation for the ability of DNA damage within nuclei to signal mitochondrial cytochrome c release *(105–107)*. It also appears as if Puma is also synthesized in a p53-independent manner in response to other agents such as glucocorticoids *(106)*.

7. GROUP I POLYPEPTIDES: KEEPING THE KILLERS IN CHECK

The effects of these group III Bcl-2 family members are inhibited by group I family members, including Bcl-2, Bcl-x_L, and Mcl-1. Over the years, several different explanations for the antiapoptotic effects of these polypeptides have been proposed. First, it was suggested that group I polypeptides inhibit apoptosis by binding and neutralizing group II polypeptides, especially Bax *(80,108,109)* or Bak *(110)*. The identification of Bcl-x_L mutants that fail to bind Bax or Bak but still inhibit apoptosis *(111)* cast doubt on this model, at least as a universal mechanism of apoptosis inhibition. Second, it was proposed that Bcl-2 prevents apoptosis by increasing the antioxidant capacity of cells *(112)*. Observations that place apoptosis-associated increases in reactive oxygen species downstream of caspase 3 activation *(113,114)* cast doubt on this model. Third, it was suggested that Bcl-2 prevents apoptosis by inhibiting the release of calcium from endoplasmic reticulum stores *(115–119)*. Unfortunately, not all proapoptotic stimuli that are inhibited by Bcl-2 induce calcium release from intracellular stores. Finally, it has been hypothesized that group I polypeptides inhibit apoptosis by binding group III polypeptides, thereby preventing the release of cytochrome c (and other polypeptides) from mitochondria *(120,121)*. This latter hypothesis has received the most attention.

Recent analysis of the binding between BH3 domains and group I polypeptides has demonstrated unanticipated specificity of these interactions *(79)*. Whereas the group I polypeptides Bcl-x_L and Bcl-w bind all tested BH3 peptides except Noxa with nanomolar affinity, other group I family members such as Mcl-1 and A1 bind Noxa, Puma, and Bim with much higher affinity than the remaining BH3 domain peptides. Coupled with the realization that different BH3-only polypeptides monitor different types of stress as described in the preceding section, these results suggest that different group I polypeptides should protect preferentially against certain types of proapoptotic stimuli. This has been documented to a certain extent *(110,122)* but requires further study.

As might be expected for polypeptides that are the final arbiters of cytochrome c release by various stresses, expression and activity of the group I Bcl-2 family members are highly regulated. Genes encoding Bcl-2, Bcl-x_L, and Mcl-1 are activated

by mitogen-activated protein (MAP) kinase signaling in some cell types *(123,124)*, cytokine-mediated activation of STAT (signal transducer and activator of transcription) factors in others *(125,126)*, and the phosphatidylinositol-3 kinase/Akt pathway, possibly acting through the transcription factor nuclear factor (NF)-κB, in others *(127–130)*. In addition, the activity of group I polypeptides is regulated post-translationally by phosphorylation. Bcl-2 is activated by protein kinase Cα-mediated or extracellular signal-regulated kinase (ERK)-mediated phosphorylation on Ser^{70} *(131,132)* and other sites *(133)*. Mcl-1 is likewise phosphorylated and stabilized in an ERK-dependent manner *(134–136)* as well as an ERK-independent manner *(130,137)*. These observations provide at least a partial explanation for the antiapoptotic effects of MAP kinase activation *(138,139)*. Further study will undoubtedly identify additional ways in which signal transduction pathways impinge on the antiapoptotic functions of Bcl-2 family members (e.g., *140,141*), adding further complexity to the regulation of apoptosis in various cells. When the observed tissue-specific differences in expression of the antiapoptotic Bcl-2 family members *(142,143)* are also considered, it starts to become clear how the ability of various stimuli to induce cytochrome c release can vary widely from one cell type to another.

Although the model depicted in Fig. 3 provides an explanation for much of the available experimental data, it is important to realize that several issues require further investigation. For example, because extremely high concentrations of isolated BH3 domains rather than physiological concentrations of native polypeptides were utilized to detect the interactions *in vitro*, it remains to be demonstrated that all the proposed interactions occur in situ. Previous studies reporting inability to detect direct binding of Bim to Bax or Bak despite extensive efforts *(102,144)* highlight the importance of this issue. In addition, it is unclear what drives Bax or Bak into the mitochondrial membrane in the presence of BH3-only polypeptides such as Puma, Noxa, Bad, and Bmf, which are unable to directly activate Bax under cell-free conditions. One possibility is that there is another stimulus for group II polypeptide activation. It has been observed, for example, that activation of Jun N-terminal kinase, which commonly occurs during apoptosis, is accompanied by the release of Bax from cytoplasmic 14-3-3 proteins and subsequent localization to mitochondria *(145)*. A second and related possibility is that group II polypeptides have an intrinsic propensity to insert in the outer mitochondrial membrane unless neutralized by group I polypeptides. Consistent with this possibility, it has been reported that Bak is sequestered by Mcl-1 and Bcl-x_L but becomes active when released from both *(110,146)*.

8. CASPASE INHIBITION BY XIAP: CLOSING THE BARN DOOR AFTER THE COW HAS ESCAPED

Whereas Bcl-2 family members regulate apoptosis upstream of apoptosome formation, the mitochondrial pathway is regulated downstream of apoptosome formation by members of the inhibitor of apoptosis (IAP) family *(147,148)*. These polypeptides, which are characterized by the presence of one or more zinc finger-like baculovirus inhibitor repeat (BIR) domains (Fig. 4), were originally identified in viruses because of their ability to inhibit apoptosis and enhance viral replication in infected cells. It is now clear that there are at least two subgroups of BIR-containing polypeptides *(150)*. One, which contains survivin and its homologues in yeast and worms,

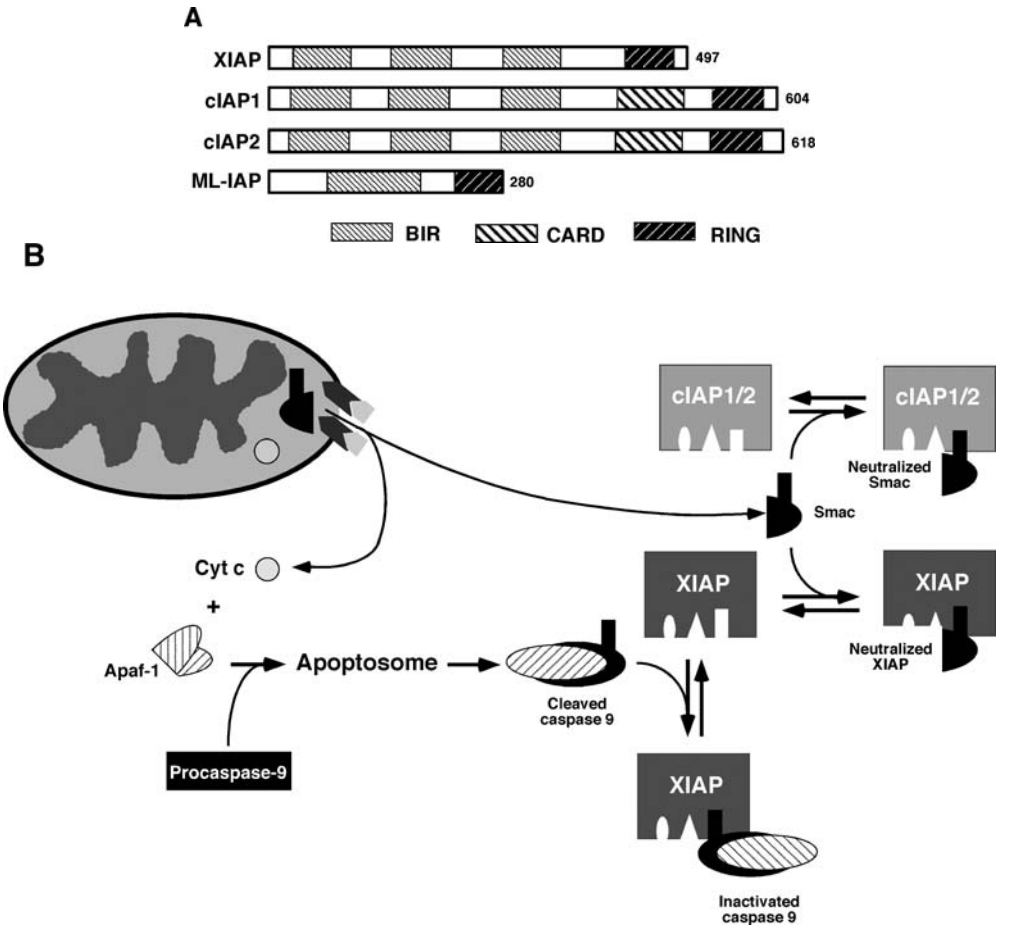

Fig. 4. Regulation of the mitochondrial pathway by inhibitor of apoptosis (IAP) proteins. **(A)** Schematic representation of several antiapoptotic baculovirus inhibitor repeat (BIR) proteins. Shaded areas depict BIR domains, caspase recruitment domains (CARDs), and RING fingers. Numbers at right indicate the number of amino acids in each polypeptide. Melanoma-associated IAP (ML-IAP) is also known as livin. **(B)** Current understanding of X chromosome-linked IAP (XIAP) and cIAP1/2 function. The third BIR domain of XIAP is known to bind procaspase 9 and prevent its activation (not depicted—see *149*) as well as inhibit partially cleaved caspase 9. Smac, which is released from mitochondria, competes for this BIR domain, freeing caspase 9. Conversely, cIAP1, cIAP2, or ML-IAP can bind and sequester Smac, liberating XIAP and facilitating caspase 9 inactivation. Because Smac can also bind to XIAP BIR2, which inhibits caspases 3 and 7, cIAP/Smac interactions likely regulate caspases 3 and 7 in a similar fashion.

is required for successful function of the mitotic spindle *(151,152)*. The other, which includes X chromosome-linked IAP (XIAP), cellular IAP1 (cIAP1), cIAP2, and melanoma-associated IAP (ML-IAP), regulates apoptosis *(147)*. In addition to one or more BIR domains, these antiapoptotic IAP proteins commonly contain a RING finger that is thought to transfer ubiquitin to bound caspases and other polypeptides, thereby marking the binding partners for proteasome-mediated degradation *(153–156)*.

The mechanism of caspase inhibition by XIAP has been extensively studied *(147,157)*. The third BIR of XIAP has multiple effects on caspase 9. First, it binds to procaspase 9

and inhibits the dimerization that is required for activation *(149,158)*. Second, BIR3 binds to a cleavage intermediate that is transiently generated when procaspase 9 is clipped between the large and small subunits *(158)*. In addition to these effects on caspase 9, XIAP also inhibits caspases 3 and 7. Structural studies suggest that the second BIR of XIAP binds the surfaces of these target caspases, orienting the molecules so that a short peptide immediately upstream from BIR2 can block access to the active sites of caspases 3 and 7 *(147,157,159)*. These observations suggest that XIAP should be able to inhibit the intrinsic pathway at multiple steps.

On the basis of these observed effects, it has been suggested that XIAP acts as a cellular buffer for small amounts of caspases that are inadvertently activated *(160)*. Consistent with this view, XIAP expression is regulated by NF-κB *(4)*, a transcription factor that enhances survival *(161)*. Nonetheless, confirming this postulated function of XIAP in intact cells or organisms has been problematic. Although XIAP overexpression inhibits apoptosis induced by various stimuli *(162–164)*, XIAP retains its antiapoptotic activity even when the binding sites for caspases 3 and 9 have both been mutated *(165)*, raising the possibility that some other critical antiapoptotic function of the polypeptide has been missed. Equally disconcerting for current models, targeted disruption of the mouse *XIAP* gene fails to yield a phenotype suggestive of increased apoptosis *(166)*. Conversely, tissue-specific XIAP overexpression *in vivo* inhibits not only thymocyte apoptosis but also thymocyte maturation *(167)*, raising the possibility that XIAP might serve multiple functions that are not yet completely understood.

9. ADDITIONAL PROAPOPTOTIC MITOCHONDRIAL PEPTIDES: THE EXECUTIONER'S ARSENAL

In addition to cytochrome c, dozens of other polypeptides are released from mitochondria during apoptosis *(168)*. One of these, endonuclease G, was initially reported to produce CAD-independent internucleosomal DNA degradation *(169)*, although subsequent observations have called this conclusion into question *(170,171)*. Apoptosis-inducing factor (AIF), a mitochondrial oxidoreductase *(172)* that is released from the intermembrane space during apoptosis *(173)*, was originally said to be critical for apoptotic changes induced by certain oxidative stimuli *(174)*, but more recent studies have cast doubt on the suggestion that AIF plays any role in apoptosis *(175)*. Perhaps more informative from the standpoint of mitochondrial pathway regulation are Smac/direct IAP-binding protein with low pI (DIABLO) and HtRA2, two polypeptides that reportedly modulate the function of XIAP, cIAP1, and cIAP2 *(176)*.

Smac/DIABLO was purified based on its ability to facilitate cytochrome c-induced caspase activation under cell-free conditions *(177)* and to bind XIAP in cells undergoing apoptosis *(176)*. Although some studies have suggested that Smac/DIABLO is easier *(178)* or harder *(179)* to release from mitochondria than cytochrome c, others have found that these two polypeptides are released in parallel *(180)*. Once in the cytoplasm, Smac/DIABLO binds to XIAP *(176–180)*, disrupting its binding to caspases *(158,180)*. X-ray crystallography and mutational analysis have demonstrated that the tetrapeptide alanine-valine-proline-isoleucine (AVPI), a sequence exposed at the N-terminus of mature Smac when the mitochondrial targeting sequence is proteolytically removed *(176,177,181)*, plays a critical role in the function of Smac/DIABLO by binding to shallow grooves on the surface of XIAP *(182,183)*.

The recognition that Smac acts by inhibiting XIAP has provided new insight into the action of cIAP1, cIAP2, and ML-IAP (Fig. 4). Earlier studies demonstrated that these polypeptides inhibit apoptosis in intact cells and under cell-free conditions *(147,184)*. Curiously, however, these polypeptides are poor inhibitors of caspases 3 and 9 ($K_i > 10\,\mu M$) compared with XIAP ($K_i < 1$ nM for caspase 9 and 10 nM for caspase 3) *(185)*. Recent experiments have demonstrated, however, that these other IAP proteins avidly bind Smac, thereby freeing XIAP from Smac inhibition and allowing caspase inhibition *(186–188)*. Thus, the regulation of proteases in the intrinsic pathway involves interactions between an inhibitor (XIAP), regulators of the inhibitor (e.g., Smac/DIABLO), and binding partners of the regulator (e.g., cIAP1, cIAP2, ML-IAP).

Despite the elegant biochemical studies implicating Smac in the regulation of XIAP, disruption of the *Smac/DIABLO* gene yields no developmental phenotype *(189)*, making it difficult to confirm that modulation of apoptosis is the sole (or even major) function of this polypeptide. The existence of additional polypeptides with a similar function could provide a potential explanation for the lack of an apoptotic phenotype in *Smac* knockout mice. Consistent with this possibility, four groups have reported that the mitochondrial protein HtRA2/Omi, a member of a family of polypeptides that act as chaperones at physiological temperature and as proteases after heat-induced activation *(190)*, also binds to and inhibits XIAP *(191–194)*. Like Smac/DIABLO, HtRA2/Omi relies on an N-terminal targeting sequence for mitochondrial uptake and is then cleaved to yield a mature polypeptide with the N-terminal sequence AVPS. Although it has been suggested that the intrinsic serine protease activity of HtRA2 might contribute to its proapoptotic effects in certain contexts *(191,194)*, possibly by degrading IAP proteins *(195)*, a missense mutation of the HtRA2 active site serine *(196)* or targeted *HtRA2* gene deletion *(197)* results in mice with a neurodegenerative phenotype rather than impaired apoptosis, hinting that the major physiological function of HtRA2 is proteolytic clearance of misfolded mitochondrial polypeptides rather than regulation of XIAP.

In addition to Smac/DIABLO and HtRA2/Omi, mammalian cells contain at least three additional polypeptides that could bind XIAP when released to the cytoplasm *(176,198)*. What is unclear at this point, however, is whether any of these XIAP-binding polypeptides are bona fide apoptotic regulators under physiological conditions *(199)*. Although a variety of studies have established that the IAP-binding polypeptides hid, grim, reaper, and sickle, all of which start with N-terminal AVPI-like sequences, act as tissue-specific IAP antagonists in Drosophila *(199)*, the importance of this mechanism of caspase regulation remains to be unambiguously demonstrated in mammalian systems.

10. ACTIVATION OF THE APOPTOTIC MACHINERY BY CHEMOTHERAPEUTIC AGENTS

Because apoptosis can be detected in circulating leukemia cells after institution of antileukemic therapy *(6–10)* and in murine solid tumors after effective antineoplastic treatment *in vivo (2,11,12)*, there has been considerable interest in identifying the apoptotic pathway(s) activated by various antineoplastic agents. Many agents, including doxorubicin, etoposide and teniposide, methotrexate, cisplatin, and bleomycin, induce

the synthesis of FasL *(200,201)* by activating the transcription factors NF-κB and AP-1 *(202,203)*. In addition, Fas receptor expression is upregulated in a p53-dependent fashion by DNA-damaging agents *(204)*. Although these observations have been used to implicate the death receptor pathway (Chap. 2) in drug-induced killing, several observations suggest that Fas/FasL interactions might not be required for killing by most agents. First, cells with defects in Fas expression or Fas-mediated signaling remain susceptible to a variety of drugs *(205–210)*. Second, blocking anti-Fas antibodies inhibit the cytotoxic effects of agonistic anti-Fas antibodies or upregulated Fas but not the previously mentioned anticancer drugs *(205–207,211)*. Third, inhibition of the death receptor pathway by crmA, an inhibitor of caspases 8 and 10 *(212)*, fails to inhibit the induction of apoptosis by these drugs *(205,209)*.

Although these observations suggest that signaling through the Fas/FasL pathway is not absolutely required for anticancer drug-induced apoptosis, the possibility that induction of Fas and FasL contributes to apoptosis, particularly after prolonged exposure to low doses of DNA-damaging agents *(135,213)*, has not been ruled out. This possibility is discussed in greater detail in Chap. 2. In addition, whereas most drug-induced apoptosis occurs independent of Fas/FasL signaling, a small group of drugs do in fact appear to trigger apoptosis by activating death receptor pathways. These include 5-fluorouracil *(214)*, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid *(215)*, proteasome inhibitors *(216)*, *trans*-retinoic acid *(217)*, CI-1040 *(135)*, and fenretinide *(218)* as well as depsipeptide *(219)* and other histone deacetylase inhibitors *(220)*. Interestingly, in instances where it has been critically examined, this reliance on the death receptor pathway appears to vary from one cell line to another *(135,218,221)*. In the case of colon cancer cells treated with fluoropyrimidines, the presence of wild-type p53 reportedly determines whether drug-induced apoptosis proceeds through the Fas pathway *(222)*. The critical determinant for other drugs is currently unknown but is almost certainly not p53.

For the majority of anticancer drugs, previous studies not only ruled out an essential role for the Fas/FasL pathway in drug-induced apoptosis (see first paragraph of this Section) but also directly implicated the mitochondrial pathway in this process. Observations that are important in this regard include the demonstration that cytochrome c release accompanies induction of apoptosis by a variety of agents *(113,120,223,224)*, the determination that Bax translocates to mitochondria in response to various drugs independent of (i.e., upstream of) caspase activity *(83,225)*, and the demonstration that combined deletion of *BAX* and *BAK* inhibits drug-induced apoptosis *(87,226)*. As might be expected, if the mitochondrial pathway plays a predominant role, dominant-negative caspase-9 constructs *(227,228)* and antiapoptotic Bcl-2 family members *(210,229)* inhibit drug-induced apoptosis. The demonstration that *Caspase9* or *Apaf-1* gene deletion delays the induction of apoptosis by staurosporine, dexamethasone, etoposide, and ionizing radiation in mouse fibroblasts or thymocytes, whereas *Caspase8* deletion does not, provides additional support for the view that most agents activate the mitochondrial pathway *(1)*. For some drugs in some cell types, the signaling immediately upstream of Bax and/or Bak activation appears to involve changes in expression or localization of BH3-only family members (see Section 6), but in other cells and for other drugs, the changes that lead to Bax and/or Bak activation still require clarification.

11. LIFE ON THE EDGE: ALTERATIONS IN THE APOPTOTIC MACHINERY IN CANCER CELLS

Changes that disable the apoptotic machinery are now considered one of the hallmarks of neoplastic transformation *(230–234)*. How inhibition of apoptosis contributes to neoplastic transformation is perhaps best understood for the oncoprotein myc, a transcription factor that is upregulated in proliferating cells but cannot induce neoplastic transformation unless an antiapoptotic Bcl-2 family member is also overexpressed *(75,235,236)*. When cells are cultured under favorable conditions, myc overexpression drives proliferation, but when cells encounter unfavorable growth conditions, myc overexpression causes apoptosis *(237,238)*. This myc-induced apoptosis appears to result from several distinct proapoptotic changes, including upregulation of $p19^{ARF}$, which binds Mdm2 and allows upregulation of p53-dependent apoptotic mediators *(239)*, upregulation of Bim *(240)*, and downregulation of Bcl-2 and Bcl-x_L *(241)*. These proapoptotic changes must be overcome if cells are to be successfully transformed by myc. According to current understanding, other proliferation-inducing oncogenes likewise require the cooperation of antiapoptotic changes *(233)*.

Because transforming oncogenes activate the intrinsic apoptotic pathway when cells encounter unfavorable growth conditions *(242,243)*, it is perhaps not surprising that the intrinsic pathway is inhibited in a number of different ways in various cancers *(233–245)*. Antiapoptotic Bcl-2 family members are overexpressed in some cancers *(15,246,247)*. In others, constitutive activation of the mitogen-activated kinase pathway *(139)* induces activation (Bcl-2) or stabilization (Mcl-1) of group I Bcl-2 family members (see Section 7). In leukemia cell lines *(248)* and a substantial portion of mismatch repair-deficient colon and gastric cancers *(249)*, the *BAX* gene is mutated, although the heterozygous nature of these mutations in clinical cancer *(249)* stands in contrast to results obtained in animal models *(250)* and by itself fails to completely account for any apoptotic defect. In other tumors, changes that upregulate the Akt pathway *(251)*, including autocrine or paracrine activation of receptor tyrosine kinases, *PI3KINASE* gene mutation *(252)* or amplification *(253)*, *PTEN* deletion, or *AKT2* gene amplification *(254)*, inhibit mitochondrial cytochrome c release *(255)*.

The most common change that impinges on the mitochondrial pathway is mutation or deletion of the tumor suppressor p53, a transcription factor that is widely reported to induce apoptosis upon forced overexpression *(256,257)*. Although many intrinsic pathway components, including Bax, Apaf-1, Bid, and caspase-6, have been identified as p53 transcriptional targets *(256–258)*, gene-targeting studies have identified the BH3-only polypeptides Puma and, to a lesser extent, Noxa, as the most important contributors to p53-induced apoptosis *(105–107)*. It currently appears that Puma plays a predominant role in p53-induced apoptosis after certain DNA-damaging stimuli *(106)*, but further study is required to determine whether similar results are obtained after all types of DNA damage and in all cell types.

Despite the frequency of p53 mutations in clinical cancer, there is reason to question whether these lesions arise to counterbalance the proapoptotic effects of oncogenes early in carcinogenesis. Current evidence suggests that p53 mutations play an important role *in vivo* to counter the proapoptotic effects of myc overexpression in Burkitt's lymphoma-like B-cell neoplasms in mice *(239)*. In epithelial neoplasms, however, p53 mutations are a late occurrence *(259,260)*, raising the possibility that other antiapoptotic

changes like those involving the PI3 kinase/Akt pathway *(251,261)*, which often occur in atypia and carcinoma in situ, might play a more important role early in tumorigenesis.

12. ARE ALTERATIONS IN THE APOPTOTIC MACHINERY DETERMINANTS OF DRUG SENSITIVITY?

The demonstration that anticancer drugs can kill cells by inducing apoptosis, coupled with the observation that cancer cells have alterations that inhibit apoptotic pathways, has led many authors to hypothesize that alterations in apoptotic pathways might contribute to drug resistance (e.g., *13,15,17,262*). Two aspects of this hypothesis are important to emphasize. First, what is being postulated is resistance of cells relative to what their sensitivity would be if apoptotic pathways were not altered, not absolute resistance to therapy. Other changes in cancer cells, e.g., their aberrant entry into the cell cycle and loss of cell cycle checkpoints, enhance sensitivity to anticancer drugs and undoubtedly contribute to the (admittedly limited) therapeutic index of these agents in some settings. Second, the hypothesis that alterations in apoptotic pathways contribute to drug resistance can only be true to the extent that apoptosis plays a role in cellular responses to various chemotherapeutic agents. As indicated elsewhere in this monograph, cells can also suffer other fates after drug treatment, and these other fates are not necessarily modulated by changes in the apoptotic machinery.

Clinical studies examining the correlation between expression of various components of the apoptotic machinery and treatment outcome have been extensively reviewed *(15,263,264)*. These studies, while potentially important for identifying predictive markers that might alter treatment and for confirming activation of various pathways that might serve as drug targets in future trials, do not establish causality. Accordingly, the following comments will focus on controlled studies that have evaluated the relationship between alterations in components of the mitochondrial pathway and drug sensitivity in model systems. These studies have focused primarily on the effects of p53 alterations and the changes in Bcl-2 family members and IAPs.

p53 alterations appear to affect sensitivity to both ionizing radiation and drugs. Studies in tissue culture have led to the conclusion that p53 deficiency can render both E1A/ras-transformed fibroblasts *(265)* and HCT116 colon cancer cells *(266)* resistant to the induction of apoptosis by various anticancer drugs *in vitro*. When the same cell lines are grown as xenografts, responses are diminished in the absence of p53. Consistent with these results, studies have also established that ionizing radiation or high-dose 5-fluorouracil induces less apoptosis in epithelial cells of the small and large intestines of $p53^{+/+}$ mice compared with that of $p53^{+/+}$ littermates *(267,268)*. Collectively, these results suggest that changes in p53 status can affect drug sensitivity in at least some cell types.

Increased expression of antiapoptotic Bcl-2 family members can also affect drug sensitivity. Virtually, all published studies agree that Bcl-2 or Bcl-x_L overexpression delays the appearance of apoptotic changes after treatment with a variety of agents *(247, 269–271)*. In addition, assays that compare the ability of isogenic cells to regrow after drug treatment *in vitro* or *in vivo* have established that forced overexpression of Bcl-2 or Bcl-x_L can enhance the ability of cell populations to recover after a variety of drug treatments *(247,271,272)*. Nonetheless, the role of group I Bcl-2 family polypeptides

in anticancer drug resistance is far from settled. Whether conclusions based on limited studies conducted predominantly in lymphohematopoietic cells can be generalized to all cell types and all therapeutic agents remains a matter of some controversy *(20)*. Moreover, the effects of other antiapoptotic Bcl-2 family members remain to be studied in a similar fashion.

At the present time, the effects of alterations in proapoptotic Bcl-2 family members also require further study. It has been reported that *BAX* deletion inhibits drug-induced apoptosis in fibroblasts *(273)* and HCT116 cells *in vitro (226,274,275)*. It has also been reported that *BAX* deletion renders mouse oocytes resistant to polycyclic aromatic hydrocarbon-induced apoptosis *in vivo (276)*. Nonetheless, the effects of *BAX* deletion on anticancer drug sensitivity remain to be established *in vivo*. Moreover, it is important to emphasize that $BAX^{-/-}$ cells represent an extreme situation that might not occur in human cancers *in vivo*. Instead, mismatch repair-deficient tumors appear to have inactivating mutations in a single *BAX* allele *(249)*, and it is unclear whether the resulting cells or cell lines completely lack Bax protein. If Bax is incompletely downregulated in human tumors, then models in which Bax is downregulated might be more pertinent to clinical anticancer drug resistance than those containing *BAX* deletions. Similar comments apply to Bak.

Information regarding the role of BH3-only protein alterations in drug resistance is likewise incomplete. In perhaps the best-studied example, *Bim* gene deletion not only renders murine thymocytes selectively resistant to paclitaxel-induced apoptosis *in vitro (100)* but also confers resistance to transformed baby mouse kidney xenografts treated with paclitaxel *in vivo (277)*. It remains to be determined whether quantitative Bim alterations that fall short of complete deletion also affect drug sensitivity. Likewise, it remains to be determined whether *Puma* deletion, which renders fibroblasts, thymocytes, or HCT116 cells resistant to a number of treatments, including doxorubicin, etoposide, and ionizing radiation *in vitro (105–107,278)*, affects long-term responses to anticancer treatments in tumor cells in vivo.

The effects of altered IAP levels also require further study. Examination of isogenic cell lines *in vitro* has established that cIAP1, cIAP2, and XIAP diminish apoptosis triggered by a number of agents, including camptothecin and menadione *(162)*. A more recent study also demonstrated that an XIAP antisense oligonucleotide sensitized NIH-H60 non-small-cell lung cancer cells to etoposide, doxorubicin, and paclitaxel *in vitro* and to vinorelbine *in vivo (279)*. Antisense oligonucleotides, however, are notorious for producing off-target effects. Further studies using additional model systems *in vitro* and *in vivo* are required to determine whether enhanced expression of cIAP1, cIAP2, and/or XIAP enhances the ability of tumor cell populations to survive drug treatment.

Moving further downstream, silencing of *APAF-1* has been reported in a large fraction of melanoma cell lines and clinical samples *(280)*. Consistent with prior observations in $Apaf\text{-}1^{-/-}$ murine fibroblasts and thymocytes *(281)*, the Apaf-1-deficient melanoma cells were reportedly resistant to doxorubicin-induced apoptosis *(280)*. Although the authors concluded that they had identified a major mechanism of drug resistance in melanoma, subsequent observations have called this conclusion into question. In particular, others have not been able to reproduce the frequent Apaf-1 downregulation in melanoma cell lines *(282–284)* or clinical samples *(284)*.

In short, alterations of p53 and certain Bcl-2 family members are clearly capable of altering long-term survival after drug treatment in some model systems *in vitro* and *in vivo*. There is currently less evidence that overexpression of XIAP or downregulation

of Apaf-1, caspase-9, or the effector caspases modulates long-term sensitivity of cells to anticancer drugs, particularly *in vivo*. On the contrary, recent evidence suggests that changes in the intrinsic pathway beyond the mitochondrial permeabilization step (Figs. 1 and 3) affect the rate of cell death but not the ultimate extent of cell death after proapoptotic stimuli *(285)*. These observations invite that speculation that proapoptotic Bcl-2 family members, once activated, might be able to kill cells through the release of cytochrome c and other caspase-independent processes even if cells are limited in their ability to activate caspases. The inability of broad-spectrum caspase inhibitors to enhance long-term survival of camptothecin-treated cells despite inhibition of apoptosis *(286)* is consistent with this notion. Collectively, these results do not negate the importance of the mitochondrial pathway in determining the response to anticancer drugs but instead suggest that future studies are likely to be most productive if they focus on upstream components of this pathway.

13. CONCLUSIONS

Previous studies have demonstrated that anticancer treatments can induce apoptosis in susceptible cell types *in vitro* and *in vivo*. Although a few agents require an intact death receptor pathway to trigger apoptosis, many agents appear to utilize the mitochondrial pathway. Despite recent improvements in our understanding of this pathway, fundamental issues currently remain unsettled. These include determination of the precise mechanism by which proapoptotic Bcl-2 family members induce outer mitochondrial membrane permeabilization as well as conclusive demonstration that other proapoptotic mitochondrial proteins such as Smac and HtRA2 play a critical role in apoptosis *in vivo*. Because the intrinsic pathway is extensively targeted for inhibition during the development of cancer, it has been speculated that changes in this pathway also contribute to drug resistance. With the exception of p53, Bcl-2, Bcl-x_L, Bim, and possibly XIAP, however, careful *in vivo* studies to establish this point remain to be performed.

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NOTE

During the two years between submission of this chapter and receipt of the page proofs, many of the unresolved issues described in this chapter have been addressed (287–289).

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2 The Extrinsic Pathway of Apoptosis

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Summary

Defects in the extrinsic pathway are linked to several disease states, including cancer. Pharmacologic manipulation of the extrinsic pathway holds exciting promise for cancer treatment. This review will discuss the current understanding of the molecular signaling events that originate from extracellular sources to initiate apoptosis, how the pathway is activated by conventional chemotherapeutic agents, and novel opportunities to exploit the extrinsic pathway for cancer treatments.

Key Words: Apoptosis; TNF; Fas; TRAIL; NF-κB; chemotherapy; death receptor; decoy receptor; extrinsic pathway; autoimmunity.

1. OVERVIEW OF SIGNALING EVENTS

1.1. Introduction

Apoptosis is essential to development and maintaining a healthy life for multicellular organisms. It is a rapid, catastrophic process that is precisely regulated in both its initiation and its execution. Although the phenomena had been described for almost a century, in 1972, Kerr, Wyllie, and Currie *(1)* first coined the term "apoptosis" to differentiate naturally occurring developmental cell death from the necrosis that results from acute tissue injury. They also noted that apoptosis was responsible for maintaining tissue homeostasis by mediating the equilibrium between cell proliferation and cell death in a particular tissue. Morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. Under normal circumstances, cells undergoing apoptosis are recognized by macrophages or neighboring cells that consume the cells' fractionated carcasses. There are two distinct molecular signaling pathways that lead to apoptotic cell death: (i) the extrinsic, or extracellularly activated, pathway and (ii) the intrinsic, or mitochondria-mediated, pathway. Both pathways activate a cascade of proteolytic enzymes called caspases that mediate the rapid dismantling of cellular organelles and architecture. Caspases are a family of proteins containing a nucleophilic cysteine

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residue that participates in the cleavage of aspartic acid-containing motifs *(2)*. There are two groups of caspases, the initiator (or apical) caspases and the effector (or executioner) caspases. Initiator caspases are capable of autocatalytic activation whereas effector caspases need activation by initiating caspase cleavage. Initiator caspases mediate the primary signaling events that result in extrinsic apoptosis activation.

Dr. P. Bruns, a German physician, first hinted at the existence of the extrinsic pathway leading to apoptosis in a paper he published in 1868 *(3)*. He reported that acute bacterial infection caused tumor regression in a subset of his patients. More than a century later, tumor necrosis factor (TNF) and lymphotoxin were isolated and found to be effective at killing cultured tumor cells *(4,5)*. After these factors were cloned *(6,7)*, it became evident they were highly homologous. The advent of automated DNA sequence analysis made possible the identification of previously unknown messenger RNA (mRNA) transcripts and brought with it the discovery of an entire family of homologous TNF proteins *(8,9)*.

The TNF family acts by binding the extracellular domains of receptor proteins: there are 19 ligands in the TNF family that can bind one or more of 29 receptors belonging to the TNF receptor (TNFR) family *(8,9)*. Members of the TNF family are primarily produced as type II transmembrane proteins, arranged in stable homotrimers. The structures of the receptors are diverse, and recent observations from crystallization analyses indicate many similarities, but also many differences, among them *(10)*. For the purpose of this review, we will focus only on the TNFR family members that can activate caspases and elicit death signals and will pay particular attention to signaling in the context of cancer therapy.

Each member of the TNF family binds to one or more receptors in the TNFR family, and some receptors bind one or more ligands. TNFRs contain one to four copies of a conserved cysteine-rich domain that follows a hydrophobic amino terminus and precedes the transmembrane domain. Ligand binding elicits a multitude of responses (including apoptosis, proliferation, and inflammation) and the given response depends upon the adapter proteins the bound receptor recruits. TNFRs signal through two classes of adapter proteins, TNFR-associated factors (TRAFs) and "death domain" (DD)-containing proteins. The subset of TNFRs that can activate apoptosis also possesses the DDs *(11)*. The DD is a conserved stretch of approximately 80 amino acids found to be essential for transducing the apoptotic signal. The death receptors that appear to play important roles in mediating apoptosis are those that bind TNF (TNF-α, TNFSF2), FasL (also known as CD95L, TNFSF6), or TNFR apoptosis-inducing ligand (TRAIL; also known as Apo2L, TNFSF10). There are four other DD-containing TNFR members, but there is little evidence directly coupling these receptors with caspase activation. They are the receptor for nerve growth factor (NGFR), the ectodermal dysplasia receptor (EDAR), DR3, and DR6. This chapter will focus only on the receptors for TNF, FasL, and TRAIL because of their major role in human disease and significant potential for therapeutic interventions.

1.2. Molecular Signaling Events

The death-inducing TNFRs recruit initiating caspases and in doing so can activate a cascade of caspase cleavage that rapidly lead to cell death (Fig. 1). The generalized sequence of events is as follows: after binding ligand, the receptors undergo conformational changes that result in recruitment of an assembly of proteins, termed the

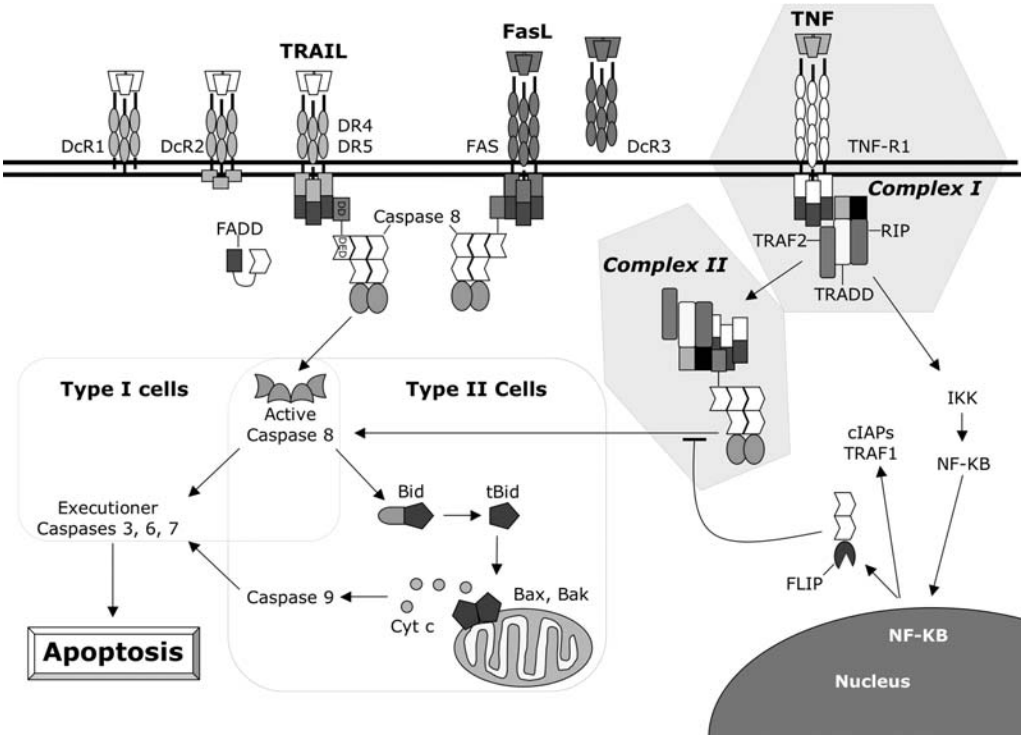

Fig. 1. The extrinsic pathway of apoptosis. Soluble tumor necrosis factor (TNF) family ligands TNF receptor (TNFR) apoptosis-inducing ligand (TRAIL), FasL, and TNF form trimers that recognize and bind their cognate death receptors. I) FasL and TRAIL: after binding ligand, DR4, DR5, and Fas undergo conformational changes resulting in assembly of the death-inducing signaling complex (DISC). Decoy receptor 1 (DcR1), DcR2, and DcR3 bind ligand with high affinity but do not induce apoptosis. DR4, DR5, and Fas then recruit Fas-associated death domain (FADD) through complementary death domains (DDs). FADD can recruit caspase 8 through their complementary death-effector domains (DEDs). Recruitment of caspases 8 to the DISC leads to its autoproteolytic cleavage, releasing two subunits that form active enzyme. In type I cells, caspase 8 cleaves and sufficiently activates effector caspases 3, 6, and 7 to fully engage the apoptotic response. In type II cells, activated caspase 8 cleaves Bid, which stimulates Bax and Bak to release factors from the mitochondria, including cytochrome c, thus activating the intrinsic pathway of apoptosis and augmenting active caspase 8. II) TNF: TNF binds TNF-R1 and recruits TNFR-associated DD (TRADD) through its DD and a complex of proteins containing receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) (Complex I). Complex I can activate inhibitor of nuclear factor (NF)-κB (IκB)–kinase complex, thereby freeing NF-κB for entry into the nucleus and rapid transcription of anti-apoptotic genes, including FLICE inhibitory protein (FLIP) and cIAP1/2. Complex I then dissociates from TNF-R1 where it binds FADD and caspase 8 (Complex II). If in sufficient abundance, FLIP can block Complex II's caspase 8 from self-activation. Otherwise, complex II triggers a caspase 8-driven apoptotic response.

death-inducing signaling complex (DISC). The DISC was first described in FasL–Fas apoptotic signaling *(12)*. TRAIL binding to its death-inducing receptors acts in a manner similar to FasL, whereas TNF-mediated signaling is more complex and will be discussed in Section 1.3 further detail. The ligand-bound Fas or TRAIL death receptors recruit a DD-containing adapter protein, Fas-associated DD (FADD) *(13)*.

FADD contains a second important death receptor-signaling motif, the death-effector domain (DED), and is the only protein in either the human or the mouse genome that contains both a DD and a DED. Bound FADD recruits initiator caspases 8 and 10 through complimentary DED domains *(14,15)*. Recruitment of caspases 8 and 10 to the DISC leads to their autoproteolytic cleavage and release of two caspase subunits that form a mature active enzyme *(16,17)*. If in sufficient abundance, activated caspase 8/10 cleaves and activates effector caspases 3 and 7, thereby fully engaging the caspase cascade. In some cells, named type I cells, activation of these effector caspases by activated caspase 8/10 alone is sufficient to induce apoptosis *(18)*. In type II cells, activated caspase 8/10 stimulates the release of factors from the mitochondria, including cytochrome c, Smac/DIABLO, and Omi/Htr2A, thereby engaging the intrinsic pathway of apoptosis (see Chap. 1 for detailed review).

Why cells behave in a type I or a type II manner is not well understood. Gene expression analysis comparing type I and type II cells using Fas activation has been performed *(19)*. The expression analysis of type I cell lines showed a preponderance of mesenchymal-like genes, whereas the type II cell lines preferentially express epithelium-like markers. A chemical screen for growth inhibition of these cells revealed that actin-binding compounds selectively inhibited growth of type I cells and tubulin-interacting compounds inhibited growth of type II cells. The functional significance of this observation may become useful in chemotherapeutic treatment selection for cancers with these types of gene expression profiles.

Caspase 8/10 connects the intrinsic and extrinsic pathways by cleaving Bid, a BH3-only member of the Bcl-2 family, which can mediate destabilization of the outer mitochondrial membrane by interacting with other Bcl-2 family members *(20,21)*. To date, Bid is the only known physiologic mediator that connects the extrinsic pathway with release of apoptotic factors from mitochondria. However, a recent report showed the tumor suppressor protein RASSF1A associated with activated death receptors to contribute to Bax activation *(22)*. The mechanism of action proposed is that RASSF1A binds to modulator of apoptosis (MAP)-1, a BH3-like protein, which can associate with Bax resulting in Bax translocation to the mitochondria. RASSF1A or MAP-1 siRNA-mediated knockdown diminished TRAIL-induced apoptosis, but this effect was shown only in the presence of cyclohexamide. RASSF1A could immunoprecipitate with TNF-R1 after a relatively long TNF treatment (2–3 h) while in the presence of cyclohexamide. Therefore, the significance of RASSF1A/MAP-1 modulation of Bax translocation in the context of a delayed death ligand response requiring protein synthesis inhibition is not clear. Nevertheless, it is interesting that a signaling pathway not involving Bid and connecting death receptors to the mitochondria has been discovered.

In humans, the *CASPASE 8* gene is found on chromosome 2q33–q34 in tandem with two other highly homologous proteins, CASPASE 10 and FLICE inhibitory protein (FLIP). Caspase 10 contains both a caspase domain and a DED. Caspase 10 is also recruited to the DISC, and whether it can functionally substitute for caspase 8 is controversial *(23–25)*. Studies of the role of FLIP recruitment to the DISC have revealed both activating and inhibitory functions depending on expression level. Although many FLIP isoforms are expressed in cells, only two are present at the protein level, a 55-kDa variant ($FLIP_L$) and a 26-kDa form ($FLIP_S$) *(26)*. $FLIP_S$ contains DEDs but lacks the caspase domain and acts as a direct inhibitor of caspase 8 cleavage. $FLIP_L$ contains tandem DEDs but lacks critical residues in its caspase domain including

the catalytic cysteine, suggesting it to be a classical dominant-negative inhibitor. However, there are reports of $FLIP_L$ acting as an inducer of caspase 8 autoproteolytic activation *(27,28)*. These results were recently challenged by a study using siRNA to selectively knockdown each FLIP isoform *(29)*. Separate knockdown of either $FLIP_L$ or $FLIP_S$ enhanced DISC formation and caspase 8 activation, suggesting the endogenous role of FLIP as primarily inhibitory.

1.3. TNF Pathway

TNF is a pro-inflammatory cytokine produced by a wide range of immune cells, including monocytes, macrophages, T cells, B cells, and natural killer (NK) cells *(8,9)*. Large amounts of soluble TNF are released in response to lipopolysaccharide (LPS) and other bacterial products. High concentrations of TNF induce septic shock, and prolonged exposure to low concentrations of TNF can result in cachexia, a wasting syndrome. TNF is involved in the progression of many human diseases, including autoimmune diseases (Crohn's disease, rheumatoid arthritis), neurodegeneration, and cancer *(30)*.

There are two receptors for TNF, TNF-R1 and TNF-R2 *(31,32)*. TNF-R1 is expressed ubiquitously and has a DD, whereas TNF-R2 has no DD and is found mainly in cells of the immune system and endothelium. TNF-R1 principally regulates the immune system by activating pro-survival signaling. TNF-R1 elicits an anti-apoptotic action by activating nuclear factor (NF)-κB, AP-1, and other transcription factor pathways. This explains why TNF-induced apoptosis using *in vitro* systems often requires the inhibition of RNA or protein synthesis. TNF-binding TNF-R1 does not bind FADD directly to activate caspase 8/10 cleavage, in contrast to Fas and the TRAIL death receptors. Instead, TNF-R1 binds to the DD-containing adapter protein TNFR-associated DD (TRADD) *(33)*. The DD of TRADD binds other DD-containing proteins, including FADD and receptor-interacting protein (RIP). TRADD can also recruit one of two TRAF proteins (TNFR-associated factor), TRAF2 and TRAF5 *(34,35)*. RIP is essential for TNF-induced NF-KB activation *(36)*. In unstimulated cells, NF-κB is held in the cytoplasm by the inhibitor of NF-κB, IκB. TNF activates NF-κB by initiating ubiquitin-mediated degradation of IκB. Phosphorylation of IκB dissociates it from NF-κB, releasing it for entry into the nucleus and initiating transcription of a large number of mostly anti-apoptotic, pro-survival genes. These include cellular-inhibitors of apoptosis (c-IAP1 and c-IAP2), FLIP, Bfl-1/A1, A20, Mn super oxide dismutase (MnSOD), and others (for a review see *37*). IκB is phosphorylated by the IκB kinase (IKK) complex. IKK activity can be purified as a complex containing two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, NF-κB essential modifier (NEMO; IKKγ). IKKβ is necessary and sufficient for phosphorylation of IκBα and IκBβ. Studies with TRAF2- and RIP-deficient murine embryo fibroblasts (MEFs) showed that both molecules are independently recruited to TNF-R1 *(38)*. It also appears that TRAF2 is sufficient to recruit the IKK complex to TNF-R1, but RIP is necessary for the activation of the IKKs *(38)*. After phosphorylation by IKKs, IκB proteins are ubiquitinated by members of the Skp1, Cullin, and F box proteins (SCF) family of ubiquitin ligases. The liberated NF-κB dimers translocate to the nucleus where they bind DNA. Activated NF-κB is then down-regulated by multiple pathways, including a negative feedback loop where newly synthesized IκBα binds to nuclear NF-κB and exports it to the cytosol. TNF can also activate other transcription

factors through c-Jun NH_2-terminal kinase (JNK) and p38/mitogen-activated protein kinase (MAPK). TRAF2 stimulates JNK through the MAPK kinase MKK7, promoting phosphorylation of c-Jun thereby increasing AP-1 activity.

This pathway of NF-κB activation is referred to as the classical, or canonical, pathway but is one of two major pathways that activate NF-κB. The non-canonical, or alternative, pathway results in the specific activation of two of the five NF-κB subunits, p52 and RelB. The other subunits are p50, RelA, and RelC, and these form heterodimers that are transcriptionally active. Unlike the classical pathway, the alternative pathway is based on IKKα homodimers that prefer the precursor of p52, p100 *(39)*. IKKα binds RelB and sequesters it in the cytoplasm; activation of IKKα results in the degradation of the carboxy-terminus of RelB and nuclear translocation of p52/RelB dimers. The alternative pathway is activated mainly by cytokines involved in development and maintenance of secondary lymphoid organs *(40)*. Another pathway of NF-κB activation is independent of IKK, and receptor signaling, and is instead based on activation of casein kinase 2 (CK2). CK2 activation can induce IκBα degradation through its phosphorylation *(41)*. This pathway only has a minor role in physiologic NF-κB activation.

TNF can also activate caspase-mediated apoptosis, but it appears that the NF-κB pathway must be disabled for this to occur *(42)*. *In vivo* TNF-induced apoptosis has a minor role in comparison with its overwhelming function in regulating inflammation. TNF-mediated caspase activation occurs when TRADD binds FADD through a DD interaction. Recent evidence shows that FADD only associates with the TNF-R1 complex after it has been internalized by endocytosis *(43,44)*. FADD-bound TRADD recruits caspase-8 and self-activates if not inhibited by NF-κB-induced anti-apoptotic proteins. The NF-κB targets, c-FLIP, TRAF1, and c-IAP1, have all been found to co-immunoprecipitate with a TNF-R1/TRADD/FADD cytosolic complex. These anti-apoptotic factors could help to dampen an apoptotic response. Therefore, apoptotic signaling through TNF-R1 includes an NF-κB-mediated rescue response that results in cell death if newly synthesized survival signals fail to be activated.

1.4. Fas Ligand

Fas plays a major role in the regulation of apoptosis of immune cells and has been implicated in immune system diseases and cancer *(45,46)*. Fas–FasL interactions are important for regulating the immune system in several ways: Fas is involved in cytotoxic T-cell-mediated killing, destruction of inflammatory and immune cells in immune-privileged sites, and deletion of self-reacting B cells and activated T cells at the end of an immune response. Dysregulation of Fas or FasL expression is associated with several disease states. Elevated serum levels of FasL have been seen in patients with NK-cell large granular lymphocyte leukemia, systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, lymphohistiocytosis, myocarditis, and acute graft-versus-host disease *(45,46)*. Some tumors have been reported to express FasL, which may be a mechanism they developed to evade attacking lymphocytes *(47)*.

Fas-mediated cell death was identified indirectly by the generation of monoclonal antibodies that recognized cell surface antigens on a malignant human lymphoblast cell line *(48)*. Once cloned, Fas was found to map to the chromosomal location of a mouse lymphoproliferative disorder known as *lpr (49)*. A point mutation near the extracellular carboxyl domain of FasL gives rise to the *gld* phenotype *(50)*. Both *lpr* and *gld* mice fail

to delete excess lymphocytes and display a lymphoproliferative phenotype including lymphadenopathy and splenomegaly. One other receptor, decoy receptor 3 (DcR3), binds to FasL. DcR3 lacks an apparent transmembrane sequence and appears to be secreted. DcR3 was found genetically amplified in several human cancers including lung and colon carcinomas and is overexpressed in several adenocarcinomas, glioma cell lines, and glioblastomas *(51–53)*. There is preliminary data suggesting that serum DcR3 level might be a useful predictive marker for cancer diagnosis *(54)*.

1.5. TRAIL

TRAIL was identified in silico using TNF sequence homology searches of the human genome database of expressed sequence tags *(55,56)*. TRAIL is unique among the TNF superfamily, and most other cytokines, because it can bind five different receptors *(57,58)*. Two of these receptors contain DD and are pro-apoptotic. They are type 1 transmembrane receptors and referred to herein as DR4 (death receptor 4/TRAIL R1) and DR5 (death receptor 5/KILLER/TRICK2/TRAIL R2). The three other TRAIL receptors lack DDs, including one that binds TRAIL very weakly at physiologic temperatures (osteoprotegerin/OPG). The remaining two receptors are also called "decoys" because they bind TRAIL with high affinity but cannot transduce the death signal. Decoy receptor 1 (DcR1/TRID/TRAIL-R3) lacks an intracellular domain (it is attached to the plasma membrane by a glycophosphatidylinositol anchor), and decoy receptor 2 (DcR2/TRUNDD/TRAIL R4) has a truncated DD in its cytoplasmic tail.

If bound to either DR4 or DR5, TRAIL and FasL exert their actions in an analogous manner. Binding of TRAIL triggers DISC formation, caspase 8/10 activation, and rapid apoptosis in sensitive cells. Similarly to Fas, apoptosis triggered by TRAIL can engage the mitochondrial pathway in type II cells or independently of the mitochondria in type I cells *(59)*. The apoptotic signaling pathway downstream of FasL and TRAIL acts through FADD and appears to be very similar. However, one significant difference between TRAIL and FasL is their potential as chemotherapeutic agents. When administered systemically in mice, FasL induces a rapid cytotoxic effect in hepatocytes whereas TRAIL appears relatively non-toxic *(60,61)*. This observation, plus TRAIL's dramatic ability to kill cancer cells while leaving normal cells unharmed, opened up an exciting new opportunity for development of a "silver bullet" for cancer therapy. TRAIL and DR4- and DR5-activating antibodies recently have entered into clinical trails.

Several mechanisms have been proposed to explain why some cancer cells are highly sensitive to TRAIL-induced death. An attractive hypothesis is differential expression of the decoy and death receptors. However, the decoy receptors do not consistently appear highly expressed in normal cells or to be absent in cancer cells *(62,63)*. Mutant death receptors or defective receptor processing has been observed in TRAIL-resistant cancer cells *(64,65)*. Additional intracellular factors leading to TRAIL resistance affect the caspase 8/c-FLIP ratio, such as loss of caspase 8 and caspase 10 because of mutations or gene methylation *(66)*, caspase-associated ring protein (CARP)-dependent degradation of caspase 8 *(67)*, or high c-FLIP expression levels *(62,63)*. Up-regulation of FLIP was detected in many tumors *(68–70)*, and expression of FLIP in transgenic mice results in escape from T-cell immune surveillance and subsequent tumor growth *(71,72)*. Further downstream in the TRAIL apoptotic pathway, Bax mutations or increased expression of IAP family members, in particular XIAP and survivin, can also cause resistance.

Recently, the human oncogene c-Myc was identified as a bio-marker for TRAIL sensitivity *(73)*. In this study, a panel of human tumor cell lines was examined, and a direct linear correlation was observed between TRAIL sensitivity and high c-Myc expression. Myc was found to bind the FLIP promoter and repress FLIP transcription *(73)*. Furthermore, Myc was isolated from an siRNA screen to identify modulators of TRAIL sensitivity *(74)*. A separate study showed Myc up-regulation of DR5 expression *(75)*, but how Myc regulates DR5 is not clear. Other oncogenic proteins can sensitize cells to TRAIL, including E1A *(76)* and oncogenic Ras *(77,78)*, but Ras-mediated TRAIL sensitization has not been observed consistently *(75)*.

2. ROLE OF THE EXTRINSIC PATHWAY IN DISEASE

The TNF family plays important functions in innate and adaptive immunity and directly activates pathways leading to cell survival, proliferation, differentiation, and death. Dysregulation of the TNF family members that can elicit apoptosis results in diseases of the immune system, neurodegenerative disorders, and cancer. Two known genetic diseases that are associated with defects in the extrinsic pathway include Fas-linked autoimmune lymphoproliferative syndrome (ALPS) and TNF-R1-associated periodic syndrome (TRAPS) *(8)*. Fas-mediated apoptosis is required for normal lymphocyte homeostasis and peripheral immune tolerance *(79)*. In Fas-deficient *lpr* mice and in patients with heterozygous dominant-interfering defective Fas alleles (encoding defective Fas proteins that complex with normal Fas), abnormal accumulation of lymphocytes often results in systemic autoimmunity *(79,80)*. Afflicted individuals develop pathogenic autoantibodies—frequently against hematopoietic cells—that cause hemolytic anemia, thrombocytopenia, or neutropenia *(81)*. Mouse knockout studies show the TRAIL pathway may also be involved in autoimmune disease *(82)*. In TRAPS, heterozygous dominant alleles of defective TNF-R1 appear to enhance the pro-inflammatory effects of TNF. This may be due in part to a decrease in TNF-R1 shedding *(83)*.

TNF and TNFR families are being targeted for therapies against a wide range of human diseases such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer. For example, pharmaceuticals to inhibit TNF have been developed to control previously recalcitrant inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease *(84,85)*. Specific TNF antagonists include the TNF antibody infliximab (Remicade) and the TNFR–immunoglobulin G (IgG) fusion protein etanercept (Enbrel) *(86)*.

What are the physiological functions of apoptosis-inducing TNF family members? The use of inhibitory antibodies and mouse knockout models has provided great insight into this question (Table 1).

2.1. Lessons from Knockout Animals

2.1.1. TNF as a Tumor Promoter

Mouse TNF and TNF-R1 knockout studies show that TNF plays an essential role in protecting against infection by pathogenic organisms. There is also growing evidence that TNF signaling is involved in fostering tumor growth. Expression studies show abnormally high concentrations of TNF in tumors. Studies of various hematopoietic and solid tumor types found an association between TNF expression, poor survival, and

Table 1
Major extrinsic pathway proteins and their official names according to the HUGO Gene Nomenclature Committee (HGNC) are shown. Knock-out mice generated for these proteins are described.

Molecular pathway molecules	*Official nomenclature (HGNC)*	*Human chromosome*	*Mouse knockout phenotype*
Death ligand			
TNF (TNF-α, Cachectin)	TNFSF2	6p21.3	Viable, highly susceptible to challenge with an infectious agent and resistant to lipopolysaccharide (LPS)-induced death following D-galactosamine treatment *(188)*
FasL (CD95)	TNFSF6	1q23	Viable but early death (50% at 4 months); FasL($^{-/-}$) mice exhibit splenomegaly and lymphadenopathy associated with lymphocytic infiltration into multiple organs and autoimmune disease *(189)*; *Gld* mice carry mutations in FasL and suffer from autoimmune disease *(50)*
TRAIL (Apo2L)	TNFSF10	3q26	Viable; susceptible to induced and spontaneous tumorigenesis *(87)*
Death receptor			
TNF-R1	TNFRSF1A	12p13.2	Viable, resistant to LPS-induced death following D-galactosamine *(190)*
Fas (CD95, Apo1)	TNFRSF6	10q24.1	No Fas$^{-/-}$ mouse published; mice carrying the lymphoproliferation *(lpr)* mutation have defects in the Fas antigen gene. The *lpr* mice develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune disease, indicating an important role for Fas antigen in the negative selection of autoreactive T cells in the thymus *(191)*
DR4 (TRAILR1, APO2)	TNFRSF10A	8p21	
DR5 (TRAILR2, KILLER, TRICK2)	TNFRSF10B	8p22–p21	Viable, but has an enlarged thymus, defective apoptotic response to ionizing radiation *(88)*
Adapter proteins			
FADD (MORT)	FADD	11q13.3	Embryonic lethal (d11.5); mice show signs of cardiac failure and abdominal hemorrhage *(89)*

(*Continued*)

Table 1
(Continued)

Molecular pathway molecules	*Official nomenclature (HGNC)*	*Human chromosome*	*Mouse knockout phenotype*
TRADD	TRADD	16q22	
Caspase 8 (FLICE, MACH, MCH5)	CASP8	2q33–q34	Embryonic lethal (d13.5); embryos exhibit impaired heart muscle development and accumulation of erythrocytes *(90)*
Caspase 10 (MCH4)	CASP10	2q33–q34	
FLIP (I-FLICE, CASPER, FLAME1, CASH, CLARP, MRIT)	CFLAR	2q33-q34	Embryonic lethal (d10.5); exhibit impaired heart development *(192)*
RIP (RIP1)	RIPK1	6p25.2	Viable, but fails to thrive; displays extensive apoptosis in both the lymphoid and adipose tissues and dies at 1–3 days of age *(193)*
TRAF2 (TRAP)	TRAF2	9q34	Traf2$^{-/-}$ mice appear normal at birth but die prematurely; atrophy of the thymus and spleen and depletion of B-cell precursors are observed *(35)*
TRAF5	TRAF5	1q32	CD27- and CD40-mediated lymphocyte activation is substantially impaired in *traf5*$^{-/-}$ lymphocytes *(194)*

resistance to therapy *(91–94)*. TNFR1$^{-/-}$ mice show reduced levels of metastatic lung disease following intravenous tumor cell injection *(95)* and reduced liver metastases following intrasplenic tumor cell injection *(96)*. TNF itself has been proposed as a tumor promoter. Using a standard mouse model of two-step chemical carcinogenesis, it was found that TNF$^{-/-}$ mice developed 10 times fewer skin tumors than wild-type mice *(97)*. Similar results indicating a role for TNF in tumor promotion were seen by using a model of hepatic carcinogenesis in TNF-R1$^{-/-}$ mice and was unaffected in TNF-R2$^{-/-}$ mice *(98)*.

Recent work by Karin and colleagues showed that TNF mediates an inflammatory response by LPS, causing metastatic growth to the lung of intravenously injected colon adenocarcinoma cells *(99)*. Inhibiting NF-κB signaling in the colon cells prevented their metastasis and resulted in their apoptosis. TRAIL expression by mouse lung tissue following LPS administration mediated the death of NF-κB-deficient cancer cells. DR5 was also found up-regulated in tumors following LPS treatment but only in cells that were NF-κB deficient. These results shed light on the interplay between TNF and

TRAIL signaling and show that if NF-κB is blocked, TNF-mediated growth can be converted into TRAIL-mediated death.

2.1.2. FasL and Negative Selection

Higher organisms have developed several mechanisms to eliminate unwanted cells rapidly, and Fas is an important mediator of this process. FasL expression at sites of immune privilege identified an important role for FasL in the interaction between non-lymphoid tissues and the immune system. The concept of "tumor counterattack" has been proposed to explain the observation that Fas is down-regulated and FasL up-regulated in the same tumor cells *(100)*. According to this concept, "immune cells are unable to destroy tumor cells since they are attacked by the FasL expressing cells." In light of this observation, significant evidence has accumulated that indicates Fas may play a major role in immune suppression of tumorigenesis. FasL expression has been reported on numerous tumors of varying origin, including colon, gastric, lung, and astrocytoma *(47)*. Disease progression is associated with increasing levels of FasL *(101, 102)*, and FasL expression has also been found higher in some metastatic tumors than in primary ones *(103,104)*. Animal studies have corroborated the FasL counterattack theory. For example, subcutaneous injection of FasL-expressing murine melanoma cells into Fas-deficient *lpr* mutant mice resulted in delayed tumor growth compared with that in wild-type mice *(105)*. However, conflicting results cast doubt on the Fas counterattack theory, instead suggesting that FasL can also play a growth stimulatory role *(47,106)*. These observations, plus those suggesting that FasL may have stimulatory effects, including induced motility of tumor cells *(107)* and growth signaling *(108)* suggest that the initial theory of tumor counterattack may be oversimplified and that FasL does more than trigger apoptosis.

2.1.3. TRAIL and Tumor Surveillance

Recent evidence from mouse knockout studies, plus TRAIL's ability to activate apoptosis in various cancer cells, led to the hypothesis that a principal function of TRAIL is to kill transformed cells. Unlike FasL, TRAIL is not expressed at detectable levels on the surfaces of T cells, NKT cells, B cells, dendritic cells, monocytes, or most NK cells *(109)*. The one exception is mouse liver NK cells, which constitutively express TRAIL in an interferon (IFN)-dependent manner *(110,111)*. Cell surface TRAIL expression increases through several pathways. Antigen-dependent activation of $CD4^+$ and $CD8^+$ T cells from peripheral blood induces expression of TRAIL mRNA, as does stimulation of macrophages with IFN-γ. The expression of TRAIL on liver NK cells and their anti-metastatic potential depend on the presence of IFN-γ and interleukin (IL)-12, as these effects were not observed in mice deficient in IFN-γ *(87,111)*. Tumor cells from cancer patients activate macrophages to produce TRAIL, and these macrophages can release molecules that induce increased expression of DR4 and DR5 on tumor cells *(112)*.

Studies with TRAIL knockout mice confirm a role for TRAIL in anti-tumor immune surveillance by NK cells, specifically in host defense against tumor initiation and metastasis *(87,111)*. $TRAIL^{-/-}$ mice were more susceptible to experimental and spontaneous tumor metastasis and were also more likely to form tumors following exposure to the chemical carcinogen methylcholanthrene *(87)*. There are two knockout mice for the receptor for TRAIL. Mice have only one receptor for TRAIL, mouse KILLER,

and it shares its highest homology with human DR5 *(113)*. Two knockout models for the TRAIL receptor have been generated and both are viable *(88,114)*. Both develop normally, but one mouse strain has an enlarged thymus *(88)*. This study showed that DR5 has a limited role during embryogenesis and early stages of development, but plays an organ-specific role in response to DNA-damage. When exposed to ionizing radiation, certain $DR5^{-/-}$ tissues show reduced apoptosis, including the thymus and spleen. Mice wild-type for DR5 show a selective increase in DR5 expression following IR in the thymus and spleen *(115)*, suggesting a significant connection between IR-induced DR5 and resulting apoptosis in these tissues.

Genetic defects in TRAIL signaling have not been strongly correlated with disease states in humans. However, deletions and mutations of DR4 and DR5 have been observed in some human tumors *(63,65,116–118)*.

An intriguing study has implicated TRAIL in mammary tissue lumen formation and suggests that dysregulation of TRAIL signaling may be a hallmark of early breast cancer lesions *(119)*. Using an *in vitro* cell culture model of 3D acinar-like structures using immortalized mammary epithelial cells, TRAIL was found to partially mediate both the apoptotic and the autophagic cell death associated with lumen formation. Autophagy is a cellular process where a multi-membrane vacuole containing cytoplasmic contents fuses with the lysosome. This results in degradation of the vacuole contents by lysosomal enzymes into recyclable macromolecules *(120)*. Recent evidence indicates that autophagy can mediate a form of programmed cell death, where there is an accumulation of vacuoles resulting in massive organelle degradation. How TRAIL is activated during mammary acinar morphogenesis is not known, but this is the first report connecting autophagy with TRAIL function *(119)*. Little is known about how and to what degree the extrinsic pathway signaling affects autophagy, but there are reports that TNF signaling can induce autophagy and possibly contribute to TNF-induced apoptosis *(121,122)*.

3. ROLE OF EXTRINSIC PATHWAY IN CHEMOTHERAPY AND RADIOSENSITIVITY

Chemotherapy and radiation, when used successfully, act to inhibit tumor growth. Ionizing radiation and DNA-damaging chemotherapeutics can elicit an apoptotic response that is principally mediated through activation of the p53 tumor suppressor protein. p53 is the most commonly mutated protein found in human cancers and is a potent transcriptional activator of genes that play principal roles in cell-cycle arrest and apoptosis *(123)*. Recent evidence suggests that p53 also influences apoptosis by directly interacting with members of the Bcl-2 family *(124)*. Members of the Bcl-2 family that p53 can activate transcriptionally include Bax, Puma, Noxa, Bnip3L, Bak, and Bid. p53 also directly contributes to activation of the extrinsic pathway. Death receptors for both TRAIL and FasL have been identified as p53 target genes *(113,125)*.

KILLER/DR5 was originally discovered as a DNA-damage-inducible p53 target gene *(113)* and is transcriptionally activated by p53 *(126)*. Certain tissues, including the spleen, small intestine, and thymus, show large increases in DR5 expression following ionizing radiation that is dependent on transcriptionally active p53 *(88,115,127)*. DR4 may also be regulated by p53 in a limited number of cell lines *(128)*. Several studies have found a p53-dependent increase in Fas or FasL, which contributes to

mediating the apoptotic response after conventional chemotherapy *(125,129–131)*, but Fas is not essential for mediating p53's effects. Lymphocytes from *lpr* mice, or those expressing DN-FADD, are equally sensitive to chemotherapy and ionizing radiation; p53 deficiency or constitutive expression of Bcl-2 markedly increased the resistance of lymphocytes to gamma radiation or anticancer drugs, but lymphocytes were still sensitive to killing by FasL *(132)*. Furthermore, apoptosis induced by chemotherapeutic drugs is not altered in embryonic fibroblasts from FADD and caspase 8 knockout mice *(89,90)*, indicating only a partial role for the death receptor pathway in response to chemotherapeutic agents. Nevertheless, partial resistance of DR5-null tissues to ionizing radiation implicates the extrinsic pathway in DNA-damage-induced apoptosis.

4. EXPLOITING THE EXTRINSIC PATHWAY FOR CHEMOTHERAPY-INDUCED KILLING

Ever since the discovery of TNF, great attention has been focused on the TNF ligands as mediators of cancer cell death *(133–135)*. Through the efforts of many scientists over the course of decades, clear pictures are emerging of the basic mechanisms of extrinsic pathway-signaling events. Understanding these events has led to exciting advances in using extrinsic pathway signaling for cancer therapy.

4.1. TRAIL

Despite the ability of TNF and FasL to induce apoptosis in cancer cells, severe toxic side effects preclude both ligands from use in systemic anticancer therapy. Systemic administration of TNF caused an inflammatory response resembling septic shock in humans *(136)*. FasL or agonistic anti-Fas antibody caused lethal liver injuries in preclinical models *(137)*. By contrast, recombinant human TRAIL showed no toxicity when systemically administered in rodents and non-human primates *(60,61,138)*. Recombinant human TRAIL has apoptosis-inducing capacity in various tumor cells in culture and in tumor implants in severe combined immune deficiency (SCID) mice *(139)*. Recombinant TRAIL (Genentech/Amgen) and activating DR4 and DR5 antibodies (Human Genome Sciences/Cambridge Antibody Technology) are currently under clinical investigation. Getting TRAIL into clinic trials was delayed by observations that certain preparations of recombinant human TRAIL had selective toxicity toward normal human hepatocytes *(140)*. Because TRAIL was toxic to cultured human hepatocytes, and not to mouse or non-human primates, it resulted in the careful analysis of different TRAIL preparations. It became apparent that TRAIL protein fused with non-physiological amino acid tags or with preparations of native TRAIL using different stabilizing chemicals resulted in multimerized, highly potent versions of TRAIL *(141–143)*. Therefore, the potential toxicity of these TRAIL versions toward normal cells can be avoided if native TRAIL is properly prepared, or if activating monoclonal antibodies specific to DR4 or DR5 are used *(139,144,145)*. Another approach to minimizing off-target TRAIL toxicity is the combination of TRAIL with an inhibitor of caspase 9, which can protect normal cells but is ineffective in protection tumor cells possessing a type I signaling mechanism *(143,146)*.

Like most normal cells, many cancer cells are resistant to TRAIL-induced apoptosis. However, many conventional and novel chemotherapies can act synergistically when combined with TRAIL. Chemotherapy or irradiation sensitized resistant cells to TRAIL

in vitro and *in vivo (63,147–150)*. Many cytotoxic chemotherapeutic agents result in DNA damage or other cellular stress that causes stabilization of the p53 tumor suppressor protein. p53 transcriptionally activates DR5 and other pro-apoptotic proteins that enhance the TRAIL signal *(113)*. Therefore, combining TRAIL with such agents should prove to be a useful therapeutic strategy in tumors harboring functional p53. However, tumor progression and resistance to chemotherapies occur because tumors select for cells defective in p53 signaling. An exciting facet of death receptor signaling is that it can occur in the absence of functional p53. Inhibitors of histone deacetylases (HDACIs) can induce apoptosis in cancer cells and are currently in clinical trials. One action of HDACIs is the increased expression of TRAIL, DR5, Fas, and FasL in leukemic cells, resulting in selective apoptosis of these cells *(151,152)*. HDACIs enhance synthesis of several proteins involved in TRAIL signaling including DR5 and when combined with TRAIL show the ability to sensitize TRAIL-resistant cells *(153,154)*. Both glucocorticoids and IFN-γ also increase DR5 expression, which may enhance TRAIL activity *(155)*. These are some of the many strategies being approached to combine TRAIL with novel agents that target proteins in both the extrinsic and intrinsic pathways, thereby increasing their sensitivity to the killing potential of TRAIL. Promising compounds identified that have been combined with TRAIL are discussed below.

4.2. TNF

Recombinant TNF was approved for isolated limb perfusion therapy against sarcomas in Europe in 1998 *(156)*. TNF combined with chemotherapeutic agents such as melphalan shows specificity toward destruction of tumor vasculature and is very effective when used for localized treatment of sarcomas and melanomas *(157,158)*. TNF plus melphalan is awaiting approval following phase III clinical trials for use in the US.

*4.3. NF-*κB

Substantial evidence indicates that NF-κB regulates oncogenesis and tumor progression. Many anticancer agents induce NF-κB nuclear translocation and activation of their target genes, which impinge on cellular resistance to anticancer agents. TNF is up-regulated by some chemotherapeutic agents, thus activating NF-κB. FasL and TRAIL also can activate NF-κB signaling *(107,159)*. Several strategies have been investigated to block pro-survival death receptor signals so that extrinsic apoptotic signals can dominate.

NF-κB inhibitors have been identified that enhance the cytotoxic effects of many conventional chemotherapies and novel anticancer agents *(160)*. Inhibiting the proteosome is an approach taken to block NF-κB activation through degradation of IκB proteins. The proteosome inhibitor Bortezomib (Velcade; PS-341) has recently been approved for treating multiple myeloma and is in clinical trials testing effectiveness against several other cancer types *(161,162)*. Bortezomib also has been effectively combined with many conventional chemotherapeutic agents and radiation (for a recent review see *163*). Proteosome inhibition results in stabilization of several critical regulators of apoptosis, including p53, Bid, and Bax; therefore, the effectiveness of Bortezomib may depend only partially on inhibiting NF-κB.

Strategies have been developed to inhibit NF-κB directly. Two compounds were identified, BAY 11-7082 and BAY 11-7085, that block IκBα phosphorylation and prevent its degradation *(164)*. Bay 11-7082 was used to enhance mitochondria dysfunction induced by UCN-01, a cell-cycle checkpoint-abrogating agent *(165)*. A recent study showed that rituximab (Rituxin), the anti-CD20 antibody approved for treatment of non-Hodgkin's lymphoma, can inhibit IKK activity and block constitutive NF-κB signaling *(166)*. Non-steroidal anti-inflammatory drugs (NSAIDs), including Cox-2 inhibitors, have been identified as inducing regression of adenomatous polyps of the colon, and NF-κB has been implicated in mediating NSAID action. Aspirin and sulindac have both been shown to inhibit IKK activity and may prove useful in targeting NF-κB *(167,168)*. Many other IKK inhibitors have been identified, but further studies are necessary to determine whether they will be clinically useful *(160,169)*.

4.4. FLIP

Whether a tumor cell is sensitive to death ligand-induced apoptosis depends on both receptor cell surface expression and an intact downstream-signaling pathway. FLIP is an important regulator of the death signal, and a compound was recently discovered that reduces FLIP expression. The synthetic oleanane triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid (CDDO) was reported to have potent differentiating, anti-proliferative, and anti-inflammatory properties and reduce tumor growth in vivo *(170,171)*. CDDO was initially constructed to mimic naturally occurring inhibitors of nitric oxide production induced by IFN-γ. The mechanism of action of CDDO and its imidizol derivative (CDDO-Im) are not fully understood, but CDDO was found to induce apoptosis involving caspase 8 cleavage *(172)*. Later, it was observed that CDDO activates a pathway resulting in FLIP degradation *(173,174)*. CDDO or CDDO-Im can cause apoptosis and cell death in a number of different human cancers, but it has shown potent synergy when used in combination with TNF or TRAIL *(175–177)*. *In vivo* studies using nude mice bearing human breast cancer MDA-MB-435 xenografts showed CDDO-induced tumor growth arrest by using daily treatments for 25 days *(178)*. A later study did not recapitulate tumor growth arrest using CDDO-Im in nude mice bearing MDA-MB-468 breast cancer xenografts but restricted tumor growth when combined with TRAIL *(179)*.

4.5. Recruiting the Intrinsic Pathway to Sensitize Cells to the Extrinsic Pathway

Because the extrinsic pathway is linked to intrinsic apoptotic signaling, combining extrinsic and intrinsic pathway activators should elicit a "double whammy." For example, TRAIL has been combined with several agents identified or specifically designed to target intrinsic signaling.

The Bcl-2 family is the major mediator of outer mitochondrial membrane depermeabilization resulting in the release of pro-apoptotic factors, such as cytochrome c, Smac/DIABLO, and Omi/Htr2A *(180)*. Overexpression of anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-Xl, A1, or Mcl-1, is frequently observed in many tumor types and contributes to chemotherapeutic resistance. Several strategies are under investigation to target these anti-apoptotic proteins. These include (i) interfering oligonucleotides to down-regulate expression; (ii) use of BH3-only peptides or controlled Bax expression

to abrogate protection; and (iii) small molecules that can inhibit protective interactions. The only agent of these categories that is currently in clinical trials are nuclease-resistant antisense oligonucleotides targeting Bcl-2 mRNA (G3139). G3139 (Genasense) is in phase II and III clinical trials treating a wide variety of adult and childhood tumors *(181)*. However, G3139 was not approved for treatment of melanoma because results from phase III trials showed it did not extend survival *(182)*. There are no reports investigating G3139 in combination with TRAIL pre-clinically, but it was shown to sensitize Fas- and IFN-γ-resistant renal cancer cell line to IFN-γ combined with an Fas-activating antibody *(183)*. Adenoviruses that express Bax and TRAIL under control of the promoter for the human telomerase protein subunit (hTERT) were used to treat nude mice bearing ovarian tumor xenografts *(184)*. Though preliminary, these strategies (or agents) diminished tumor growth while maintaining relatively low toxicity.

Anti-apoptotic Bcl-2 family members are held in check by Bcl-2 family proteins that contain only the BH3 member of the four Bcl-2 homology domains, the so-called BH3-only proteins. A novel approach was taken to generate stablilized BH3 peptides termed SAHBs (stabilized α-helix of Bcl-2 domains) *(185)*. These peptides proved to be protease-resistant and cell-permeable molecules that bound with high affinity to multidomain BCL-2 member pockets. A SAHB of the BH3 domain from BID was effective in inhibiting growth of human leukemia xenografts *in vivo* in short-term assays. A small molecule BH3 mimetic, ABT-737, shows promise in the treatment of Bcl-2- or Bcl-XL-overexpressing tumors *(186)*. ABT-737 was identified using a structure-based combinatorial chemical approach to target Bcl-XL and binds Bcl-XL, Bcl-2, and Bcl-w with high affinity *(186)*. ABT-737 synergized with paclitaxel and the activated BH3-only protein Myr-Bid to cause apoptotic cell death. Because overexpression of Bcl-2 and Bcl-XL is the key to many cancers' resistance to apoptotic stimuli, Bid SAHBs or ABT-737 will very likely synergize with other chemotherapeutic agents, including TRAIL and other extrinsic pathway activators. Whether Mcl-1 overexpression will mediate resistance of tumors remains to be determined in clinical trials.

Other classes of apoptotic targeting agents have also been combined with TRAIL resulting in significant tumor regression. A small molecule SMAC mimic potentiates TNF- and TRAIL-induced death *(187)*. Treatment of glioblastoma cells with a combination of TRAIL and the SMAC mimic resulted in apoptosis of tumor cells, but normal cells were not harmed. Data from animal studies have not been published yet.

5. CONCLUDING REMARKS

Despite being investigated for decades, the TNF and the TNFR family of proteins continue to provide important insights into human health and disease. Here, we focused on the current understanding of their role in activating the extrinsic pathway of apoptosis, how this affects oncogenesis, and how this knowledge can be used for targeted chemotherapeutic design. Several promising cancer-killing agents that engage the extrinsic signaling pathway are in clinical trials, and several more appear promising in preclinical studies. The next stage of clinical research must include rational combination of chemotherapeutic agents that both activate apoptotic signaling pathways and block pro-survival mechanisms, while minimizing off-target toxicities. A major challenge to be overcome is determining whether a patient will respond to agents that activate the extrinsic or intrinsic pathways of apoptosis prior to their treatment.

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3 Evaluating the Importance of Apoptosis and Other Determinants of Cell Death and Survival

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Summary

Apoptosis, the cell's intrinsic death program, has emerged as a key regulator in physiological growth control and regulation of tissue homeostasis. Research into the underlying mechanistic basis and its importance in disease and treatment has progressed tremendously. However, it is important to note that apoptosis is only one form of cell death and that we should not overlook the importance of other death mechanisms. The particular death pathway that is the most important determinant for cancer therapy is not necessarily that which has the fastest kinetics, as is the bias in many laboratories, but rather that which displays the most sensitive dose–response relationship. In this chapter, we argue that in many situations apoptosis is not the most important determinant of treatment response. Furthermore, we provide a summary of the different forms of cell death and discuss their contribution to cancer development and treatment response.

Key Words: Apoptosis; clonogenic survival; senescence; cancer therapy.

1. INTRODUCTION

The realization that cell death often occurs as part of an active, programmed process has significantly altered our understanding of both cancer development and cancer treatment. Cell death is now often considered as a "choice" made by a cell that has become irreparably damaged or that has become potentially dangerous because of the activation of a growth-promoting oncogene. Apoptosis, a well-described form of programmed cell death characterized by the activation of caspases *(1)*, is often used synonymously with cell death itself. This is understandable, as this form of cell death results in rapid and normally complete destruction and removal of the cell. However,

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when we consider cell death in the context of both the development and the treatment of cancer, it is perhaps more useful to expand the definition to include other processes that lead to the inactivation of the proliferative or "clonogenic" capacity of the cell. These other processes include senescence, mitotic catastrophe, autophagy, and necrosis *(2)*.

It has become clear that apoptosis represents a powerful and important cellular defense against cancer development. Correspondingly, loss of apoptotic sensitivity is considered one of the six hallmarks of cancer *(3)*. The propensity of individual cells to undergo apoptosis is markedly influenced both positively and negatively by a variety of different genes, many of which become mutated and/or deregulated in human cancers *(2)*. Furthermore, apoptosis can be highly influenced by the local microenvironment of a tumor, which is characterized by extreme heterogeneity in oxygenation and nutrient supply *(4)*. Consequently, the cellular apoptotic sensitivity can vary remarkably among different tumors and even within the same tumor. As anticancer agents, including the most commonly used chemotherapy agents and radiation, are capable of activating apoptosis, it has become widely assumed that apoptotic sensitivity is also an important contributor to overall treatment sensitivity *(5,6)*. This has led to the search for novel agents capable of inducing apoptosis in human cancers and also for mechanisms of increasing the apoptotic sensitivity of tumor cells. However, as already eluded to above, apoptosis represents only one form of cell death that can contribute to the inactivation of tumor cells. The importance of other non-apoptotic pathways are frequently overlooked with respect to their role in preventing cancer and, even more so, in determining treatment sensitivity. In fact, several critical analyses of the literature have concluded that apoptosis and the genes controlling it, such as p53, are not major contributors to treatment response *(7–19)*. How then has the widely held view, that apoptosis is a major determinant of treatment sensitivity, become so persuasive? In part, this may be due to the difficult or in some cases impossible task of separating treatment response from patient prognosis. Apoptosis, and the genes controlling it, may alter several other tumor parameters that are important for patient prognosis but unrelated to cellular treatment sensitivity. These parameters include the rate of tumor growth, invasiveness, and metastasis. A second and perhaps even more important reason results from the fact that many laboratory experiments fail to consider all the potentially relevant forms of cell death *(2,8,20,21)*. Each process of cell death is activated with different kinetics and with a different dose-response following treatment. The most important form of cell death is that which displays the most sensitive dose–response relationship and not necessarily that which occurs with the fastest kinetics. Many laboratory assays are biased to detect apoptosis or other forms of rapid cell death. This can lead to dramatic misinterpretations of cellular sensitivity to treatment.

2. FORMS OF CELL DEATH

Understanding and evaluating the contribution of different forms of cell death to cancer development and treatment requires consideration of their individual mechanisms of action and their genetic determinants. For the purposes here, we discuss each of these briefly.

2.1. Apoptosis

The most well-defined form of cell death is apoptosis, an active, complex, and highly regulated process initiated within the cell in response to either an intracellular or

an extracellular cue. Apoptosis is an essential and normal part of many physiological processes including embryonic development, the immune system, and maintenance of tissue homeostasis *(22)*. Consequently, alterations in the control of apoptosis lead to several human disorders including cancer and autoimmune disease (lower apoptosis) or neurodegenerative disease or immunodeficiency (higher apoptosis). Apoptotic cell death is characterized by the sequential activation of several different cysteine proteases known as caspases *(23)*. These proteins are initially expressed in an inactive procaspase form *(24)* and are additionally kept in check by a family of inhibitor of apoptosis (IAP) proteins *(25)*. Apoptosis begins following the activation of an "initiator" caspase such as caspase 8 or 9. These caspases subsequently activate other effector caspases, which then cleave a large set of cellular proteins leading to the ultimate destruction of the cell. Caspase 8 activation is termed the "extrinsic" pathway because it is normally activated on the binding of a ligand and subsequent activation of a death receptor present in the cellular membrane *(26)*. Caspase 9 activation is part of the "intrinsic" pathway that is activated within the cell in response to various forms of cell damage or cell stress. Conditions within the tumor microenvironment such as hypoxia *(4)* and nutrient deprivation *(27)* can activate apoptosis through this intrinsic pathway. DNA damage caused by chemotherapy agents and radiation also can lead to caspase 9-dependent apoptosis *(28–30)*.

What then determines whether a particular cell will initiate apoptosis in response to cellular stress? Another way to pose this question is to ask what determines the dose–response relationship for the induction of apoptosis to a particular form of damage or cell stress. Critical in this relationship is the balance of pro- and anti-apoptotic proteins, chief among which are the family of BH3-containing proteins such as BCL2 and BAX *(31,32)*. These proteins control permeabilization of the outer membrane of the mitochondria and the subsequent release of cytochrome c *(33)*. Cytochrome c release leads to formation of the apoptosome and caspase activation. Thus, activation of apoptosis in response to cancer treatment depends highly on the specific balance of these pro and anti-apoptotic proteins. This balance is known to vary widely among both different cell types in the body and different tumors. For example, activation of apoptosis through the intrinsic pathway can be highly influenced by p53, which is itself activated in response to DNA damage and hypoxia in human tumors. The ability of p53 to induce apoptosis is due in large part to its transactivation of pro-apoptotic proteins such as BAX and PUMA. Many tumors show loss of p53 function and are thus unable to initiate apoptosis through this pathway. However, in those tumors that retain p53 function, its ability to induce apoptosis is still highly dependent on the relative level of other pro- and anti-apoptotic proteins in the cell *(34)*. Consequently, some cells may require significantly higher levels of stress or damage than others to tip this balance in favor of apoptosis. This is also true of normal tissues. Although p53 is activated in response to DNA damage in most normal cell types, apoptosis is much more easily activated in certain cell types, such as those of lymphoid origin *(35)*. Many other normal cell types almost never undergo apoptosis even after very high amounts of cell damage. Thus, the importance of p53 as an inducer of apoptosis is context dependent. This example applies to most regulators of apoptosis and leads to the conclusion that evaluation of the dose–response relationship is critical for evaluating the relative importance of apoptosis in response to damage. However, it is important to remember that apoptosis may occur independently of p53 as well.

2.2. Senescence

Senescence is a term that reflects the permanent inactivation of proliferation that, like apoptosis, is imitated by a genetic program. Cells undergoing senescence remain metabolically active yet do not actively cycle and thus for cancer will not contribute to further growth of the tumor. Senescence is also a normal part of physiology that increases as organisms age. Senescence was first visualized in cultured primary cells that exhibit an initial period of exponential growth, followed by a permanent arrest termed replicative senescence *(36)*. Replicative senescence correlates with the gradual shortening of telomeres during the exponential growth period. In addition to this replicative form of senescence, "premature" senescence can also be elicited by various cellular stresses such as those caused by oncogene activation or by DNA damage *(37,38)*. In both situations, the cells enter a permanent cell-cycle arrest characterized morphologically by a flattened cytoplasm and increased granularity or biochemically by stimulating senescence-associated β-galactosidase expression *(39)*. Senescence-inducing stresses typically do not induce shortening of the telomeres but instead are controlled by a number of gene products that are only beginning to become understood. The best understood part of senescence induction involves the activation of cell-cycle inhibitor proteins. Senescence activation following DNA damage is influenced by p53, and its target gene CDKN1A, also known as p21 *(40)*. This is thought to initiate a cell-cycle checkpoint that leads eventually to permanent arrest, perhaps through widespread gene silencing *(41)*. Also critical in the induction of senescence is the *p16* gene from the INK4A locus and the retinoblastoma (RB) gene product *(42,43)*. In cells that retain p53 function, p16 has been shown in some cases to be necessary for the induction of senescence after DNA damage *(44)*. However, this may not be universally true as other studies suggest that the maintenance of the senescent state and not its induction requires p16 *(45,46)*. Like apoptosis, avoidance of senescence is thought to play a role during tumor development. However, the considerable overlap between the genes that control apoptosis and those that control senescence make the situation difficult to assess. Nonetheless, recent evidence from several model systems indicates that tumors do indeed downregulate senescence through a variety of mechanisms *(47–50)*.

2.3. Mitotic Catastrophe (Chromosome Aberrations)

Mitotic catastrophe is a term that has evolved to encompass the type of cell death that results from or following aberrant mitosis. This is often morphologically associated with the accumulation of multinucleated, giant cells containing uncondensed chromosomes. This process is thought to occur when cells proceed through mitosis in an inappropriate manner. For example, mitotic catastrophe frequently occurs if cells enter mitosis with unrepaired or misrepaired DNA damage. Death because of mitotic catastrophe contrasts with that described for apoptosis and senescence in that it is more of a trigger for cell death rather than a genetic program for cell death. Consequently, mitotic catastrophe resulting in cell fusion, polyploidy, or failure to perform cytokinesis may subsequently lead to cell death by apoptosis, senescence, or necrosis *(51)*. The important distinction is that cell death is caused by the mitotic catastrophe rather than the initial cell damage itself.

Several checkpoints in G2 and throughout mitosis exist to prevent mitotic catastrophe. These include two genetically distinct G2 checkpoints that are activated by

DNA damage and block mitosis by preventing the activation of the mitotic kinase CDK1. Cells that show deregulated expression of CDK1 enter into mitosis prematurely and die through mitotic catastrophe *(52)*. An "early" G2 checkpoint is regulated by activation of the DNA damage sensing protein ataxia telangiectasia mutated (ATM) *(53)*. This checkpoint is activated within minutes of DNA damage and prevents cells that are in G2 phase at the time of damage induction from entering mitosis. Inhibition of CDK1 occurs following ATM- and CHK2-dependent phosphorylation of the CDK1 inhibitor CDC25C. A "late" G2 checkpoint is controlled in a similar way through initial activation of the ATM- and Rad3-related (ATR) protein and subsequent phosphorylation of CHK1. This late G2 checkpoint prevents cells that arrive in G2 phase with remaining DNA damage from entering mitosis. In addition, p53 can contribute to G2 arrest by upregulation of its targets p21 and 14-3-3σ *(54)*. Bypass of this checkpoint permits premature entry into mitosis even if the DNA has not been fully replicated or repaired, leading to mitotic catastrophe.

Several mitotic checkpoints are also thought to exist to ensure proper spindle assembly and attachment prior to cytokinesis. The spindle checkpoint is regulated by a number of different kinases, including the aurora kinases (A, B, and C), polo kinases (PLK1, 2, and 3), as well as the BUB1 and BUBR1 spindle checkpoint kinases. Deregulation of these kinases has been shown to lead to mitotic catastrophe, although it is still unclear precisely how this occurs *(55)*.

Many of the abovementioned genes and the pathways that control them are modified during cancer. For example, transmission of the signals from ATM and ATR to CHK1 and CHK2 is dependent on the tumor suppressor genes *BRCA1* and/or *NBS (56)*. Consequently, the propensity to undergo mitotic catastrophe can vary significantly among different cell types that may have lost these tumor suppressor gene functions. For example, breast cancer cells with aberrant BRCA1 proteins would be expected to exhibit increased sensitivity to mitotic catastrophe. Moreover, some chemotherapeutic drugs such as paclitaxel induce this form of cell death by directly disrupting the mitotic spindles or damaging microtubules *(57)*.

2.4. Autophagy

Autophagy is a process in which cells digest their own cytoplasm to generate small macromolecules that are essential for cell survival. Autophagy is induced in response to growth factor removal and is thought to sustain overall survival during times of low nutrient environment *(58)*. The molecular basis of autophagy, and its relationship to cell survival mechanisms, is an active area of current research. DNA-dependent protein kinase (DNA-PK) was recently demonstrated to play a critical role in ionizing radiation autophagy *(59)*. The mammalian target of rapamycin (mTOR) pathway, which is a general sensor of nutrient status, appears to be critically required to regulate autophagy *(60)*. Intracellular structures called phagophores engulf parts of the cytoplasm, forming cytoplasm-filled vacuoles. These are subsequently delivered to the lysosomes for degradation. Cell death can result in a distinct manner in cells that undergo excessive autophagy. This form of cell death is distinct from apoptosis or necrosis and is thus unique to the autophagic process *(61,62)*. The precise function for death by autophagy in mammals is not fully understood *(63,64)* but has been implicated in several pathologies including cancer *(65)*. Indeed, the involvement of the

mTOR pathway, which is activated by PI3 kinase signaling, suggests that autophagy may often become differentially regulated during cancer.

2.5. Necrosis and Others

Necrosis is considered to be inappropriate or accidental death that usually occurs under extremely unfavorable conditions that are incompatible with a critical normal physiologic process. Examples of conditions that can activate necrosis include extreme pH, energy loss, and ion imbalance *(66)*. Necrosis has been typically thought of as an uncontrollable, an irreversible, and a chaotic form of cell death. More recently, however, studies have suggested that necrosis is also a regulated process that can be modulated *(67)*. It is a characterized by cellular swelling, membrane deformation, and organelle break down and is usually the consequence of such conditions as infection, inflammation, or ischemia *(22)*. However, whether active signaling pathways for this form of death exist is uncertain.

3. INVOLVEMENT OF CELL DEATH DURING TUMOR DEVELOPMENT AND TREATMENT

From this brief overview, it is already clear that various mechanisms of cell death can contribute to cancer development and treatment response in largely varying degrees. The loss or downregulation of cell death pathways clearly occurs during cancer development, not only in the case of apoptosis but in other mechanisms of cell death. In the case of cancer development, it is easy to see how even subtle changes in cell death can contribute to tumor growth and development. A carefully regulated balance between cell proliferation and cell death is absolutely required for maintenance of tissue homeostasis. This equilibrium needs to be only modestly affected to allow proliferation to exceed cell death and consequently to the development of a tumor. Selection against apoptosis may be especially important, as many oncogenes not only promote proliferation but also sensitize the cell to death by apoptosis *(68)*. Consequently for the cell to realize the true potential of an oncogene, it must also counter-balance this increased apoptotic sensitivity.

However, it is less clear whether the changes in sensitivity to cell death that occur during carcinogenesis will also significantly alter the response of these same tumors to treatment. There are certainly compelling experiments that have clearly demonstrated that apoptosis can influence tumor response. This has been perhaps most elegantly studied in the Eμ-myc lymphoma model developed by Lowe and colleagues *(69)*. Tumors arising because of the presence of myc activation in the B-cell lineages are highly influenced by secondary pathways that affect apoptosis. For example, when these mice are crossed with p53 +/− heterozygous mice, all the lymphomas that develop demonstrate loss of heterozygosity for p53 *(34)*. Furthermore, loss of apoptosis through knockout of p53, overexpression of BCL-2, or loss of INK4A/ARF dramatically accelerates tumor onset and tumor growth. This model clearly demonstrates that apoptosis acts as a barrier to cancer development. This model has also provided evidence that apoptosis is important for cancer therapy. When mice-harboring lymphomas were treated with chemotherapy agents, the tumors with loss of p53, INK4A/ARF, or overexpression of BCL2 also responded much more poorly than those expressing only

myc *(34)*. These data clearly demonstrate that in this model, the propensity to undergo apoptosis influences tumor response.

Despite this compelling data, we cannot necessarily extrapolate this result to human tumors, especially those of non-lymphoid origin. The model described above displays an extreme sensitivity to apoptosis because of two distinct factors. First, the cells of lymphoid origin generally show a much higher propensity to die by apoptosis—in other words, the balance between pro- and anti-apoptotic proteins is already tipped in favor of apoptosis in these cells *(35,70)*. Second, overexpression of myc also results in an increased sensitivity toward apoptosis *(71)*. Consequently, the control tumors in this case are extremely sensitive to apoptosis. This is perhaps best exemplified by the fact that a single dose of cyclophosphamide is sufficient to cause a long-term remission in the majority of mice. In solid human tumors, which develop primarily from epithelial cells, the situation may be substantially different. Indeed, in a comprehensive review of the literature, Brown and Wilson concluded that "there is little or no support that apoptosis, and the genes govern it, determine the response to therapy" *(9)*. In contrast to the results with the lymphoma model, they found that the propensity to undergo apoptosis in human tumors of epithelial origin played no role in predicting treatment sensitivity.

Despite the aforementioned review of clinical data, the concept that selection of cells with resistance to apoptosis during carcinogenesis results in a co-selection of cells that will be resistant to treatment has become a persuasive one in the research community. Why has this concept become so widespread? We argue that this has arisen in part due to the use of inappropriate assays to evaluate treatment responses. It is important to evaluate all potential forms of cell death that may contribute to treatment response *(8,20)*. Furthermore, for each type of cell death, it is critical to consider both the kinetics of cell death and its dose–response relationship with the treatment.

4. CONSIDERATION OF CELL DEATH KINETICS AND DOSE RESPONSES IN RELATION TO SURVIVAL

It is often said that "you only live once," but it is equally true that "you only die once." In many cells, several forms of cell death may be possible in response to cancer treatment. The form of cell death that actually inactivates any particular cell is obviously the one that occurs first, that is, that with the fastest kinetics. In cells that retain considerable apoptotic sensitivity, the most rapid form of cell death is often apoptosis, even though it may not be the most sensitive. This is particularly true when treating cells with high doses of a DNA-damaging agent. However, this does not imply that other mechanisms of cell death would not have occurred if given the opportunity (Fig. 1). The important consideration in such a case is whether the probability that the cell survived would be changed if that rapid form of cell death was eliminated. Differences in the rate of cell death have complicated our understanding of the importance of apoptosis for two distinct reasons. The first is because many assays are biased to detect rapid changes in cell number. Rapid forms of cell death are thus more easily detected by these types of assays (discussed in Section 5). The second is because the most rapid form of cell death can mask other forms of death. Consequently, if multiple forms of cell death are activated in the same cell, the one

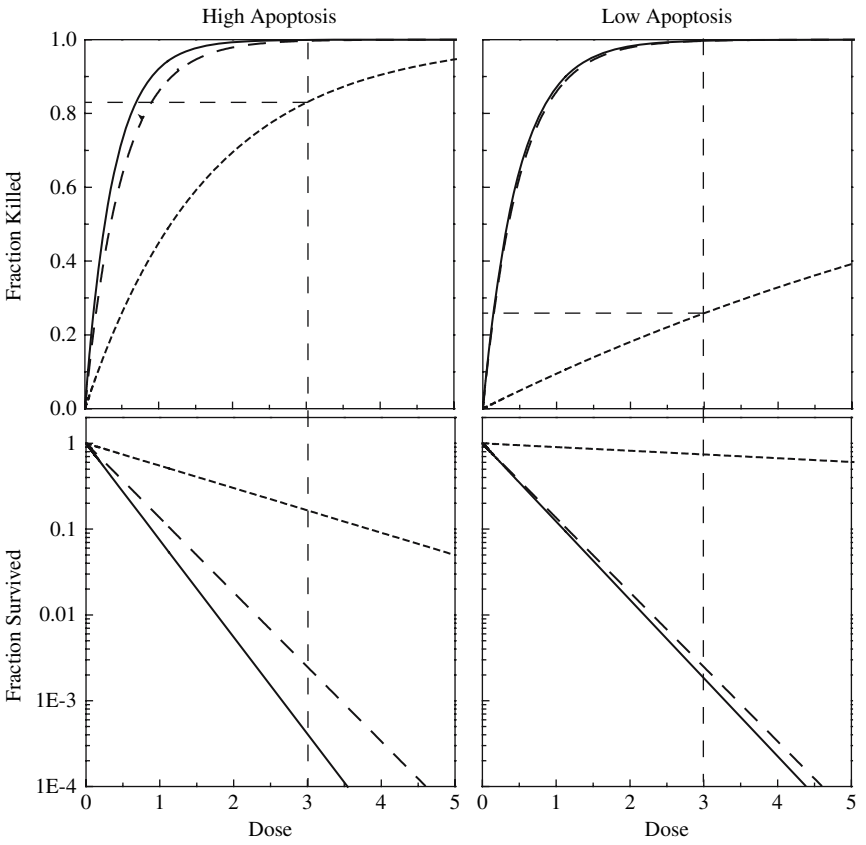

Fig. 1. Importance of dose–response relationships for different modes of cell death. Shown are hypothetical dose–response curves for death (upper frames) and survival (lower frames) in response to an anti-cancer agent. The dose–response relationships for overall (solid), apoptosis only (short dash), and non-apoptosis mechanisms (long dash) are shown separately. Two important concepts are illustrated here. First, a significant change in the sensitivity to one mode of cell death, in this case apoptosis, does not necessarily impact significantly on overall survival. The two examples have substantially different sensitivities to death by apoptosis, but much smaller differences in overall survival. The mode of cell death that is the most sensitive (in this case non-apoptotic death) is the one most important for determining the overall response in both cases. Second, even though apoptosis may not be an important determinant of the overall level of cell kill, the vast majority of cells may still die through this process. In the examples above, doses that are sufficient to kill more than three logs of cells induce apoptosis rates of more than 80% in the apoptosis sensitive cells and more than 25% in the resistant cells. However, in both cases, this is of little consequence to overall survival. Thus, simply because cells die by apoptosis does not imply that apoptosis is an important determinant of outcome.

that is manifested first will be the only one observed. The most rapid form of death will not necessarily be the most important (sensitive).

Interestingly, studies with the Eμ-myc model of lymphoma also provide one of the best examples of how disabling one cell death pathway can reveal another that is equally as sensitive *(72)*. In this model, the development of lymphoma (time to onset/aggressiveness) is very strongly influenced by loss of apoptotic sensitivity. Loss

of the INK4A/ARF locus reduces apoptosis and accelerates tumor development in this model. This locus encodes two tumor suppressors: ARF, which is responsible for activating p53 in response to oncogene activation, and INK4A, which encodes the p16 CDK inhibitor. Thus, the loss in apoptotic sensitivity following deletion of the entire INK4A/ARF locus is due entirely to ARF. Indeed, when the Eμ-myc transgenic mice were crossed with a INK4A *(p16)* knockout mouse, no reduction in apoptosis or acceleration of tumor development was observed. As expected, when p14ARF knockout mice were crossed with the Eμ-myc mice, lymphomas demonstrated significantly reduced apoptosis and rapid tumor onset. Loss of p14ARF in this case appeared equivalent to loss of p53 or overexpression of BCL2. However, the tumors that arose in the Eμ-myc p14ARF mice, despite showing low levels of apoptosis, were found to be just as chemosensitive as those tumors arising in the control mice. It was convincingly demonstrated that instead of dying by apoptosis, the tumor cells from the p14ARF knockouts entered premature senescence. Thus, in these mice, loss of apoptosis simply revealed an equally sensitive form of cell death that took longer to manifest—premature senescence. It is likely that this principle is also present for other forms of cell death in a variety of other human tumors. This concept may explain in part the poor correlation between apoptotic sensitivity and treatment response that is found in the literature *(7,9,11,12,15–19)*.

The example shown above also illustrates the importance of the dose–response relationship between apoptosis and the treatment agent. Modifying the dose–response relationship for a particular form of cell death will only affect overall treatment sensitivity if this is the most sensitive form of cell death (Fig. 1). There are many examples in the literature using genetically matched cell lines in which dramatic changes in apoptotic sensitivity do not lead to any change in overall treatment sensitivity as assessed by clonogenic survival. For example, we have shown that HCT116 cells can be dramatically sensitized to undergo apoptosis in response to a variety of DNA-damaging agents following knockout of the *p21* gene *(18)*. However, when treatment sensitivity is assessed by a clonogenic assay to these same agents, no differences are found between the matched cell lines. Clearly in this case, induction of cell death by alternative modes of cell death occurred at doses far lower than those required to induce apoptosis—even in the cell line that exhibited increased apoptosis because of loss of the *p21* gene.

5. LABORATORY EVALUATION OF TREATMENT RESPONSES AND THEIR INTERPRETATIONS

As mentioned in Section 4, one of the principle reasons for the widely held view that apoptosis plays an important role in treatment sensitivity arises from the use *in vitro* and *in vivo* assays that are biased or inappropriate for assessing overall treatment sensitivity. Outlined below is a brief description of some of these assays and their strengths and limitations with respect to evaluation of treatment response.

5.1. In Vitro

A number of *in vitro* assays are used to assess treatment sensitivity, including those based primarily on the cell growth. The MTT assay, which measures the activity of a mitochondrial enzyme, or assays based on cellular protein or DNA content are often

considered measures of viability and surrogates for treatment sensitivity. However, these assays are chiefly based on measurements of cell number. These assays are normally executed several days after exposure of cells to a damaging agent and as such are influenced not only by cell death but also by transient changes in the rate of cell growth. As many of the same genes that influence cell death also influence cell proliferation, especially in the case of apoptosis, it is difficult or impossible to interpret overall treatment sensitivity from these types of assays. A good example is data from the NCI60 cell line database suggesting that p53 is a strong predictor of treatment sensitivity *(73)*. In this study, treatment sensitivity was evaluated by a short-term (2-day) growth assay. As p53 elicits temporary cell-cycle blocks in response to DNA damage, it is not surprising that this assay would produce such a result. Regardless of how it is performed, any assay that is performed at such short times after treatment will by definition ignore any forms of cell death that occur after longer times. Indeed, critical analysis of the role of p53 in response to radiation *(74)* and other treatment stresses has shown that it is not a reliable predictor of response.

As an alternative, several *in vitro* assays are based on the detection of specific modes of cell death including apoptosis. The least effective assays do not evaluate treatment at the cellular level but instead measure an average or population response. These assays include those that measure changes in the average level of caspase activity or the amount of DNA fragmentation. These assays are useful to demonstrate that apoptosis is occurring but are relatively non-quantitative. Somewhat better are cell-based assays for apoptosis that evaluate the known features of apoptosis such as DNA condensation or exposure of phosphatidyl serine at the cell surface. Other modes of cell death can similarly be identified using assays based on the known morphological and/or biochemical features of that particular form of cell death. For example, senescence is often detected by an increase in cellular β-galactosidase activity.

There are two fundamental limitations of all these death-based assays that limit their usefulness as measures of treatment sensitivity. The first is that they give a picture of the response only at the point in time—the time at which the cell population is evaluated. Given the fact that cells in a population can die by several different processes that each operate with different kinetics that may further vary in different cell types, one can never be sure that the cells that remain viable at the time of assessment will not subsequently die by some form of cell death. This can be partially solved by making continuous measurements as a function of time. However, this introduces further complication as surviving cells begin to proliferate and dilute the dead cells. Furthermore, as the end stages of apoptosis can result in complete cell destruction, the apoptotic cells are also eliminated from assessment as time progresses. This is particularly a problem when attempting to assess cell death by these assays *in vivo*. The second major problem is that it is difficult or perhaps even impossible to simultaneously assess all possible modes of cell death. Consequently, these death-based assays can never give a full picture of treatment response.

The solution to the problem of identifying all forms of cell death was solved in 1956 by Puck and Markus, who developed an assay based on the ability of a single cell to grow into a colony. This "clonogenic assay" has formed the basis of *in vitro* cellular response studies in tumors and also some normal tissues *(75)*. The clonogenic assay tests for the ability of a cell to recover from treatment in such a way that it can proliferate again and form a clone of substantial size (normally evaluated 10–20 days after

treatment). It thus can measure the ability of a cell to survive from all possible (known and unknown) forms of cell death. This assay is analogous to the well-accepted and well-proven assays of treatment sensitivity in other organisms such as yeast and bacteria.

Clonogenic survival shows a typical log-linear relationship with treatment dose implying that the probability of cell kill increases in a roughly linear way with dose (Fig. 1). In other words, each incremental increase in dose kills the same percentage of remaining cells. This relationship is well supported by *in vivo* dose responses of tumors containing large numbers of cells, which correspondingly require a very low percentage of cell survival for cure. The relationship between treatment dose and clonogenic survival established *in vitro* has successfully predicted the doses required to cure transplanted tumors *in vivo* even when rates of apoptosis do not correlate *(18,76,77)*. For example, given the fact that a $1\,cm^3$ tumor contains more than 10^9 cells, treatment requires killing more than 99.9999999% of the cells to have a chance at being effective. The log-linear relationship of cell survival and treatment dose allows one to predict the curative doses required to reach these levels.

This numerical example also exemplifies the importance of comparing dose–response relationships for the various death processes when evaluating their relative importance. For example, if a particular treatment dose results in 0.1% survival (99.9% death) as assessed by a clonogenic assay, but causes only 35% apoptosis, one can conclude that apoptosis is not an important contributor to cell death. The fact that 99.9% of cells are killed implies that cell death is equally effective in the 65% of cells that do not die by apoptosis. One can easily imagine in this case that substantial increases or decreases in apoptosis induction would not affect clonogenic survival. The literature is full of examples in which modest levels of cell death are assessed using treatment doses that will inactivate several logs of cell kill if assessed by the clonogenic assay. Obviously, the relevance of such studies is highly questionable.

The clonogenic assay is not without problems. The assay often involves plating of dilute concentrations of cells under conditions that are significantly different from those found *in vivo*. Furthermore, the *in vitro* conditions ignore the unique microenvironmental parameters of a tumor that can be important contributors to treatment sensitivity such as oxygen and cell-to-cell contact. Several investigators have also pointed out that the long-term culture of cells *in vitro* can result in selection of resistant clones that are not reflective of the original cells *in vivo*. In some instances, it is thus desirable to use primary cells derived from tumors *in vivo*. In this case, it is important that these primary cells can also tolerate *in vitro* culture conditions without substantial death even without treatment. This can be assessed by the plating efficiency—a measure of the number of cells that retain clonogenic capacity in the absence of treatment. When this value is very low (less than 5%), the predictive power of the clonogenic assay comes into question. However, such a low-plating efficiency is indicative of the fact that the cells do not tolerate *in vitro* culture well and thus place doubts on any *in vitro* assays that may be used with these cells. Thus, we feel strongly that the clonogenic assay is far and away the best *in vitro* tool for assessing treatment response.

5.2. In Vivo

There is clearly a need to evaluate cellular treatment sensitivity within the context of the normal environment, a so-called *in vivo* assay. However, even in this context, several common assays can be highly biased or inappropriate for evaluating treatment

response. In particular, assays that are based primarily on evaluating or comparing tumor size (or presence) at fixed times after treatment are difficult to interpret and heavily biased toward tumors that display rapid forms of cell death like apoptosis. For example, investigators may treat either spontaneous or transplanted tumors and then evaluate the size of the tumor at one or more times after treatment. This type of experiment is heavily influenced by the intrinsic tumor growth rate, which is itself influenced by cell loss that occurs through death mechanisms such as apoptosis. A tumor that suffers a high loss factor will grow much more slowly than a similar tumor with a lower cell loss factor. To illustrate this problem, we can consider two different tumors that behave identically with the exception that one has a much slower growth rate because of a higher rate of spontaneous apoptosis (Fig. 2). If we treat each of these two tumors with a dose that produces the same amount of overall cell kill (the same level of survival), it will take substantially longer for the slow-growing tumor to reappear. This may cause misinterpretation of the treatment sensitivity as the "time to relapse" was significantly longer in the slow-growing (apoptotically sensitive) tumor, even though treatment sensitivity and curability are identical. In fact, at any time after treatment, the slow-growing tumor will appear smaller than the other tumor leading one to this mistaken conclusion.

Misinterpretations of treatment sensitivity can also result from differences in the kinetics of cell death following treatment. Again, let us consider a hypothetical situation in which two tumors have equal growth rates and equal overall responses to treatment (Fig. 2). However, in this case, cell death in one of the tumors occurs principally through a rapid apoptotic mechanism and in the other through a much slower mechanism based on mitotic catastrophe. If one follows the size of the tumors after treatment, the apoptotic tumor will regress rapidly because of the induction of apoptosis. The other tumor, although it will eventually show an equivalent loss in cell number, will not regress quickly and thus "appear" to respond more poorly. Again, assessment of tumor size at a fixed time after treatment will not yield the correct information on treatment sensitivity. Owing to both of the factors illustrated in these examples, comparison of tumor size at fixed times after treatment is nearly impossible to relate to overall treatment sensitivity.

A much better way to evaluate the treatment sensitivity of tumors is to measure the growth *delay* that results from treatment. The growth delay is a measure of the difference in times for treated and untreated tumors to reach a certain size (e.g., three times the starting volume). For this value to be meaningful, it must be evaluated at a sufficiently long time after treatment such that all forms of cell death have had an opportunity to have taken place. At this time, the tumor should be growing at a rate that is equivalent again to the untreated tumor (Fig. 2). The difference in time required for the control and treated tumors to reach equivalent sizes directly reflects the percentage of cells that survived the treatment. As seen in Fig. 2, this value is completely independent of the rate of tumor regression after treatment. To remove the problems associated with comparing tumors that have different intrinsic growth rates (our first example above), one must use the specific growth delay *(78)*. This is simply equal to the growth delay expressed as a fraction of the growth rate (e.g., time to reach three times starting volume) of the untreated tumor.

Although measurements of growth delay correct for the two errors outlined in our example, they do suffer from other potential problems. Tumor growth rate can be

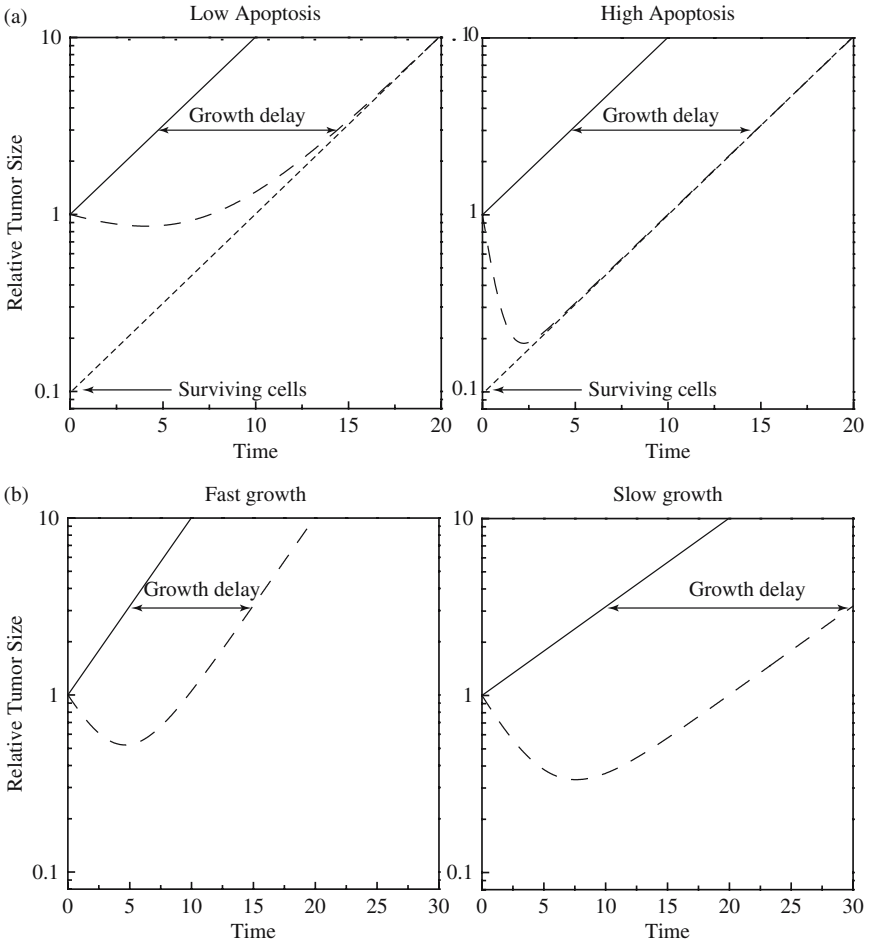

Fig. 2. Interpretation of tumor responses *in vivo*. These illustrations highlight two distinct ways that apoptosis can result in misinterpretation of *in vivo* treatment responses. (A) Plotted are the hypothetical responses of two tumors with the same growth rates and same overall response to treatment. In the left panel, the tumor cells die primarily through non-apoptotic pathways. In the right panel, cells die primarily through apoptotic pathways. Comparison of the size of the tumors after treatment leads one to the incorrect conclusion that the apoptosis-prone tumor is more sensitive. In this case, the tumor cells die quickly leading to a large tumor regression. However, in both cases, the same numbers of cells die, and after a sufficiently long period of time (when the size of the tumor is determined solely by the survivors), the two tumors are identical. The correct evaluation of response is made by comparing the growth delay at a point where the tumor has restored its normal growth rate. (B) Plotted are the hypothetical responses of two tumors that again have an identical overall treatment sensitivity (the same survival) but very different rates of growth. In this case, the slow growth of the tumor in the left panel could be attributed to high spontaneous rates of apoptosis. Despite the fact that the same numbers of cells survive, the slow-growing tumor has a much longer growth delay. This difference arises not because of a difference in sensitivity but rather to the trivial explanation that the tumor simply grows slower and thus takes a longer time to reach its evaluation size. The correction to make in this case is to use specific growth delay (see text).

affected by treatment in ways that are unrelated to the overall amount of cell death of the tumor cells. For example, damage to tumor vasculature may slow tumor growth in a way that is unrelated to killing the tumor cells *(79)*. This extra growth delay may be incorrectly interpreted as increased cell death even though no additional tumor cells may be killed.

The gold standard of all assays of treatment sensitivity is thus those that evaluates tumor cure. This is evaluated by a so-called TCD50 assay, in which the dose required to cure 50% of the tumors is calculated. In these experiments, cure typically follows a sigmoidal function of treatment dose. Although this is the best of all assays, there has been a steady decrease in its use. This is likely due to the fact that a large number of mice must be used to accurately define the dose–response relationship. Furthermore, this assay takes a comparatively long time, typically requiring many months to be sure that cure has been achieved. The TCD50 assay is somewhat analogous to the *in vitro* clonogenic assay, as it ultimately measures the probability that a single cell can survive and reform the tumor. For example, at doses that will cure 50% of treated tumors, the average number of surviving cells per tumor is just slightly above 1. The assay is unaffected by rates of tumor growth or rates of cell death. When performed correctly, this assay provides the ultimate measure of overall treatment sensitivity.

The TCD50 assay also allows one to consider the possible existence of tumor stem cells. This is an old concept that has received renewed interest and implies that only a small fraction of tumor cells are truly clonogenic and capable of unlimited proliferation *(80,81)*. This concept may also explain the poor "plating efficiency" that has been observed when primary tumor cells are cultured *in vitro*. The bulk of the tumor cells thus represent somewhat more differentiated cells that lack this clonogenic capacity. Obviously in such a case, it becomes important to evaluate the response of the tumor stem cells and not the response of the bulk of the tumor. As the TCD50 is influenced only by those cells that have the ability to both survive and reform the tumor, it accurately reflects the treatment sensitivity of the relevant cells in the tumor. The behavior of the non-stem cells in this case is irrelevant.

6. CONCLUSIONS

Our understanding of the molecular basis and regulation of the apoptotic pathway and its importance in cancer has, somewhat paradoxically, clouded our ability to understand the important molecular determinants of treatment response. Experiments demonstrating that apoptosis could indeed influence treatment sensitivity have contrasted with other studies showing that dramatic changes in apoptosis can occur without affecting the overall treatment response. Thus, the question of whether apoptosis, or the genes controlling it, is important for cancer therapy remains controversial.

We have argued that conflicting conclusions over the importance of apoptosis results both from studies with model systems that cannot be extrapolated to most human tumors and from the use of assays that may be heavily biased toward detection of specific modes of cell death. Several elegant genetic models have convincingly demonstrated that it is possible to create transformed cells that become, and retain, extreme sensitivity to apoptosis. In these cases, apoptosis becomes the most sensitive form of cell death in response to treatment and is thus a dominant determinant of overall response. However, in real human cancers, apoptotic sensitivity is dramatically

lower and correspondingly also less important (or even unimportant) for treatment response. The important point is that all the different forms of cell death outlined in Section 2 are potentially important in tumors of various types. Proper evaluation of treatment response thus requires careful analysis of each of these forms of cell death prior to making any conclusions. This is most accurately assessed *in vitro* by use of the clonogenic assay that is capable of integrating all forms of cell death. Similarly, *in vivo* assays of tumor response must also be conducted in ways that allow evaluation of all forms of cell death. Short-term assays that are influenced by tumor growth rates or the rates of regression should be avoided. Proper selection and interpretation of these assays is necessary for future experiments aimed at evaluating the importance of apoptosis and other determinants of cell death and survival.

The finding that apoptosis can, but rarely does, contribute to tumor response has important implications on how to move forward with research into better treatments for cancer. Especially important is to not make the mistake to generalize this finding to all forms of cell death. Just because apoptosis, and the genes controlling it, may not be important for treatment sensitivity in many tumors does not mean that the genotype of cancer cells is an unimportant factor during treatment. Cells that die as a result of non-apoptotic mechanisms may still be highly influenced by cancer-associated mutations. Particularly important in many tumors of epithelial origin are the molecular determinants of mitotic catastrophe. These include the more than 130 known DNA repair proteins that play crucial roles in determining treatment sensitivity *(82)*. The importance of the DNA damage response and in particular DNA repair on treatment sensitivity has been demonstrated repeatedly for a vast number of DNA-damaging agents *(83)*. Loss of critical proteins that participate in the DNA damage response, for example, the proteins required for the repair of double-strand breaks by ionizing radiation, almost universally cause sensitivity to DNA-damaging agents *(84)*. This finding also supports the argument that activation of apoptosis and/or other forms of cell death in response to *initial* DNA damage is rarely important as a determinant of treatment sensitivity. DNA repair, which may require several days for completion, remains a critical determinant of cell survival. In these cases, cell death results only in the cells that are incapable of repair or that undergo misrepair. These cells tend to die at comparatively long times after treatment and importantly after mitotic catastrophe *(74)*. Continued research aimed at improving our understanding of this and other forms of cell death will undoubtedly lead to new approaches for improving cancer treatment.

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4 Mitotic Catastrophe

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Summary

Mitotic catastrophe (MC) is the result of premature or inappropriate entry of cells into mitosis, usually occurring because of chemical or physical stresses. MC is characterized by changes in nuclear morphology and the eventual appearance of polyploid cell progeny in affected cell populations, is markedly enhanced in cells lacking p53 function, and is the result of overaccumulation of cyclin B1 in cells delayed late in the cell cycle by the inducing agent. Thus, MC is considered to be the predominant mechanism underlying mitotic-linked cell death. Along with characteristic features associated with MC, a delayed DNA damage phenotype has been noted in these populations, suggesting a potential role for MC in mutagenesis and the acquisition of genomic instability. Although generally lethal, some cells can survive MC through mechanisms that are incompletely understood. Cytological features associated with meiotic cell division have been noted in polyploid cell populations produced through MC, a finding that might be particularly relevant in the understanding of tumor progression and that might provide a novel mechanism for the generation of quasi-diploid progeny from MC-induced polyploid cell populations. This review summarizes the literature pertaining to MC and describes current lines of research in this interesting research area.

Key Words: Mitotic catastrophe; cell cycle regulation; cyclin B1; endopolyploid cells; mitosis; meiosis; carcinogenesis; tumor progression; delayed DNA damage; SPCC.

1. MOLECULAR MECHANISMS UNDERLYING MITOTIC CATASTROPHE

1.1. Mitotic Catastrophe is the Result of Premature Entry into Mitosis Following Abrogation of G2/M Checkpoint Function

Exposure of some cell types to a broad class of agents can lead to a loss of regulation of cell division, such that cells enter into a premature mitosis, an event that culminates in a phenomenon called mitotic catastrophe (MC). MC is characterized by the aberrant

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nuclear morphology observed following premature mitotic entry *(1)* and often results in the generation of aneuploid and polyploid cell progeny. Ultrastructural studies of HeLa cells *(2,3)* during long-duration hyperthermia characterized a general disorganization of cellular organelles that occurs in cells undergoing spontaneous premature chromosome condensation (SPCC). Cells undergoing SPCC can either fail to achieve cytokinesis (early-SPCC) or divide and fuse shortly thereafter (late-SPCC) *(4)*. The early and late classifications arose based on two different observations: in the case of Chinese hamster ovary (CHO) and HeLa cells heated for up to 24 h at 41.5 °C, cells arrest in mid-S phase and SPCC figures observed in cell preparations have the characteristic appearance of cells in S phase undergoing chromosome condensation due to mitotic factors *(5)*, thus producing early-SPCC. Following radiation and other agents that block primarily in late S and G2 phases, the condensed chromosome morphology is more fibrillar, looking identical to the morphology obtained when a G2 phase cell is induced to prematurely enter mitosis *(5)*; thus, these cells are termed late-SPCC. The difficulty in distinguishing SPCC from normal mitoses in some cell lines, especially late-SPCC, has lead to the use of nuclear fragmentation as an endpoint indicative of MC; studies have shown that these two quantities correlate well with SPCC *(4,6)*.

Stress-induced SPCC and subsequent MC are found following activation of cyclin B1/cdc2 kinase complex occurring while cells are delayed in S or G2 phases *(4,7)*, indicating that stress-induced MC is the result of abrogation of cell cycle regulatory pathways, in particular the G2 checkpoint *(8–10)*. MC has been found to occur in cells exposed to a variety of treatments, including adriamycin *(11)*, 5-fluorouracil *(12)*, etoposide *(13)*, temozolomide *(14)*, topotecan *(15)*, camptothecin *(16)*, combretastatin *(17)*, paclitaxel *(13,18)*, phytoestrogens *(19)*, overexpression of c-H Ras *(20)*, prolonged mild heat shock *(17,21,22)*, UV radiation *(23)*, methyl-methanesulfonate *(23)*, ionizing radiation *(4,24–26)*, and hyperthermia combined with radiation *(27)*. Specific effects on mitotic spindle formation and mitotic microtubule stability, mediated by cyclin B1/cdc2 kinase activity, have been identified as playing an important role in MC *(28,29)*. Furthermore, there is evidence in support of the notion that cell cycle delays play a critical role in the development of SPCC and MC. All the aforementioned studies reported that delays late in the cell cycle were observed prior to MC. In SPCC and MC induced by long-duration mild heat shock of HeLa cells, blockage in G1 during the heat treatment using either cycloheximide or caffeine (both agents known to induce a G1 block in HeLa cells) results in a reduction in S delay, SPCC, and cytotoxicity *(30)*. Inhibition of cell cycle progression following radiation exposure leads to protective effects on cell survival, which has prompted some authors to consider that repair of potentially lethal damage can occur during the arrest interval *(31)*. It has been proposed that one of the cellular functions of mutations in the tumor-suppressor gene p53 is to promote MC as a mechanism for removing damaged cells from populations following genotoxic stress *(32)*. The mechanisms underlying this function of p53 involve its activity as a modulator of G2 checkpoint mechanisms.

1.2. p53 is an Important Repressor of MC

All living organisms have evolved mechanisms to regulate the timing of cell division within the context of the completion of DNA replication. Events associated with mitosis, such as nuclear envelope breakdown, chromosome condensation, and assembly of the mitotic apparatus are mediated through the specific phosphorylation of cellular proteins

by large multi-protein complexes containing, in addition to other proteins, the cdc2 and cyclin B1 gene products. The activity of this complex is stimulated through the action of the cdc25C phosphatase; inhibition is mediated by wee1 and other inhibitory kinases; in both cases, changes in the phosphorylation state of key residues of cdc2 result in changes in cyclin-dependent kinase activity. Cdc25C activity is inhibited in the presence of unreplicated DNA, whereas inhibitory kinases such as wee1 are constitutively active. Cyclin B1 biosynthesis also contributes to the regulation of mitotic entry, as cyclin B1 levels are cell cycle regulated, with the gene being expressed only in late S and G2 phases in human cells *(33)*; proteosome-mediated degradation of cyclin B1 begins in anaphase, resulting in undetectable levels of the protein by the time cells enter the next S phase *(34)*. These pathways act in concert to ensure that mitosis does not commence prior to the completion of DNA replication. Other mechanisms function to inhibit entry into mitosis if DNA is damaged, resulting in a G2 block; there are many pathways involved here, some are p53-dependent, and others are not. The end result is to reduce the activity of cyclin B1/cdc2-containing complexes, and the mechanisms that have been elucidated demonstrate the incredible diversity at play in biological organisms. Chk1, chk2, atm, and atr gene products all contribute to the activation of p53 in response to genotoxic stress, whereas p53-mediated inhibition of cyclin B1/cdc2 activity can occur through expression of the cdk inhibitor p21, direct effects on cyclin B1 or cdc2 biosynthesis, inhibition of cdc25C nuclear transport through chk1/chk2- and 14-3-3-σ-dependent pathways *(35)*, or destabilization of cyclin B1/cdc2 complexes by gadd45 *(36)*. One study *(37)* has also implicated chk2 in the phosphorylation-mediated inactivation of cdc25A and radiation-induced inhibition of DNA synthesis that is generally thought to be responsible for S phase delays following irradiation *(38)*.

In the absence of checkpoint activation, mitosis can occur very rapidly, provided the right activating pathways predominate over the inactivating pathways (Fig. 1). When cells are damaged or otherwise perturbed from the usual sequence of cell cycle events, many of these regulatory pathways serve to check further progress through the cell cycle, until either a sensed deficiency (e.g., incomplete DNA replication) is ameliorated or some other imbalance is fixed. This balance between inhibitory and stimulatory pathways relies on the abundance of these same regulators, as it has been demonstrated that overexpression of cyclin B1 in G2-arrested cells leads to MC in genetic manipulation studies *(8)*, independent of the activity of the inhibitory pathways. So, under ordinary conditions, these checkpoints serve to ensure that orderly progress through the cell cycle occurs. Under extraordinary conditions, however, these regulatory pathways can become abrogated, resulting in a deviation from the expected cellular behavior. MC is just one of these deviations.

1.3. MC Has Been Associated with Overexpression of Cyclin B1 in p53-Deficient Cells

Previous studies of heat- and radiation-induced MC in HeLa cells *(4,7,27)* have demonstrated that a common feature of the effect of the two modalities is the high accumulation of cyclin B1 that occurs prior to the appearance of MC. Further studies indicate that this phenomenon is not limited to HeLa cells, being also observed in hamster and mouse cell lines, as long as p53 is non-functional *(24,39)*, and in human glioblastoma U87MG cells transduced with a dominant-negative p53 adenovirus

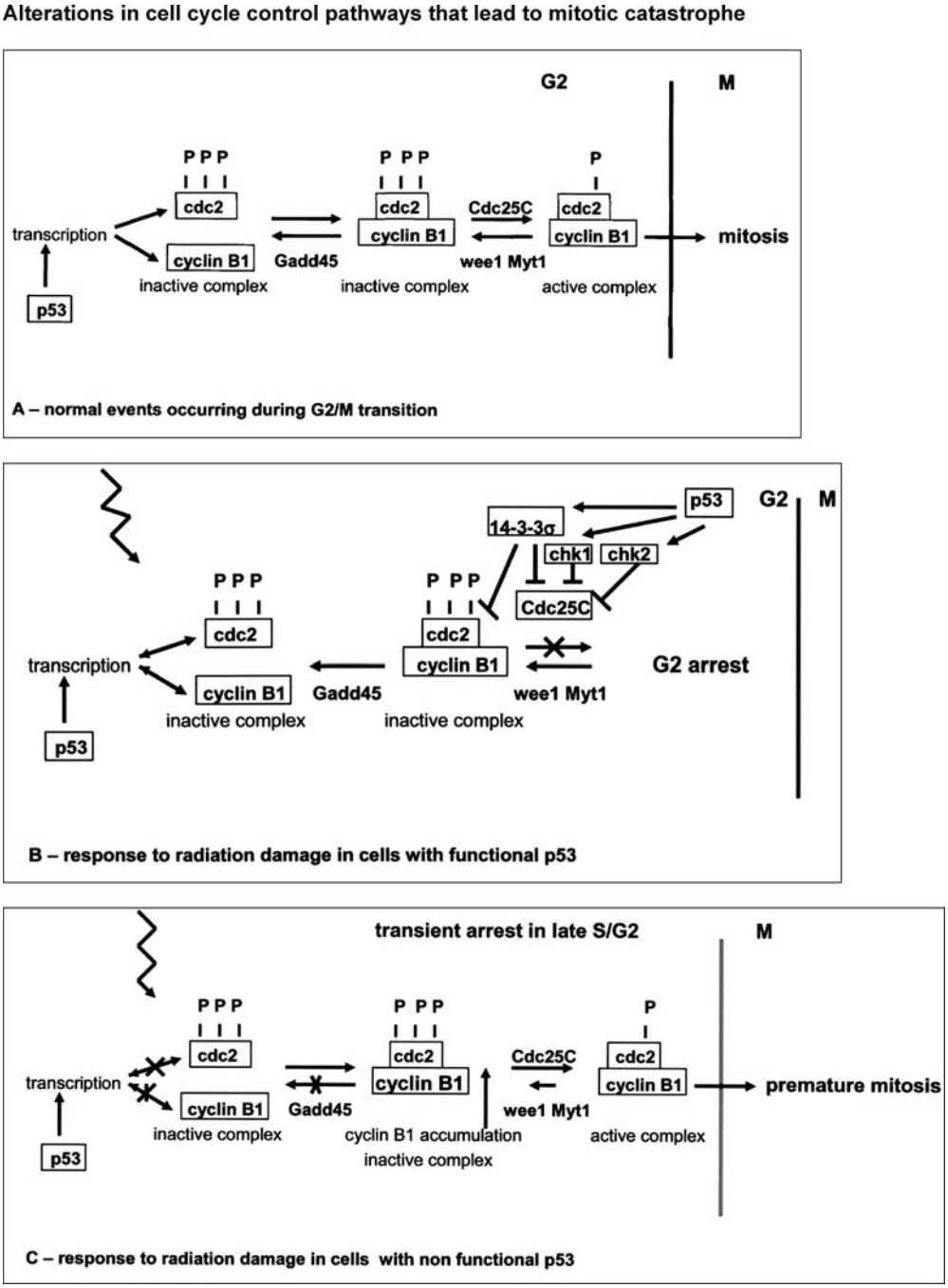

Fig. 1. A complex set of pathways acts to regulate entry of cells into mitosis, where active cyclin B1/cdc2 kinase complexes initiate nuclear envelope breakdown, chromosome condensation, assembly of the mitotic spindle, and other events required for cell division. Control of nuclear levels of activated cyclin B1/cdc2 complexes is accomplished through pathways related to biosynthesis, post-transcriptional modification, and intracellular transport of the component proteins in these complexes. In the nucleus, cyclin B1/cdc2 is normally maintained in an inactive state by the tyrosine kinases

construct that rendered the cells, otherwise wild-type p53, impaired in their p53 function *(40)*. Furthermore, experiments have shown that in some cell lines, this elevation of cyclin B1 levels persists for several cell generations in the treated population. Although studies have demonstrated that p53 deficiency can lead to the disruption of many cell cycle regulatory pathways *(9,41)*, other reports have demonstrated that effects of p53 on cyclin B1 gene expression alone can contribute significantly to MC *(42)*. p53 acts to regulate cellular levels of cyclin B1, through a transcriptional repression mechanism that is not completely understood *(43)*. Nevertheless, studies have shown that inhibition of p53-dependent regulation of cyclin B1 transcription results in MC in G2-arrested cells *(14,42)*, and overexpression of cyclin B1 can overcome the G2 block *(44)*. Other studies *(45)* reported that MC is independent of p21 (Waf-1) and 14-3-3-σ, but p21 can lead to G1 arrest in tetraploid cells produced during MC. Another study *(46)* demonstrated that p53-mediated transcriptional regulation of cyclin B1 requires a short region at the C-terminal regulatory domain of p53. Thus, it appears that, in addition to those p53-dependent pathways related to cyclin B1/cdc2 activation, at least one pathway has evolved that functions to simply repress cyclin B1 synthesis, and abrogation of this pathway can lead to MC and other subsequent effects on cell cycle regulation. Control of cyclin B1 levels during a G2 arrest is an important function, as elevation of cyclin B1 can reduce the level of control of other cell cycle regulators simply by mass action, if their levels are not increased accordingly *(47)*. Nevertheless, the generality of this phenomenon as a toxicological mechanism underlying radiation-induced MC has not been established in a variety of human tumor cell lines. Radiation is an important treatment modality for a variety of human cancers. According to the American Cancer Society, in 2003, over a million persons were diagnosed with cancer in the USA. Radiation therapy will be an important clinical treatment for a significant fraction of these patients. However, an important complication associated with radiation therapy is the appearance of tumors containing radioresistant cells *(48)*. Although mechanisms underlying this phenomenon are unknown, it has been suggested that radiation-induced chromosomal instability

Fig. 1. wee1 and myt1: wee1 inactivates cdc2 kinase through an inhibitory tyrosine phosphorylation, whereas myt1 inhibits cdc2 through phosphorylation at threonine 14 and tyrosine 15 residues in a cyclin-dependent manner. Dephosphorylation of cdc2 at threonine 14 and tyrosine 15 in late G2 by cdc25C promotes entry into mitosis. DNA damage activates the DNA-PK/ATM/ATR kinases triggering two parallel cascades of events that inactivate the cyclin B1/cdc2 complex. In the first cascade, the chk kinases phosphorylate and inactivate cdc25C, thus preventing activation of cdc2. In the second cascade, p53 induces a set of genes that either inactivate or inhibit nuclear translocation of cyclin B1/cdc2complex: 14-3-3-σ binds to the phosphorylated cyclin B1/cdc2 complex and prevents its transport into the nucleus (14-3-3-σ also acts to sequester cdc25C in the cytoplasm, thus preventing cyclin B1/cdc2 complex activation in the nucleus); Gadd45 binds to and dissociates the cyclin B1/cdc2 complex; and p21Cip1 inhibits cdc2 kinase activity. In the absence of functional P53, those events usually leading to a decrease in nuclear cyclin B1/cdc2 activity, along with the loss of P53-mediated transcriptional regulation of cyclin B1 and cdc2 gene expression, results in an abnormally high accumulation of cyclin B1/cdc2 complexes in the nucleus. Once there, we hypothesize that the negative regulatory effects of wee1 and myt1 are effectively diluted by the high levels of cyclin B1/cdc2 present, resulting in premature mitotic entry and subsequent mitotic catastrophe. Panels: simplified cascade of events occurring at the border of G2/M in **(A)** an untreated cell; **(B)** an irradiated cell having functional P53; **(C)** an irradiated cell having non-functional P53.

may contribute to induced radioresistance *(49)*. Moreover, as most human tumors have defects in p53 or p53-related pathways *(32)*, they are resistant to treatments aimed at inducing death through apoptotic pathways. Thus, it is conceivable to infer that if MC is a major mode of radiation-induced cell death in cell lacking p53 function, clinical interventions designed to enhance its production in tumor cells might lead to a therapeutic gain. The elucidation of the mechanisms underlying radiation-induced MC might therefore have a major impact on the clinical effectiveness of radiation therapy.

2. STRESS-INDUCED MC

2.1. Cell Cycle Delays

Results have been reported *(1,6,7)* on the effects of MC induced by long-duration 41.5 °C hyperthermia treatment in HeLa cells, and a role for increases in cyclin B1/cdc2 activity occurring during delays in S phase in the appearance of MC has been identified *(7)*. Experimental conditions that inhibited the ability of cells to undergo the heat-induced block in S phase eliminated the induction of heat-induced MC and were protective toward cell survival *(30)*. It was also reported that MC is a phenomenon distinct from apoptosis in HeLa cells *(2)*. Reports on the effects of ionizing radiation in the induction of MC have also been published, in which the extent of cell cycle delays have been characterized following 0, 5, 10, and 20 Gy of X-ray exposure. These data *(4)* demonstrated that radiation-induced MC shares many features with heat-induced MC. Radiation-induced cell cycle delays occurred primarily in late S and G2 phases, as opposed to the mid-S delays observed at 41.5 °C, and the morphology of chromatin in cells undergoing premature mitosis was different from that observed in the heat experiments, where heat produced primarily early-SPCC and radiation exposure led to the appearance of late-SPCC. Although the details of the phase-location of cell cycle delays and the morphology of SPCC were different for the two treatments, both heat and radiation exposure resulted in significant MC. For both stresses, cyclin B1 levels increased prior to the appearance of MC; for radiation, this increase in cyclin B1 was dose-dependent up to 10 Gy. The increased levels of cyclin B1 protein correlated well with increases in cyclin B1-dependent kinase activity; both increases appeared prior to MC in the treated populations. Although the yield of MC was not dose-dependent in these experiments, the extent of MC was (measured as the number of fragments per fragmented cell). In these experiments, cyclin B1 levels also apparently peaked again in later cell generations after the induction of MC, suggesting that cyclin B1 gene expression might be persistently altered in the treated populations. The observed increase in cyclin B1 levels was thought to occur because expression of this gene in human cells is known to occur only in late S/G2 phases *(33)*; simple arrest of cells at this part of the cell cycle might allow for a longer time for transcription and accumulation of cyclin B1 *(7)*. Although such an explanation might seem simplistic, recent reports have shown that transcriptional regulation of cyclin B1 is mediated by p53 during a radiation-induced G2 block and is apparently not related to effects on mRNA or protein stability *(43)*. As HeLa cells are deficient in p53 function because of human papilloma virus (HPV) infection, these developments prompted investigations on the effects of p53 status on radiation-induced MC.

Evaluation of other cell lines for the induction of MC by irradiation began in the authors' laboratory; a thorough analysis is still ongoing using the Large-Scale Digital

Cell Analysis System (LSDCAS) *(50,51)*. LSDCAS holds a promise of being quite useful in these studies, as the analysis of many cell lines using cytological assays is complicated by low plating efficiencies, and it is only the proliferative cells in the culture that are at risk of radiation-induced MC. LSDCAS ameliorates this situation by allowing for the analysis of proliferative cells, even in the presence of a high fraction of a non-proliferative cell population. LSDCAS has advantages over other time-lapse systems currently in use *(52)*, as this system allows for the monitoring of thousands of microscope fields for up to a month following irradiation. A summary of the findings related to radiation-induced MC within the context of p53 status by using the cytological approach is presented in Table 1. Although it was found that all cell lines were delayed late in the cell cycle, it was only those cell lines with non-functional p53 that exhibited high levels of cyclin B1 during these delays, and the subsequent yield of MC was much higher in these cell lines. These results suggest that lack of p53 function is critical to the induction of radiation-induced MC.

Table 1
Summary of the Incidence of Radiation-Induced Mitotic Catastrophe (MC) in Various Cell Lines

Cell line	*Origin*	*p53 status*	*Induction of MC by irradiation*	*Delays late in the cell cycle?*	*Overexpression of cyclin B1?*
HeLa S3	Cervical carcinoma	Non-functional (HPV)	+++	Yes	+++
HeLa Clone 3	Derived from HeLa S3	Non-functional (HPV)	+++	Yes	+++
V79	Chinese hamster lung	Mutant	+++	Yes	+++
GM10115	CHO hybrid w/human chromosome 4	Mutant	+++	Yes	++
115	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++
CS9	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++

(Continued)

Table 1
(Continued)

Cell line	*Origin*	*p53 status*	*Induction of MC by irradiation*	*Delays late in the cell cycle?*	*Overexpression of cyclin B1?*
LS12	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++
138	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++
U87-MG	Human glioblastoma	Wildtype	+	Yes	−
GM00037F	Primary human fibroblasts		+	Yes	−
MEF-12(1)	Mouse embryo fibroblasts	Wildtype	+	Yes	+
MEF-10(1)	Derived from MEF-12(1)	Mutant	+++	Yes	+++

2.2. LET Effects on the Induction of MC

Experiments studying the induction of MC in V79 Chinese hamster cells by low-energy protons *(24)* demonstrated that high Linear Energy Transfer (LET) protons are much more effective in causing MC than X-rays. In particular, doses as low as 0.5 Gy produce detectable MC in these cells, whereas the limit of detection following X-ray exposure is about 2 Gy using cytological assays. Radiation-induced MC shows an Relative Biological Effectiveness (RBE) effect that appears to be similar to that observed for survival, indicating the importance of MC as a mechanism of radiation-induced cell killing *(24)*. Other studies *(53,54)* have shown that the cells are delayed for longer intervals late in the cell cycle following high-LET proton irradiation, when compared with delays caused by X-irradiation, in accord with earlier studies using alpha particles *(55)*. Thus, the longer duration of cell cycle delays induced by high-LET protons is thought to contribute to the increased yield of radiation-induced MC in these studies.

3. DELAYED DNA DAMAGE ASSOCIATED WITH MC

3.1. p53-Deficient Cells Undergoing MC Later Present a Novel Form of Delayed DNA Damage

It is generally thought that abrogation of G2 checkpoint function in irradiated populations is a lethal event; yet, the mechanisms underlying these processes are poorly understood. Beginning some 24 h following irradiation, DNA degradation has been observed in irradiated HeLa cells *(56)*. It has been reported that a novel form

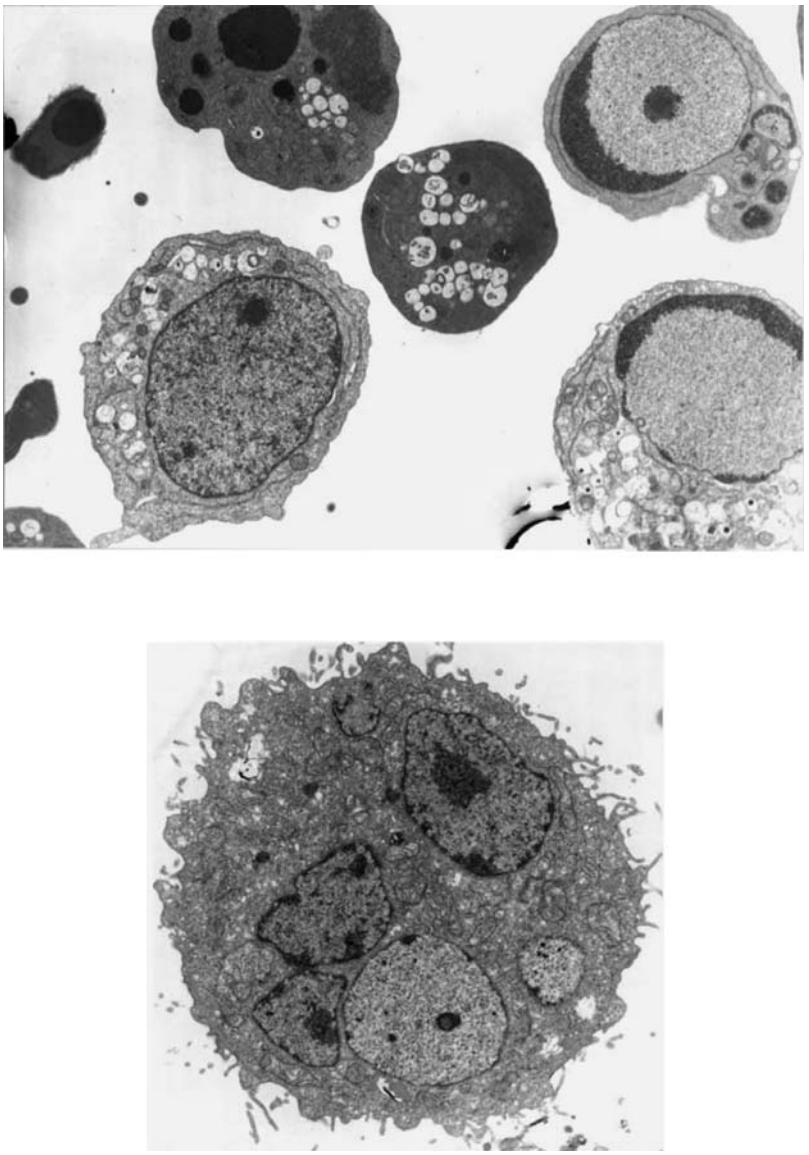

Fig. 2. Morphological comparison of cells undergoing apoptosis (top panel) and mitotic catastrophe (bottom panel). Glutaraldehyde-fixed cells were embedded in resin, sectioned, and stained with osmium tetroxide, as described in Swanson et al. *(2)*. Top panel: HL-60 cells undergoing etoposide-induced apoptosis fixed at 4 h post-etoposide treatment (68 μM) (×2950). Apoptotic cells can be seen presenting blebbing of the membrane, extensive vacuolization, and condensation and marginalization of chromatin. Bottom panel: HeLaS3 cells irradiated with 10 Gy X-irradiation were fixed at 24 h post-irradiation (×3800). Multiple nuclei can be seen in this giant cell; each nuclear fragment is enveloped in an intact nuclear membrane, a characteristic feature of mitotic catastrophe as reported in Swanson et al. *(2)*.

of DNA damage is found in HeLa cells following radiation-induced MC *(57)*. This damage produces "non-sticky" free 3′-OH groups in cellular DNA (a chemical form of DNA damage not directly produced by ionizing radiation) and is thus recognized by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay, yet apoptosis does not occur under these conditions *(17,22,26,58,59)*. These results were preceded by a study using transmission electron microscopy that demonstrated in cells undergoing heat-induced MC that the reformation of the nuclear membrane occurred within chromatin fragments *(2)*, consistent with observation that delayed DNA damage is also observed following heat-induced MC (unpublished data from the authors' laboratory), and that morphological changes associated with apoptosis and other modes of cell death were not associated with MC (Fig. 2 and Table 2). Reformation of the nuclear membrane itself is unlikely to cause chromatin disaggregation; rather, it has been suggested that cytoplasmic nucleases partially digest incompletely condensed chromatin during SPCC, thus producing the observed damage *(57,60)*. Alternatively, aberrant re-condensation of chromatin in mitosis-arrested cells through degradation of cyclin B1, mediated by proteosome activities that usually occur in late

Table 2
Modes of Cell Death

Event	*Definition*	*Morphology*	*Assays*
Apoptosis	Programmed cell death. A distinct mode of cell destruction. Represents a major regulatory mechanism in eliminating abundant and unwanted cells during embryonic development, growth, differentiation, and normal cell turnover	Rapid cell shrinkage; condensed and marginalized chromatin; convolution or "blebbing" of the plasma membrane	Agarose gel electrophoresis; electron microscopy; TUNEL; FCM; WB; IP; IF
Anoikis	Detachment-induced apoptosis occurring when cells lose contact with the extracellular matrix	Same features as for apoptosis, except that it occurs in rounded-up cells detached from the extracellular matrix	Agarose gel electrophoresis; electron microscopy; TUNEL; FCM; WB; IP; IF
MC	Post-mitotic event driven by abrogation of the G2/M checkpoint in cells that have non-functional p53 and are exposed to various damaging agents. Often results in the generation of aneuploid and polyploid cell progeny	Giant cells; spontaneous premature chromosome condensation (SPCC); nuclear fragmentation	Light microscopy; Electron microscopy; TUNEL; FCM; WB; IP; IF

anaphase, may contribute to this effect *(61)*. Regardless of the mechanism(s) underlying this late-appearing DNA damage, cells that are able to survive this process might possess a high level of gene mutations. In fact, it has been demonstrated that exposure of cells to various non-genotoxic stresses (including heat exposure) can lead to persistent genetic instability in surviving cells *(62)*. In another study using LSDCAS *(50)*, 50% of the colonies derived from cells surviving a 5 Gy radiation exposure were found to be abnormal with continuous production of MC for many cell generations following irradiation; moreover, the incidence of MC in some surviving clones did not diminish during colony formation.

4. PROMOTING MC MAY ADVANTAGEOUSLY INCREASE CLINICAL OUTCOME

Some types of human glioblastomas have functional p53 but fail to undergo apoptosis because of radiation treatment rendering the prognosis extremely unfavorable. Somatic mutations in phosphatase and tensin homologue on chromosome 10 gene (PTEN), a phosphatase tumor suppressor protein *(63)* that dephosphorylates phosphatidylinositol-3-phosphate (PIP3), a lipid second messenger that is produced by activated phosphatidylinositol-3-kinase (PI3K) *(64–66)*, occur in a vast number of tumors and most markedly in glioblastomas; these mutations represent one of the culprit in the gliomas' resistance to radiation treatment. In general, PTEN mutants retain protein tyrosine phosphatase activity but are not able to dephosphorylate PIP3 *(66,67)*; thus, PTEN-deficient tumors have elevated levels of PIP3. Activation of PI3K through mitogens or growth factors, both in normal and in cancer cells, starts a cascade of events that includes activation of Akt/protein kinase B, a serine-threonine kinase that phosphorylates and inhibits pro-apoptotic BAD *(68)*, caspase 9 *(69)*, and members of the fork head family of transcription factors *(70)*, and increases in nuclear factor-κB activity, thus facilitating the induction of genes that suppress apoptosis *(71–73)*. Moreover, PI3K/Akt favors translocation of Mdm2 from the cytoplasm onto the nucleus where it negatively regulates the pro-apoptotic function of p53 *(74)*. Another protein, survivin, has also been shown to play a role as suppressor of apoptosis in gliomas. Survivin is a 16.5-kDa protein member of the inhibitor of apoptosis (IAP) family; it is expressed in G2/M phases of the cell cycle *(75–77)* and is associated with gliomas progression from low to high grade *(78–82)*. It has been shown that survivin enhances survival of tumor cells through suppression of apoptosis through inhibition of the caspase cascade pathway *(83–85)*. It has also been reported *(86)* that radiation increases levels of phosphosurvivin, a more stable survivin isoform than its unphosphorylated counterpart *(87)*, and that radiation causes survivin to compartmentalize in the nucleus, a phenomenon that has been associated with enhanced double-strand DNA repair capability of exposed cells *(86)*. Thus, survivin appears to be implicated in radiation resistance through the activation of more than one pathway in human glioblastomas. A preferential upregulation of genes involved in the DNA repair/replication pathways following radiation exposure of U87MG cells has also been reported *(88,89)*. In both normal and neoplastic astrocytic cells, DNA repair, not programmed cell death, is responsible for p53 induction in response to cytotoxic agents *(90)*. Thus, it appears that upregulation of DNA repair is a fundamental property of astrocytes exposed to clastogenic agents. The result of this capability is that these cells are resistant to the action of cytotoxic agents.

Recent studies *(40)* have shown that transduction of a dominant-negative p53 adenovirus construct in p53 wild-type U87MG cells leads to the induction of MC through overaccumulation of cyclin B1 and abrogation of the G2/M checkpoint. These results suggest that although mutant adenovirus transduction methodology cannot be used as anticancer therapy because of the inherent damage to the surrounding normal cells, interventions aimed at facilitating abrogation of the G2/M check point and at inducing MC may represent a novel mechanism to augment radiation efficacy in wild-type p53 cells that are resistant to apoptosis.

5. MC AND TUMOR PROGRESSION

5.1. Endopolyploid Cell Formation and Reductive Cell Division in Populations that Have Undergone MC

Illidge and collaborators *(91)* have reported polyploid giant cell formation (what the authors of this review would refer to as MC) after irradiation of human lymphoblastoid Namalwa cells. The polyploid cell population escapes death and is capable of releasing reproductive descendants as it enters an endocyclic path in which the polyploid cells divide and give rise to diploid or tetraploid cells during a restitution period. Data from the same group *(92)* also showed the presence of chromosome double loops in the giant cells by the second week post-irradiation. These formations are referred to as polyploid "bouquets." Cells bearing these features return to an interphase state and subsequently separate into several secondary nuclei within the giant cell. These subnuclei eventually resume DNA synthesis and sequester cytoplasm around their genetic material giving rise to secondary cells that are able to continue mitotic propagation. Using the same cell line, Ivanov and collaborators *(93)* have also reported that a proportion of lymphoblastoid Namalwa cells exposed to severe genotoxic treatment undergo MC and endoreduplication giving rise to endopolyploid cells that resemble cells in meiotic prophase. Homologous DNA recombination repair occurs in these cells, and they replicate their DNA for more than a few rounds of endomitotic cycles producing mitotic descendants. Thus, endopolyploid cells, originated from MC cells, present a survival advantage over the remaining proportion of cell population that can die through apoptosis.

During megakaryocyte differentiation, the immature megakaryocyte (promegakaryoblast) undergoes a series of endocycles increasing its ploidy to a 2X DNA content *(94)*. Megakaryocytes of 8–16N DNA content express cyclin B1 and cyclin B1-dependent H1 kinase activity with the cells retaining the ability to enter subsequent endomitotic cycles. It has been reported *(95)* that chromosome segregation did not require cyclin B1 degradation, whereas inhibiting the inactivation of cyclin B1/cdc2 complexes stopped chromosome decondensation and prevented migration of telophase chromosomes *(96)*. Studies performed using a non-destructible form of cyclin B1 in prometaphase kidney cells of normal rats have shown that the principal effect of inactivation of cyclinB1/cdc2 complexes is on the spindle dynamics that regulate chromosome migration and cytokinesis *(97)*. Thus, de-regulated degradation of cyclin B1 could partly explain the processes occurring during endomitosis. Interestingly, overaccumulation of cyclin B1 protein and activation of cyclin B1/cdc2 complexes were also described by the authors of this review *(4,7,27,57)* in irradiated and mild-heated HeLa cells undergoing MC.

These findings point out that MC and endomitosis might share common pathways of activation.

Apart from the megakaryocyte in which polyploidization is a phenomenon occurring during normal differentiation, the other reported findings pertain to cells that lack p53 function. These results contribute to the notion that cancer cells that escape cell death after genotoxic treatment and undergo MC and endoreduplication are not reproductively dead but retain proliferative potential.

Moreover, work carried out by our group *(98)* shows that irradiated HeLa cells that have undergone MC and have been cultured for up to 20 days post-irradiation present similar morphological features as those present in meiotic prophase and described in ref. *21*. Meiosis or reduction division occurs in germ cells, and it results in haploid cells with new combination of genes in the daughter cells because of chromosome crossover; mitosis occurs in somatic cells, and it involves the division of one diploid mother cell into two identical diploid daughter cells. Although meiosis is considered a rare event in normal somatic cells, this might not be the case in cancer cells where a highly unstable genome is present *(99)*. In particular, we report that following irradiation, HeLa cells undergo MC and form endopolyploid cells. By the end of the first week post-irradiation, the majority of the cells that underwent MC die. At 7–9 days post-irradiation and later, the polyploid cells that have escaped death stop DNA synthesis and initiate a de-polyploidization process. A portion of the endopolyploid cells is then able to segregate its nuclei and to produce viable descendants. DNA image cytometry reveals that when mitoses are resumed, the cells' DNA content is reduced from 8N to quasi-diploid in anaphases by subsequent reduction divisions that appear to occur omitting S phase passage. These data imply that endopolyploid tumor cells conserve their original individual genomic integrity and can re-initiate mitosis through reduction division *(98)*. Thus, the ability of a cancer cell exposed to genotoxic agents to first overcome death and then to put in place an array of pathways that imply the occurrence of MC, polyploidization and endoreduplication, reduction division, and restitution to mitotic division needs to be looked at very closely as these pathways are likely to be responsible for the acquisition of resistance to treatment and to confer a higher chance for long-term survival and might, therefore, be important mechanisms in tumor progression in vivo.

5.2. Cells that Undergo MC and Escape Death Later are Thought to Divide Through Neosis

Sundaram and collaborators *(100)*, using irradiated mouse embryo fibroblasts and human tumor cell lines, have reported what they define as a novel type of cell division in cancer cells called neosis. After radiation treatment, cells undergo MC and while the majority of them die, a small proportion of cells divide through neosis. Neosis is characterized by karyokinesis through nuclear budding followed by asymmetric, intracellular cytokinesis from which several small mononucleated cells originate. These small mononucleated cells have extended mitotic life span and were termed by the authors Raju cells. Raju cells are thought to be progenitors of transformed cells. In the human metastatic neuroblastoma HTB11 cell line, neosis was preceded by an absence of mitotic activity for about 8 weeks; the onset of mitotic crisis was accompanied by the formation of large polyploid cells (MC cells) followed by neosis and production of small Raju cells with an extended mitotic life span *(100)*.

The results reported above are intriguing and interesting, and mirror preliminary results of work in progress in the laboratories of the authors of this review. We are analyzing late MC events in irradiated HeLa cells, and we find that by day 18 post-irradiation, the HeLa cell population that has undergone MC and has escaped death consists of polyploid cells, multi-nucleated cells, small mononucleated cells, and endomitotic giant cells that are in the process of extruding small mononucleated cells that resemble the Raju cells described by Sundaram and collaborators *(100)*. Thus, a captivating parallel seems to exist between the phenomenon described by Sundaram and collaborators *(100)* and that noticed by us. Whatever the name used to designate delayed division of MC or giant cells and their progeny, it will be important to determine how this progeny of apparently normal mononucleated cells arising from reduction division contributes to tumor progression. We have begun extensive analysis of genotoxically treated human cancer cells using LSDCAS. Experiments performed using LSDCAS will allow for the quantitative determination of the ultimate fate of irradiated cells that undergo MC and escape death. Understanding the mechanisms underlying the appearance of clonogenic cells from polyploid cell populations might lend further light on the mechanisms involved in cancer resistance to genotoxic treatment.

6. CONCLUDING REMARKS

MC is a common mechanism underlying mitotic-linked cell death, and it is the result of premature entry into mitosis occurring during delays late in the cell cycle. A predominant effect in cells lacking p53 function, MC has been observed after a variety of treatments with cytotoxic agents and generally results in the production of polyploid cell populations. The mechanism underlying the premature entry into mitosis, which immediately precedes MC, involves the overaccumulation of cyclin B1, which is thought to overcome mitosis inhibitory pathways through mass action. Although most cells die because of MC, there is evidence that a small fraction of cells can survive this event. The mechanism underlying the restoration of clonogenicity in populations that have undergone MC is still an active research area, but some reports in the literature suggest a potential role for meiotic cell division mechanisms in the generation of quasi-diploid cell fractions from polyploid parental populations. Understanding the specific mechanisms underlying the temporary change from a pro-mitotic to a pro-meiotic division regimen might lend important insights into phenomena occurring during human tumor progression.

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5 Autophagy and Autophagic Cell Death

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Summary

Macroautophagy or autophagy is a degradative pathway terminating in the lysosomal compartment after the formation of a cytoplasmic vacuole that engulfs macromolecules and organelles. During the last decade, progress made in our understanding of the molecular controls of autophagy has uncovered the importance of tumor suppressor molecules in the stimulation of autophagy. Downexpression of autophagy is an early event during tumorigenesis. However, the relation between autophagy and tumor progression seems to be more complex because cancer cells are able to trigger autophagy in response to various situations including changes in their extracellular environment and cancer treatments. The role of autophagy in cancer cells balances between two apparently opposite outcomes. Autophagy as a stress response mechanism can protect cancer cells from various insults. But autophagy can eliminate cancer cells by triggering autophagic cell death. These two aspects of autophagy will be discussed in this review.

Key Words: Autophagy; apoptosis; cell death; cancer; macroautophagy; lysosome; signal transduction.

1. INTRODUCTION

Autophagy is a lysosomal degradative mechanism occurring in different modes (chaperone-mediated autophagy, microautophagy, and macroautophagy) *(1)*. This chapter is dedicated to macroautophagy (hereafter referred as to autophagy). Autophagy is a general and evolutionarily conserved vacuolar catabolic pathway terminating in the lysosomal compartment *(2–4)*. It contributes to the quality control of cytoplasmic components by recycling macromolecules (autophagy is responsible for the turnover of long-lived proteins) and removing organelles when damaged or in excess (peroxisomes, mitochondria). From a cell biology standpoint, autophagy is characterized by the formation of a multimembrane-bound autophagosome that engulfs portions of the

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cytoplasm. The delimiting membrane of the autophagosome is derived from an 'isolation' membrane or phagophore of unknown origin *(5)*. In mammalian cells, most autophagosomes can fuse directly with lysosomes or merge with endocytic compartments *(6,7)* to form an amphisome where the sequestered material is denatured because of the acidic environment. The final step is the fusion of amphisomes with the lysosomal compartment where the sequestered material is degraded.

The discovery of autophagy by de Duve and Wattiaux *(8)* was contemporary with that of lysosomes. The physiological importance of autophagy in maintaining cell homeostasis in organs such as liver and in cultured cells rapidly emerged *(9,10)*. At the same time, the term autophagic cell death or type II programmed cell death (PCD II) was introduced to describe a cell death different from apoptosis or type I PCD (PCD I) *(11,12)*. From these data, it appeared that autophagy is a cell response to stress, which under certain circumstances can lead to cell death. However, the precise role of autophagy as a cell death mechanism remains to be explored *(13)*.

Identification of its molecular machinery and signaling pathways has shed some light on the importance of autophagy in physiological processes and diseases *(1,2,14)*. Among the autophagy-related (*ATG*) genes, discovered in yeast and almost integrally conserved in all eukaryotic phyla, which control the formation of the autophagosome *(15)*, *beclin 1* (the mammalian ortholog of the yeast *ATG6*) is a tumor suppressor gene that contributes to a complex with the class III phosphatidylinositol-3-kinase (PI3K) to the formation of the autophagosome *(16,17)*. Other tumor suppressor gene products [p53, PTEN, TSC1/TSC2, death-associated protein kinase (DAP kinase)] are involved in the control of autophagy *(18)*. Interestingly, autophagy is also stimulated in cancer cells by ceramide *(19,20)*, a tumor suppressor lipid *(21)*.

The aim of this chapter is to discuss the role of autophagy in cancer cell survival and death. Readers interested in a complete review of autophagy are referred to the different chapters of the book *Autophagy* edited by Daniel Klionsky *(22)*. In addition, several recent reviews were dedicated to the role of autophagy during cell death *(13,23–25)* and tumor progression *(26–28)*.

2. MOLECULAR CONTROL OF AUTOPHAGY

In this section, we will briefly summarize the molecular machinery involved in the formation and maturation of autophagic vacuoles and signaling pathways that modulate autophagy. Readers who want a more complete view of the role of Atg and signaling of autophagy can consult several recent reviews on these topics *(29–32)*.

2.1. ATG Complexes Implicated in the Formation of Autophagosome

The discovery of *APG* (autophagy, *33*) and *AUT* (autophagocytosis, *34*) genes that regulate autophagy in the yeast *Saccharomyces cerevisiae* has contributed to our understanding of the molecular control of autophagy. Many *APG* and *AUT* genes are identical to *CVT* genes involved in the cytoplasm-to-vacuole (the yeast lysosome) targeting of some enzymes (aminopeptidase I, vacuolar α-mannosidase) under nutrient-rich conditions *(35)*, whereas autophagy directs them to the vacuole under starvation conditions. Recently, a unique nomenclature *ATG* for these genes has been introduced *(15)* and will be used hereafter in this review.

2.1.1. Conjugation Systems

The formation of the autophagosome is dependent on two conjugation systems reminiscent of the ubiquitination of proteins in the proteosomal pathway that function in a coordinated manner *(29,30,32,36)*. The first conjugation system involves four Atg proteins 5, 7, 10, and 12. The final complex is formed by Atg5–Atg12 non-covalently associated with the coiled-coil protein Atg16. Membrane-associated multimerization of Atg16 and Atg5–Atg12 conjugate is required for autophagy. In this conjugation system, Atg7 and Atg10 play the role of the E1 and E2 enzymes in the ubiquitin pathway, respectively. This conjugation system is conserved in mammalian cells where it initiates the formation of the sequestering membrane before its retrieval to cytosol *(37)*. The second conjugation system is characterized by the conjugation of a protein Atg8 with a phospholipid, phosphatidylethanolamine. This system is dependent on the activity of Atg7 and Atg3, which have functions similar to those of the E1 and E2 enzymes in the ubiquitin pathway, respectively. Atg4, which is a protease belonging to the cysteine protein family of caspases, is required for the processing of Atg8 before the conjugation reactions and for its release from the phospholipid anchor *(38)*. Several Atg8 mammalian homologs GATE-16 Golgi-associated ATpase enhancer of 16KDa, MAP1-LC3 Microtubule associated protein 1 light chain 3, GABARAP γ-aminobutyric acid A receptor-associated protein (GATE-16, MAP1-LC3, GABARAP) can be engaged in Atg3-dependent conjugating reactions *(36,39)*. One of these homologs, MAP1-LC3, is engaged in the formation of the autophagosome after a processing similar to that of yeast Atg8 *(40)*. As the lipid-modified form of Atg8 (MAP-LC3) remains associated with the autophagosome's outer and inner membrane, these proteins are useful to monitor autophagy either morphologically or biochemically because of the change in electrophoretic mobility due to the lipid modification of the protein during autophagy *(41,42)*. The role of other Atg proteins in biogenesis of the autophagosome has been discussed in recent reviews *(32,43)*.

2.1.2. Class III PI3K

The first evidence for the implication of this enzyme stemmed from the discovery that 3-methyladenine (3-MA) is able to block the formation of autophagosomes *(44)*. After this seminal observation, it was demonstrated that wortmannin and LY294002, two PI3K inhibitors, interfere with the formation of autophagosomes in rat hepatocytes *(45)*. Further studies have shown that 3-MA inhibits autophagy by interfering with the activity of class III PI3K *(46)*.

Class III PI3K is associated with the membrane-anchored p150 adapter, which tethers the enzyme to cytoplasmic membranes *(47)*. In *S. cerevisiae*, the class III PI3K (Vps34) and its membrane adaptor Vps15 have been shown to control autophagy *(48)*. Vps34 and Vps15 exist in two complexes that control different membrane transport processes. Complex I, which also contains Atg14 and Atg6(Vps30), controls autophagy, whereas complex II, which is composed of Vps38 and Atg6(Vps30) in addition to Vps34/Vps15, governs the vesicular transport to the vacuole (yeast lysosome). In mammalian cells, class III PI3K forms a complex with Beclin 1 (the ortholog of the yeast Atg6) *(17)*.

In addition to a role in membrane transport processes (reviewed in *49*), class III PI3K controls autophagy by its interaction with Beclin 1 *(50,51)*. Moreover, this interaction is essential for tumor repressor function of Beclin 1 but does not interfere with the class III PI3K-dependent vacuolar protein sorting *(50)*.

3. REGULATION AND SIGNALING OF AUTOPHAGY

3.1. Overview

As a great variety of stimuli are able to modulate autophagy, it is not surprising that numerous signaling pathways have been shown to control this process: tyrosine kinase receptors, protein kinase A, casein kinase II, MAP kinases, and calcium have been shown to interfere with autophagy *(31,52)*. However, the mechanisms by which these signaling pathways contribute to the control of autophagy are still largely unknown and require the identification of downstream targets. This variety probably reflects the diversity of stimuli able to modulate autophagy and also the cell-type specificity needed to integrate these stimuli. It can be envisioned that stimuli are *in fine* transduced to a common target upstream of the molecular machinery engaged in the formation of the autophagosome. The kinase TOR (target of rapamycin) occupies this privileged position in autophagic signaling in all eukaryotic cells *(53,54)*. The class I PI3K-signaling pathway, which acts upstream of mammalian Target of Rapamycin (mTOR), plays a major role in the control of autophagy in metazoans *(46,55,56)*.

3.2. Class I PI3K/mTOR Pathway

Class I PI3K enzymes phosphorylate PtdIns4P and PtdIns$(4, 5)P_2$ to produce PtdIns$(3, 4)P_2$ and PtdIns$(3, 4, 5)P_3$, which, through Pleckstrin homology (PH) domains, recruit protein kinase B (Akt/PKB) and its activator phosphoinositide-dependent kinase-1 (PDK1) *(57,58)* at the plasma membrane. The PI3K/PKB-signaling pathway modulates the function of numerous substrates involved in cell survival, cell cycle progression, and cellular growth, which are frequently altered in human cancers *(59,60)*. The plasma membrane-associated class I PI3 kinase transduces a negative signal for the biogenesis of autophagic vacuoles in HT-29 cells *(46,61)*. Through its lipid phosphatase activity, the tumor suppressor PTEN, which is mutated in various cancers, dephosphorylates the phospholipid produced by class I PI3 kinase *(60,62,63)*. Overexpression of wild-type PTEN, but not of a phosphoinositide phosphatase-deficient mutant of PTEN, counteracts the down-regulation of autophagy in HT-29 cells after activation of PI3 kinase *(61)*. PTEN positively regulates autophagy by inhibition of the PI3K/PKB-signaling pathway in human intestinal cancer cells. In addition, a dominant-negative mutant of PKB increases autophagy and a constitutively active form of PKB decreases autophagy *(61)*, suggesting a critical role of the PKB oncogene in the negative regulation of autophagy in HT-29 cells. The expression of a mutant of Ras (Val12Y40C) that specifically activates the class I PI3K-signaling pathway decreases autophagy in transformed fibroblasts *(64)*. These different data allow us to speculate that genetic disruption of autophagy control can stimulate oncogenesis.

Further experiments on colon and breast cancer cells showed that the lipid tumor suppressor ceramide stimulates autophagy by down-regulation of the PI3 kinase/PKB pathway *(19)*. Interestingly, programmed autophagy is induced by ecdysone in the fat body during *Drosophila* development through the inhibition of the class I PI3K pathway *(65)*. Another study in *Drosophila* shows the importance of the class I PI3K and its downstream effector the kinase TOR in regulating starvation-induced autophagy *(55)*. However, TOR can sense starvation independently of the class I PI3K pathway (reviewed in *53*). In fact, the first evidence for a role of mTOR in the control of autophagy was obtained in rat hepatocytes *(66)*, where the inhibitory effect of amino

acids on autophagy could be relieved by rapamycin, an inhibitor of TOR. Accordingly, the inhibition of TOR in yeast, where the class I PI3K pathway is absent, stimulates autophagy *(67)*. The role of events downstream of TOR controlling autophagy has been discussed in recent reviews *(31,68)*.

4. AUTOPHAGY IN CANCER CELLS

Autophagy may suppress tumor development by causing cell killing that would limit cell population size or otherwise by maintaining a low DNA mutation that would prevent oncogenesis *(69)*. Although autophagy has been found to occur in various types of tumor cells *(70)*, cancer cells often display reduced autophagic capacity *(16,27,28,71)*. A reduced rate of autophagic activity has also been reported in chemically transformed rat pancreatic cells in which autophagic capacity increases at precancerous stages and then diminishes during adenoma to carcinoma transition *(72)*. This suggests that autophagic activity is decreased during malignant progression.

4.1. Autophagy in Tumor Development

The link between autophagy and tumor development is also supported by the fact that some tumor suppressor proteins and oncoproteins are involved in the control of autophagic activity in cells (reviewed in *27,28,31*). Beclin 1, PTEN, PKB/class I PI3-kinase pathway, DAPK, Ras, c-Myc, p53, and Bcl-2 are examples of these categories of proteins (Fig. 1). The implication of *beclin 1* in tumor progression was suggested by the observation that this gene is monoallelically deleted in a high percentage of human cancers and tumor cell lines *(16)*. Stable transfection of *beclin 1* into MCF-7 human breast carcinoma cells reduced their tumorigenicity in nude mice and slowed their proliferation rate *(16)*. Furthermore, *beclin 1*-haploinsufficient mice displayed an increased incidence of spontaneous tumors *(73,74)*, and the cell-proliferative capacity was markedly increased in some of their tissues.

The increased expression of Akt/PKB and the loss of function of PTEN protein, an inhibitor of the PI3K/PKB pathway, are frequently observed in cancer cells. As discussed in Section 3, the PTEN tumor suppressor can induce autophagy in human

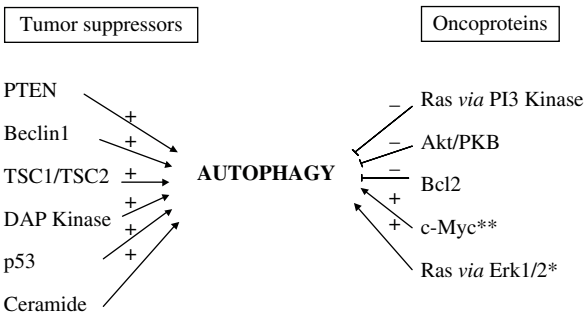

Fig. 1. Control of autophagy by tumor suppressors and oncoproteins. Autophagy is stimulated by tumor suppressors and inhibited by oncoproteins. **The proto-oncogene c-Myc has been shown to induce autophagy independently of its oncogenic property *(75)*. *The stimulation of autophagy through the ras-dependent ERK1/2-signaling pathway has been shown in human colon cancer cells *(76,77)*.

colon cancer cell lines whereas the activation of Akt/PKB has an inhibitory effect on autophagy *(61)*. These findings suggest that PTEN and the Akt/PKB pathway may participate in tumor progression by interfering with autophagy. Furthermore, Akt/PKB-induced inhibition of autophagy is mediated through mTOR, which is regarded as a "gate keeper" of the autophagy pathway (see Section 3). This inhibition requires the expression of two tumor suppressors, TSC1 and TSC2 *(88,89)*. The role of mTOR in oncogenesis is supported by the fact that the well-known inhibitor of mTor, rapamycin, displays antitumor effects in several tumor models *(80)*.

DAP kinase and its homolog Drp-1, which have tumor suppressor properties and control apoptotic membrane blebbing, have been shown to stimulate autophagy in response to amino acid starvation, tamoxifen, and interferon (INF)-γ *(81)*.

Recent observations indicate that p53, a known regulator of cell growth and proliferation, is also involved in the modulation of mTOR activity and autophagy *(82)*.

4.2. Autophagy in Established Tumors

Cancer cells are able to induce autophagy in response to stress conditions by adapting their metabolism to survive. This might occur when tumors are poorly vascularized and cancer cells have to survive in low oxygen and reduced nutrient conditions *(83–85)*. In agreement with this assumption, it has been reported that endostatin, an inhibitor of angiogenesis, induces autophagic cell death in endothelial cells *(86)*. Although these

Table 1
Autophagy as a Response to Cancer Therapy

Effectors	*Cells*	*References*
Epidermal Growth Factor Receptor EGFR inhibitors	Glioma	*(87)*
Platelet derived growth factor Anti-PDGF	Glioma	*(88)*
Interferon (INF)-γ	Cervix cancer cells	*(81)*
Tumor necrosis factor (TNF)-α	Leukemic cells	*(101)*
Peroxysome proliferator activated receptor PPAR-γ activation	Neuroblastoma	*(89)*
Radiation	Breast, colon, prostate	*(99)*
Arsenic trioxide	Glioma	*(90)*
Celecoxib	Colon cancer cells	Unpublished results
Lipooxygenase inhibitors	Pancreatic cancer cells	*(91)*
Resveratrol	Ovarian cancer cells	*(92)*
Tamoxifen	Breast cancer cells	*(97)*
Temozolomide	Glioma	*(93)*
Etoposide	Murine pluripotent hematopoietic stem cells	*(111)*
Rapamycin	Breast cancer cells	*(94)*
	Glioma	*(95)*
Histone deacetylase inhibitors	Cervix cancer cells	*(96)*

studies point out the role of autophagy in solid tumor development, the relationship between angiogenesis and autophagy needs to be investigated further.

4.3. Autophagy and Cancer Therapy

Although many anticancer drugs have shown to induce apoptosis in cancer cells, autophagy is also frequently observed in response to these treatments *(26)* (Table 1). Anticancer drug-induced autophagy can lead either to cell death or alternatively to protection against cell death induced by these treatments (Table 1). For example, tamoxifen, a well-known anticancer drug, induces autophagic cell death in human mammary carcinoma MCF-7 cells *(97,98)*. By contrast, autophagy is a cell survival mechanism in response to certain drugs and treatments such as low radiation, sulindac, and tumor necrosis factor (TNF)-α *(99–101)*. However, the mechanisms by which anticancer drugs promote autophagy remain to be fully elucidated.

5. AUTOPHAGIC CELL DEATH

Although apoptosis (PCD I) is well characterized, less is known about the autophagic cell death (PCD II) that can also occur in cells *(24,25)*. Apoptotic cells display nuclear fragmentation, membrane blebbing, externalization of phosphatidylserine at the outer leaflet of the plasma membrane, and activation of caspases. The latter proteases mediate cell death by cleaving several cellular components that are eliminated by phagocytosis. Programmed autophagic cell death is characterized by the appearance of autophagic vacuoles. This self-digestion mechanism of cells causes a reduction of cell mass in tissues and may especially take place in situations in which the number of dead cell bodies exceeds phagocytosis capacity. It is worth nothing that the induction of autophagic vacuoles in cells may reflect autophagy-induced cell death or alternatively autophagy-induced cell survival, but the molecular distinction between these two types of autophagy is currently not defined. In addition, the distinction between apoptosis and autophagic cell death is not so clear-cut because autophagic vacuoles can be observed in dying cells with apoptotic features *(27,102)*.

5.1. Autophagy and Apoptosis

Numerous reports reveal that apoptosis and autophagy pathways are linked (reviewed in *25,27,103,104*). This interpretation is based on results showing that (i) autophagy and apoptosis share some common molecular signaling pathways, (ii) several inducers of apoptosis can promote stimulation of autophagy, and (iii) in some instances, specific inhibition of each cell death pathway (apoptosis or autophagy) can switch cells to the other form of death.

It has been reported that several actors of both death receptor- and mitochondria-mediated apoptosis are also involved in the regulation of autophagy. For example, activation of a receptor-interacting protein (RIP) and c-Jun N-terminal kinase (JNK) pathway induced autophagic cell death in L929 cells in response to inhibition of caspase activation *(105)*. TRAIL, a well-known ligand of death receptor-mediated apoptosis, induces autophagy in a model of the formation of lumens in mammary acini *(106)*. Stimulation of autophagy is observed in human T-lymphoblastic cells in response to TNFα, another ligand of the death receptor pathway *(101)*. Recently, the interaction of

Atg5 with the death domain of Fas-associated death domain protein (FADD) has been shown to play a crucial role in INFγ-induced cell death independently of detectable activation of caspase 8 *(107)*. Moreover, ceramide, a well-known mediator of death receptor-induced apoptosis, is also an actor of autophagy activation and autophagic cell death *(19,20)*. The kinase Akt/PKB, which is involved in the insulin-like growth factor (IGF) receptor-signaling pathway, exerts negative control in both apoptosis and autophagy *(28,59)*. In addition to its pro-apoptotic activity, DAP kinase can mediate autophagic vacuolization in a caspase-independent manner *(27)*. Inhibition of caspase-8 activity, which is known to be mainly stimulated by death receptor signaling, has been shown to induce autophagic cell death in L929 cells associated with the up-regulation of the *atg7* and *beclin 1* genes *(105)*.

Mitochondria are another common mediator of apoptosis and autophagy *(104,108–110)*. Their removal by autophagy in response to etoposide has been observed *(98)*. In addition, Tolkovsky and co-workers *(112)* reported that the blockade of apoptosis leads to selective elimination of mitochondria in neuronal cells in response to several apoptotic inducers including the withdrawal of nerve growth factor (NGF). This effect is prevented by 3-MA treatment, indicating that the loss of mitochondria was caused by their sequestration by autophagosomes. Similarly, Kirkland et al. *(113)* demonstrated that autophagy and lipid peroxidation are required in the reduction of mitochondrial mass in NGF-deprived sympathetic neurons. It has been shown that depolarized mitochondria can be selectively sequestrated by autophagosomes in primary hepatocytes *(114)*. This observation led to the suggestion that autophagy may protect against apoptosis by elimination of damaged mitochondria that release pro-apoptotic signaling molecules such as cytochrome c, Apaf, and reactive oxygen species. It has been shown in yeast that the autophagic degradation of mitochondria induced by rapamycin or nitrogen starvation requires the protein Uth1p mainly localized in the outer mitochondrial membrane of these cells *(115)*. The lack of Uth1p was found to induce resistance to both Bax expression and rapamycin, a well-known inducer of autophagy, suggesting it plays role in a death pathway related to autophagy. Another link between autophagy and mitochondria is the interaction of Beclin 1 with the antiapoptotic Bcl-2 protein *(116)*. Recently, it has been shown that this interaction negatively regulates autophagy during cell starvation *(117)*. These findings are consistent with previous observations showing that overexpression of Bcl-2 prevents autophagy *(110)*, whereas its down-regulation stimulates this process *(118)*. The BH3-like domain protein BNIP3 and the BH3-interacting protein Hspin1 stimulate autophagy in cells *(20,119,120)*. As the mechanisms involved in the recognition of mitochondria by the autophagic machinery are unknown at present, the literature data suggest that the protein Uth1p in yeast, the BNIP3 protein in mammalian cells, or lipid oxidation of the mitochondrial membrane are possibly involved in this process.

The existence of a double switch between apoptosis and autophagy has been demonstrated by experiments involving the blockage of one or another death pathway. There are examples in the literature in which the inhibition of caspase activation can lead to autophagic cell death *(105,112)*. Reciprocally, it has been shown that impaired autophagy can trigger apoptotic cell killing *(121)*.

5.2. Autophagic Cell Death

Autophagy related to cell death can occur in response to stress conditions, such as anticancer treatments, as summarized in Table 1. Tamoxifen is one anticancer drug whose induction of autophagic cell death is well characterized in MCF-7 cells *(97,98)*. Tamoxifen-induced autophagy is associated with increased expression of Beclin 1 *(19)*. Both these responses are mediated by ceramide, which is considered a tumor suppressor lipid *(21)*. The association of autophagic vacuoles with cell death is also observed in various cell types during development and aging and also in several diseases, notably neurological disorders *(13,122–124)*. In *Drosophila* development, the involution of the salivary glands requires apoptosis associated with autophagic features *(125)*. Neuronal development is associated with both apoptosis *(126)* and autophagy-related cell death *(127)*.

The link between autophagy and cell death was first demonstrated by experiments showing that 3-MA, a pharmacological inhibitor of autophagy activity, inhibits autophagy and cell death in MCF-7 cells *(97)*. More recently, the role of autophagy in cell death has been shown by RNA interference silencing of the *Atg* genes in different cellular models *(105)*. In mouse L929 fibroblastic cells, reduction of the expression of the *atg7* and *beclin 1* genes inhibits autophagic cell death induced by Z-VAD-fmk, a caspase inhibitor with broad specificity. Similarly, in cells lacking apoptotic machinery ($Bax^{-/-}Bak^{-/-}$ cells), etoposide treatment causes non-apoptotic cell death through *atg*-dependent mechanism *(128)*. In *Dictyostelium discoideum*, disruption of the *atg1* autophagy gene impairs autophagy vacuolization but cells still die by a non-vacuolar mechanism *(129)*, supporting the notion that distinct cell death mechanisms may co-exist in a given cell.

6. CONCLUSION

The importance of autophagy during tumor progression has become increasingly clear in recent years *(27,28,130)*. The expression of several tumor suppressors including tumor suppressor gene products and tumor suppressor lipids is required to stimulate autophagy. Accordingly, a defect in autophagy is observed in many cancer cells and is probably an early event during tumor progression. However, the mechanism by which some tumor suppressor molecules control autophagy remains to be clarified. Uncovering the mechanism by which *beclin 1*, the only identified tumor suppressor gene product among *atg*, regulates autophagy is an important step toward the understanding of its tumor suppressor capacity *(76,77)*. Suppression of autophagy probably contributes to the proliferation of cancer cells and to the increase in tumor mass through the inactivation (complete or partial) of tumor suppressor gene products that restrain the activation of mTOR.

Although it is generally agreed that autophagy is low in cancer cells, the presence of autophagic vacuoles is observed, *in situ*, in many tumors *(70)* and autophagy is stimulated by chemotherapeutic treatments and radiation in a large panel of cancer cells *(28)*. These observations suggest that gene amplification and alternative control of autophagy are effective in cancer cells. Alternatively, one cannot exclude that in the tumor mass, cancer cells with unaltered autophagic potential are selected by stresses such as limitation in nutrient and oxygen supply and the use of non-lethal doses of anticancer treatment. In this scenario, autophagy would be a cell survival mechanism

that contributes to the adaptation of cancer to adverse conditions. Alternatively, autophagy is a cell death mechanism (PCD II), but in many cases signs of both apoptosis and autophagy can be observed in cancer cells *(27,102)*. In this latter case, the temporal intervention of autophagy is important to consider. When autophagy prevails before the point of no return of apoptosis (mitochondrial depolarization), it can be assumed that autophagy would counteract or delay cell death. However, when autophagy is triggered after mitochondrial depolarization, in cells doomed to die, it can contribute to cell dismantlement. This last function is probably a heightened version of the autophagic removal of faulty mitochondria observed in non-apoptotic cells *(109)*. In this context, investigation of the molecular relation between autophagy and mitochondria would better define the role of autophagy during apoptosis but also in mitochondrial diseases and aging. As previously mentioned, cancer cells can trigger autophagic cell death in response to various cancer treatments. However, the mechanisms by which these treatments stimulate autophagy are largely unknown. Elucidation of these molecular controls would be of value in establishing whether autophagic cell death is simply an intensified version of autophagy as classically defined (responsive to amino acids, controlled by mTOR) or a mechanism with alternative controls. Knowing more about the key mechanisms that regulate autophagy may provide some clues about the consequences of manipulating autophagy in cancer cells.

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6 Regulation and Function of Detachment-Induced Cell Death (Anoikis) in Cancer Progression and Metastasis

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Summary

Epithelial cells and possibly all normal cell types must receive adhesion-dependent signals from their microenvironments to survive. The cell death that is triggered when adherent cells detach has been termed "anoikis," and the appreciation that tumor metastasis involves the suppression of anoikis has prompted aggressive investigation into the biochemical and molecular mechanisms that control the process. Much of this work has focused on integrins and the integrin-mediated signaling events that mediate attachment-induced normal cell survival and the molecular mechanisms that are activated in metastatic tumor cells that uncouple these responses. However, it also appears that unique cell death pathway(s) can be activated in metastatic tumor cells when conventional caspase-dependent apoptotic pathways are disrupted, some of which resemble those activated in cells undergoing autophagy (auto-digestion). Here, we will review what is known about the signaling pathways that control attachment-mediated survival in normal cells and the molecular alterations that appear to disrupt these pathways in metastatic cells. We will also review the evidence that alternative cell death mechanism(s) can be activated in metastatic tumors when apoptosis is disrupted.

Key Words: Integrin; AKT; caspase; Bim; autophagy.

1. SOCIAL CONTROLS ON CELL SURVIVAL AND CELL DEATH

Over a century ago, Paget first proposed the "seed and soil" hypothesis, whereby tumor metastasis depends not only on specific tumor cell properties (the "seed") but also on appropriate characteristics found in the host microenvironment (the "soil") *(1,2)*. Work performed by cancer metastasis researchers over the past three decades

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has confirmed Paget's hypothesis and identified some of the specific host factors involved *(2,3)*. Indeed, one important early observation demonstrated that tumor cells are remarkably heterogeneous with respect to their abilities to survive in the circulation and colonize particular host microenvironments, such that the vast majority of cells die en route *(2)*.

Martin Raff *(4)* first proposed the idea that cell death (apoptosis) is the default pathway in all mammalian cells beyond the blastomere stage of development. In a pioneering opinion piece on the subject, he proposed that this default cell death was suppressed in normal cells through signals generated by their specific microenvironments, involving cytokines, hormones, and adhesion molecules *(4)*. He also argued that migration from a normal cell's tissue of origin would strip it of these survival signals, resulting in apoptosis. He suggested that this response would serve as a safeguard to the inappropriate cellular migration that occurs during tumor metastasis, the process that results in the vast majority of the mortality observed in patients with solid tumors.

These observations prompted Frisch and Francis *(5)* and other investigators to directly characterize the effects of detachment on cell death in populations of cells that normally require attachment for their expansion (epithelial and endothelial cells and fibroblasts). These studies demonstrated that detachment results in rapid induction of apoptosis, and Frisch and Francis coined the term "anoikis" to describe the process *(5)*. Anoikis is studied *in vitro* by plating cells on supports that do not allow for cellular attachment (bacterial plates and plates coated with poly-L-lysine) or by rotating them in continuous suspension culture. It is also likely that the classical approach for isolating tumorigenic and metastatic cells by growing them in soft agar also selects for cells that resist anoikis. Whether any of these conditions accurately model the pressures metastatic tumor cells are subjected to *in vivo* remains an unanswered question. Furthermore, it is not clear how long a tumor cell must resist anoikis in transit between the primary tumor and the distant site, and it is possible that the *in vitro* conditions exaggerate the stress exerted upon tumor cells in vivo.

2. ADHESION-DEPENDENT SURVIVAL SIGNALING

Integrins play a central role in mediating adhesion between adherent cells and the extracellular matrix, and the signal transduction pathways they activate to suppress apoptosis in attached cells have been the focus of most of the investigation in the field overall *(6–9)*. Although several different protein kinases are independently activated by integrins, the work performed to date indicates that they converge on the phosphatidylinositol-3′ (PI-3) kinase/AKT (AKR mouse thymoma transforming oncogene) pathway to promote survival *(7,10,11)*. In addition, many of the genetic alterations that accumulate in metastatic tumors, and in particular the mutational inactivation of the lipid phosphatase on chromosome 10 (PTEN) *(12)* and activation of the Ras pathway *(3,11,13)*, also collaborate to promote AKT activation and metastasis and to suppress anoikis.

2.1. Role of AKT

AKT activation is mediated by the generation of 3′-polyphosphoinositides, which bind to AKT's pleckstin homology (PH) domain and recruit it to the inner surface of the plasma membrane *(14)*, and by phosphorylation of AKT at T308 and S473 *(10,15)*.

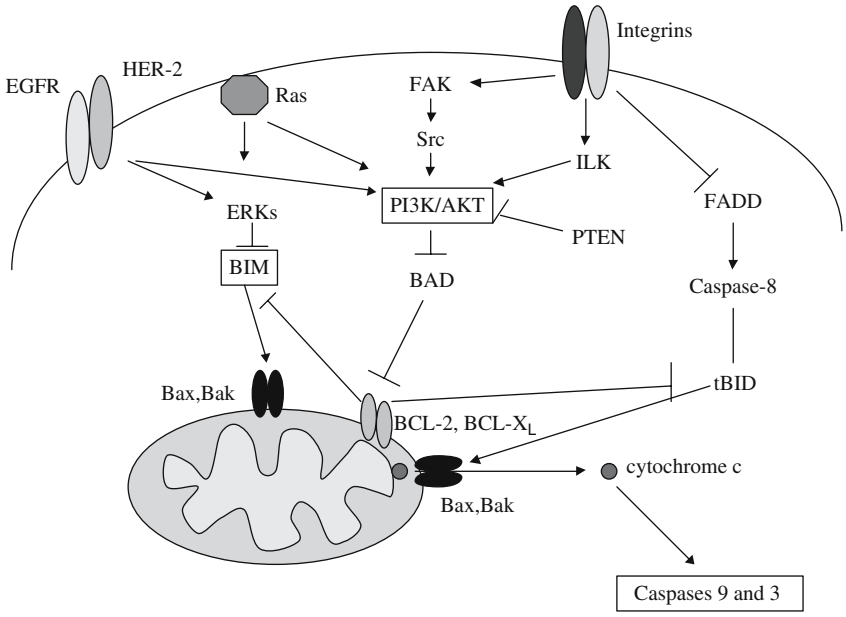

Fig. 1. Control of anoikis in apoptosis-proficient cells. Integrins and members of the erbB family of growth factor receptors (EGFR, HER-2), as well as mutant forms of Ras, drive activation of the ERK and AKT pathways, leading to phosphorylation of BH3-only members of the BCL-2 family (Bim, Bad) and suppression of cytochrome c release. Thus, detachment from the matrix disengages integrin signaling and unleashes these proteins to activate Bax and Bak, resulting in cytochrome c release and cell death. Under these circumstances, effector caspases (caspases 3, 6, and 7) carry out the proteolysis that is required for DNA fragmentation.

Integrin-linked kinase (ILK) is a serine-threonine, PH domain-containing kinase that binds to the cytoplasmic tails of β1-integrins *(16,17)* and functions to suppress anoikis *(18)* (Fig. 1). Several studies have demonstrated that ILK promotes phosphorylation of AKT on S473, and siRNA-mediated knockdown of ILK blocks integrin-mediated AKT activation and survival signaling *(19–22)*. Whether ILK directly phosphorylates S473 or indirectly promotes phosphorylation by activating another AKT kinase remains unclear. Nonetheless, there is consensus that ILK-mediated activation of AKT plays an important (if not exclusive) role in adhesion-mediated cell survival (Fig. 1).

2.2. Role of Focal Adhesion Kinase

The focal adhesion kinase (FAK) protein tyrosine kinase is a second integrin-associated signal transduction intermediate that plays a central role in integrin-mediated survival *(8,23)* (Fig. 1). Dominant active forms of FAK block anoikis *(23,24)*, whereas inhibition of FAK by various approaches induces cell rounding and detachment and promotes cell death *(24,25)*. The molecular mechanisms involved in FAK's effects are still under investigation but appear to involve crosstalk with Src *(26,27)* (Fig. 1), and parallel studies have established that active Src can also block detachment-induced cell death *(26,28)*. Furthermore, FAK also regulates the activity of the death receptor-associated protein serine/threonine kinase receptor-interacting protein (RIP) *(29,30)*,

which regulates survival by promoting activation of the transcription factor, nuclear factor (NF)-κB *(31–34)*. FAK is cleaved by caspases, resulting in inactivation of its survival-promoting activity and reinforcing cell death signaling *(35–38)*. Direct contacts between FAK and its cytoskeletal interacting proteins (vinculin, talin, paxillin, and the actin cytoskeleton) also appear to be crucial for survival signaling, presumably because they control the activation of the BH3-only members of the BCL-2 family that appear to control commitment to cell death (see Section 3.3).

2.3. Role of PTEN

The lipid PTEN is a third important signaling intermediate that controls AKT activation and anoikis *(39,40)* (Fig. 1). The protein was originally cloned at a frequent site of loss of heterozygosity on chromosome 10q in advanced solid tumors *(41–43)*, and early analysis of its sequence suggested that it might be a protein phosphatase *(41)*. However, subsequent studies demonstrated that PTEN is more active as a lipid phosphatase that removes phosphate groups from the 3′ position of polyphosphoinositides *(12,42)*. Thus, PTEN is a direct antagonist of PI-3 kinase and downstream targets that contain PH domains (e.g., ILK and AKT). Loss of wild-type PTEN is a relatively common event not only in glioblastomas but also in a variety of other solid malignancies, leading to elevated basal AKT activation *(12)*. Reintroduction of wild-type PTEN into cells that lack it tends not to have a dramatic effect on spontaneous apoptosis but does dramatically sensitize cells to anoikis *(39,40)*.

2.4. Role of Ras

Ras pathway activation leading to activation of the extracellular signal-regulated kinases (ERKs) can also suppress anoikis *(44–46)* (Fig. 1), but it is not clear that the ERKs are as directly regulated by adhesion-dependent signaling as the PI-3 kinase/AKT pathway is. Rather, ERK activation appears to be more commonly driven by either mutations in the Ras proteins themselves (particularly K-ras in lung and pancreatic cancers) or constitutive growth factor receptor [erbB family of growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER-2)] signaling (Fig. 1). Interestingly, detachment results in downregulation of EGFR expression in MCF-10a cells, and it is possible that this effect is required for subsequent cell death *(44)*.

3. MOLECULAR MECHANISMS UNDERLYING DETACHMENT-INDUCED APOPTOSIS

The observation that loss of matrix attachment results in apoptosis was first reported by Frisch and Francis in 1994 *(5)*. Since then, studies aimed at understanding the molecular mechanisms underlying the phenomenon coupled with advances in the field of apoptosis overall have helped to elucidate several of the key players and to create hypotheses for how they are regulated. Overall, there is good consensus that AKT and the ERKs play important roles in suppressing anoikis and that the cytoskeleton-associated, proapoptotic, BH3-only BCL-2 family members, BCL-2-interacting mediator of cell death (Bim) and Bcl-2-modifying factor (Bmf), are critical for commitment to cell death, at least in certain cell types (Fig. 1). However, parallel studies have implicated AKT and Bim more broadly in apoptosis regulation, raising

the question of whether they are especially important for this specialized pathway of cell death. Furthermore, we are finding that metastatic tumors that possess defects in the regulation of caspase activation (overexpression of BCL-2, inactivation of caspase-3) can still die just as efficiently as non-metastatic cells that do not possess these defects, and death in these cells is still associated with "classical" oligonucleosomal DNA fragmentation (so-called DNA laddering) *(47)*. We will therefore begin with a discussion of the consensus that exists regarding the roles of caspases and BCL-2 family proteins in anoikis and conclude with a section describing the "unorthodox" anoikis that is often observed in metastatic tumors.

3.1. Anoikis: Extrinsic or Intrinsic?

Historically, apoptotic responses have been described as "extrinsic" or "intrinsic" based on whether initiator caspase activation is driven by cell surface (death) receptors or by Bax/Bak-mediated release of cytochrome c from mitochondria *(48,49)*. Thus, extrinsic pathway stimuli initiate apoptosis through formation of a "death-inducing signaling complex" (DISC) and Fas-associated death domain (FADD)-dependent aggregation of procaspase-8, whereas intrinsic pathway stimuli initiate apoptosis through formation of an "apoptosome" and Apaf-1-dependent aggregation of procaspase-9. Complicating things is the observation that activated caspase-8 can trigger the intrinsic pathway by cleaving the BH3 protein, Bid, which translocates to mitochondria to induce cytochrome c release *(50,51)*. Thus, extrinsic pathways have been further subclassified as "type I" or "type II" *(52)* based on whether induction of apoptosis requires this so-called mitochondrial amplification loop (as it does in type II cells).

Abundant evidence implicates caspase-8 as an important contributor to cell death during anoikis *(53–60)* (Fig. 1), although precisely how it is activated remains the subject of some controversy. Several groups have demonstrated that caspase-8 is activated early in cells undergoing anoikis in a manner that is insensitive to inhibitors of caspase-9 or caspase-3 *(53,54,61)*. Furthermore, dominant-negative forms of FADD and other molecular strategies to inhibit FADD-dependent caspase-8 activation are strong inhibitors of anoikis-associated cell death, and proteins that modulate the FADD-dependent activation of caspase-8 [death-associated protein 3 (DAP3), Fas-associated death domain-like interleukin-1beta converting enzyme (FLICE) inhibitory protein (FLIP)] also regulate anoikis *(54)*. Beyond these observations, there is less consensus with respect to how FADD and caspase-8 are activated. One study concluded that detachment induces increased Fas and FasL expression in endothelial cells and that FasL was required for cell death *(55)*. However, another group disputed these conclusions and suggested instead that FADD-dependent activation of caspase-8 occurs independently of death ligand-induced aggregation of death receptors *(7)*. Other complicating issues include the suggestion that Bid is not processed to tBid in cells undergoing anoikis but rather the full-length protein translocates to mitochondria during the response *(62)*. Furthermore, caspase-8 activation is at least partially blocked by BCL-2 and BCL-X_L *(54)*, proteins that are thought to act primarily at the level of mitochondrial cytochrome c release *(49)* and downstream of FADD-dependent activation of caspase-8. Other work argued that caspase-8 activation occurs downstream of cytochrome c release in human intestinal epithelial cells *(63)*, and evidence has been advanced that Bax translocates to mitochondria within 15 min in detached primary breast epithelial

cells and subsequently forms large aggregates within 1 h that are associated with loss of cytochrome c and mitochondrial membrane potential, suggesting that the intrinsic pathway might be activated directly *(64)*. Whether an orthodox or unorthodox extrinsic pathway or an intrinsic pathway predominates could also be cell-type dependent.

3.2. BCL-2 Family Proteins in Apoptosis

Members of the BCL-2 family of cell death regulators play evolutionarily conserved roles in promoting or inhibiting apoptosis in eukaryotes *(49,65,66)*. The BCL-2 family can be divided into three structure-based subcategories. The founding member of the family (BCL-2) and its anti-apoptotic structural cousins (BCL-X_L, MCL-1, A1) contain four so-called BCL-2 homology (BH) domains, and they inhibit apoptosis (at least in part) by directly binding to and neutralizing the proapoptotic effects of other members of the family (Fig. 1). The proapoptotic, multidomain Bax, Bak, and Bok proteins contain only three of the four BH domains found in their anti-apoptotic relatives, and they can aggregate to form pores in the outer mitochondrial membrane, thereby facilitating cytochrome c release and downstream caspase activation (Fig. 1). The third subcategory consists of a group of proapoptotic proteins that only share their BH3 domains with the other members of the family. The BH3-only proteins appear to function at the interface of upstream signaling events and commitment to cell death *(65,66)* (Fig. 1). Thus, the mechanisms that control the activation or inactivation of the BH3-only proteins have become the subject of intensive investigation.

The best worked out of these mechanisms involves the activation of the BH3-only protein Bid by activated caspases 8 or 10 *(50,51)* (Fig. 1). Full-length Bid is a largely inert protein that localizes to the cytosol in resting viable cells. Active caspase-8 promotes the N-terminal cleavage of Bid, forming an active form of the protein (tBid) that translocates to mitochondria to promote cytochrome c release and possibly other ultrastructural events *(67)* that facilitate this release. Bid-deficient cells fail to display cytochrome c release in response to death receptors and other caspase-8 activators *(68,69)*, indicating that Bid and/or tBid plays a non-redundant role in this pathway. There is also evidence that Bim can be regulated by proteolytic cleavage *(70)*, but additional investigation is required to establish the general relevance of these observations.

Phosphorylation-mediated inhibition of BH3 protein function represents a second major mechanism for the control of these proteins by upstream signaling pathways. The best-characterized example of this mechanism is found in the case of Bad *(71,72)*, which is phosphorylated on S132 by AKT *(73)*, on S112 by cAMP-dependent protein kinase (PKA) *(74)*, and on S132 by p70S6K *(75)* (Fig. 1). Phosphorylation of Bad promotes its association with 14-3-3 proteins, thereby sequestering it away from the anti-apoptotic proteins it neutralizes (BCL-2 and BCL-X_L) *(71)*. Not surprisingly, Bad has been implicated in the cell death that is induced by cytokine withdrawal *(72)*, presumably because growth factors suppress Bad activation through their effects on AKT activation. The BH3-only protein Bim also appears to be regulated by phosphorylation *(45,46,76, 77)* (Fig. 1). However, as we will discuss below (Section 3.3), most of the available evidence implicates the ERKs rather than AKT in the suppression of Bim function. Bim phosphorylation appears to promote its ubiquitylation and degradation through the proteasome.

There is general consensus that apoptosis-associated cytochrome c release is dependent on the multi-domain proapoptotic BCL-2 proteins (i.e., Bax and Bak) *(78)*,

but precisely how they are activated remains unclear. Letai and co-workers *(79)* used isolated mitochondria to obtain evidence that a subset of the BH3-only members directly bind to and activate Bax and Bak to form oligomeric pores in the outer mitochondrial membrane, and this result was subsequently confirmed and extended by Kuwana et al. *(80)* in reconstituted lipid bilayers (Fig. 1). However, it has also been argued that the effects of the BH3-only proteins are really limited to disrupting interactions between the pro- and anti-apoptotic multi-domain family members *(65)*, as convincing evidence for direct binding of the BH3-only proteins to either Bax or Bak has been difficult to obtain. Thus, it is possible that the functional differences noted in the previous studies *(79)* had more to do with differences in the spectrum of BH3-binding partners rather than to differential effects on Bax or Bak activation *(65)*.

3.3. BCL-2 Family Proteins in Anoikis

Bmf is a BH3-only member of the BCL-2 family that was isolated in a yeast two-hybrid screen for MCL-1-interacting partners *(81)*. It interacts with the myosin V actin motor in resting cells by binding to dynein light chain 2 (DLC2). Yeast two-hybrid studies indicated that Bmf interacts with all the major anti-apoptotic BCL-2 family members interrogated but does not interact stably with Bax or Bak *(81)*. The protein is expressed at high levels in hematopoietic cells as well as in epithelial cells of the kidney, liver, and pancreas. Transient overexpression of the protein in Jurkat cells induced apoptosis, and stable combined overexpression of Bmf and BCL-2 sensitized hematopoietic cells to growth factor withdrawal-induced apoptosis relative to cells expressing BCL-2 alone *(81)*. These observations support the idea that Bmf plays a role in monitoring the integrity of the actin cytoskeleton and could orchestrate the cell death that occurs following disruption of integrin signaling. Consistent with this idea, Bmf was released from DLC2 and formed complexes in human MCF-7 breast adenocarcinoma cells cultured on poly-Hema plates *(81)*. Whether Bmf is actually required for detachment-induced cell death remains to be determined, for example, by silencing its expression with siRNA.

The BH3-only protein Bim also interacts with the cytoskeleton in resting cells through its interaction with cytoplasmic DLC1 *(82)*. However, this interaction appears to promote the interaction of Bim with microtubular dynein motor complexes through DLC1-mediated binding to dynein intermediate chain. Thus, studies with Bim-deficient thymocytes established that the protein plays a central role in cell death induced by the microtubule-stabilizing drug, paclitaxel *(83)*, whereas paclitaxel appears to have little to no effect on mobilization of Bmf *(81)*. Conversely, in the original study that described the involvement of Bmf in anoikis, detachment did not induce release of Bim from microtubule dynein motor complexes in MCF-7 cells under conditions that promoted release of Bmf *(81)*.

Nonetheless, several more recent studies strongly suggest that Bim does play a critical role in anoikis (Fig. 1). Detachment leads to a rapid and sustained increase in Bim protein levels, and a Bim-specific siRNA construct inhibited anoikis-associated cell death *(44)*. Bim stabilization is associated with a decrease in EGFR and ERK signaling *(44)* (Fig. 1), and in fact, upstream ERK antagonists (MEK inhibitors) promote sensitivity to detachment-induced cell death in human MDA-MB-231 breast adenocarcinoma cells *(76)*. Overall, it appears that the Ras–Raf–ERK pathway controls cellular Bim expression by phosphorylation of S69, leading to ubiquitylation of the

protein and its degradation via the proteasome *(76)*. Interestingly, cells arrested at the G_1/S transition may also display resistance to anoikis *(45)*. This mechanism also paradoxically appears to involve ERK activity, but in this case ERK activation is apparently not controlled by upstream Ras–Raf–MEK activation but rather by inhibition of ERK phosphatase activity *(45)*.

The BH3-only protein Bid has also been implicated in anoikis, but its involvement may be somewhat unorthodox. Typically, as discussed above (Section 3.2), Bid is activated by caspase-8-mediated cleavage, which generates a truncated form of the protein (tBid) that possesses an enhanced ability to translocate to mitochondria to activate Bax or Bak (Fig. 1). Thus, Bid is a central player in cell death that is driven by caspase-8 activation, most notably in response to death receptor engagement. However, it has been suggested that full-length Bid translocates to mitochondria in cells undergoing anoikis, where it may be capable of activating Bax and/or Bak independently of cleavage *(62)*. Further investigation is required to establish the prevalence of these effects and to define the biochemical mechanisms involved. For example, it is conceivable that unique posttranslational modification(s) induced during anoikis are required for the response. It is also interesting to note that full-length Bid has recently been implicated in DNA repair *(84)*, arguing that, as is true of FADD *(85)* and caspase-8 *(86)*, its spectrum of physiological activities extends beyond the regulation of cell death.

3.4. Caspase-Independent Mechanisms in Anoikis

Given their evolutionarily conserved roles in cell death, most investigators equate caspase dependence with apoptosis *(87)*. Similarly, most of the investigations into the molecular mechanisms that regulate anoikis have focused on pathways that directly or indirectly regulate caspase activation (Fig. 1). However, the bulk of this work has been conducted with non-transformed cells (HUVEC endothelial cells, MDCK cells, human MCF10a breast epithelial cells), and it is not clear that anoikis becomes a barrier to cancer progression before solid tumors become metastatic. Indeed, growth in suspension does lead to induction of apoptosis in many of the commonly studied human solid tumor cell lines, although perhaps not surprisingly the kinetics of these responses may be somewhat delayed ($>$8 h) relative to those observed in the non-transformed cells. Because solid tumors acquire defects in the control of apoptosis as they progress, the relative resistance of frank carcinomas with respect to what is observed in normal epithelial cell lines is to be expected.

In an effort to directly assess the importance of anoikis resistance in tumor metastasis, we have compared isogenic non-metastatic and metastatic variants of various human solid tumor cell lines with respect to their abilities to undergo anoikis *in vitro*. Our work has focused on variants of two commonly studied human prostate cancer cell lines (LNCaP and PC-3) that were selected for enhanced spontaneous metastasis through orthotopic "recycling" in nude mice *(47)*, although we have performed experiments with human colon cancer cell lines as well *(28,88)*. Others have performed similar studies with human oral squamous cell carcinoma lines *(89)*. We previously reported that LNCaP cells selected for increased metastatic potential (LNCaP-LN3) were resistant to thapsigargin- and doxorubicin-induced apoptosis *(90)* and displayed androgen-independent growth *in vitro* and *in vivo (91)*. Apoptosis resistance appeared to be driven by increased expression of BCL-2 *(90)*, consistent with the observation that androgen-independent primary tumors also display elevated BCL-2 levels.

In subsequent experiments, we measured apoptosis-associated DNA fragmentation in the non-metastatic and metastatic LNCaP subclones produced when the cells were cultured in suspension by two different strategies (rotation or plating on agarose) *(47)*. We expected that the elevated BCL-2 expression and global apoptosis resistance displayed by the metastatic subclones would translate into increased resistance to anoikis. Unexpectedly, rates of DNA fragmentation were indistinguishable in the non-metastatic and metastatic cells. In subsequent experiments, we were also surprised to find that even enforced stable expression of BCL-2 failed to inhibit anoikis-associated DNA fragmentation in the non-metastatic or metastatic cells *(47)*.

Further characterization of the biochemical mechanisms that mediated DNA fragmentation in the cell lines did reveal important differences between the non-metastatic and metastatic cells (V.Bondar, manuscript submitted). In the former, suspension culture induced cytochrome c release from mitochondria and caspase-3 activation, and DNA fragmentation was partially blocked by the pan-caspase inhibitor zVADfmk. However, in the metastatic cells or the BCL-2 transfectants, anoikis did not trigger significant cytochrome c release or caspase-3-like activity, and zVADfmk had no effect on "DNA laddering." Similar results were obtained in human MCF-7 breast adenocarcinoma cells that lack caspase-3 because of mutation. In these apoptosis-resistant cells, a chemical inhibitor of cathepsin B (CA0-074Me) attenuated DNA fragmentation (Fig. 2), and we are currently exploring the possibility that the response is mediated via lysosomal proteases and/or an alternative physiological cell death pathway known as "autophagy" that, like apoptosis, may also be relevant to tumor progression and metastasis *(92)* (Fig. 2).

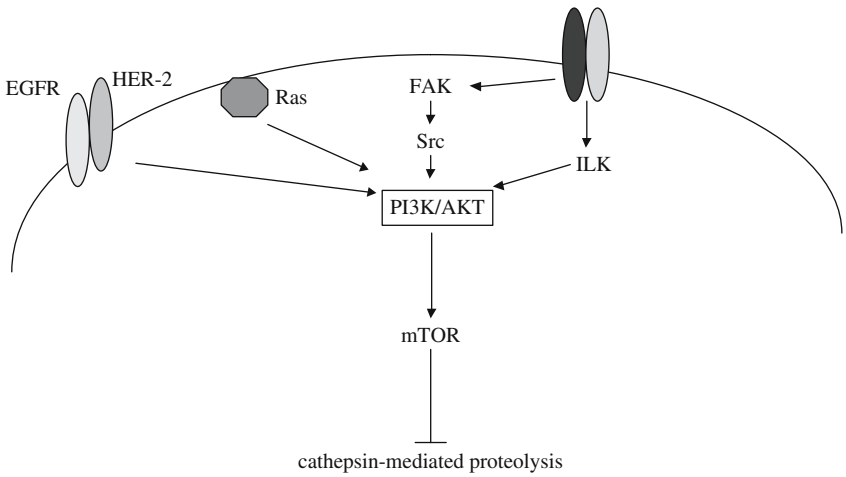

Fig. 2. Hypothetical model: cathepsins mediate anoikis in apoptosis-resistant cells. Many metastatic tumors acquire defects in the control of apoptosis that prevent cytochrome c release and/or effector caspase activation. However, these cells can still undergo a form of anoikis-associated cell death that involves classical "DNA laddering." We speculate that in these cells, lysosomal proteases (cathepsins) carry out the proteolytic events that are required for cell death, because a specific chemical inhibitor of cathepsin B can inhibit DNA fragmentation under these circumstances. Cell death is still associated with decreased AKT phosphorylation, but in our model, this allows for cathepsin mobilization, perhaps by relieving molecular target of rapamycin (mTOR)-mediated inhibition of autophagy.

Although our data create an interesting story, their physiological significance remains unclear. Because the LNCaP cell line was isolated from a prostate cancer lymph node metastasis, the parental cell line had already acquired molecular defects that rendered it metastasis-competent in the patient, and the further increase in metastatic potential generated by the orthotopic "recycling" might be a laboratory phenomenon. Furthermore, there is no way of knowing how long a tumor subclone must resist anoikis to allow it to seed a distant organ site: if the process takes place in less than an hour, then all the solid tumor cell lines studied by our group and others already possess enough resistance to survive the process. Finally, suspension culture often stimulates these cell lines to form aggregates, and previous work has shown that three-dimensional culture provides survival support that involves Ras pathway activation.

Nonetheless, independent studies have also implicated caspase-independent mechanisms in anoikis. An interesting example of this can be found in the recent implication of the Bit-1 protein in anoikis in endothelial cells *(93)*. Bit-1 apparently localizes to the mitochondria in resting cells but is released during anoikis. It forms a complex with the Groucho family member and transcriptional coregulator, AES (amino terminal enhancer of split), which in turn interacts with the groucho/transducin-like enhancer of split (TLE) transcriptional repressors. Thus, AES binding to TLE proteins releases their transcriptional repression activities *(94,95)*. Bit-1 in turn appears to be an inhibitor of AES, displacing the protein from preexisting AES–TLE complexes to facilitate transcriptional repression. Bit-1 also possesses peptidyl-tRNA hydrolase activity, and it is possible that this activity or other functions of the protein participate in cell death. It is still not clear how Bit-1 promotes caspase-independent cell death, because the targets identified to date (BCL-2, NF-κB) are classical inhibitors of caspase activation and apoptosis.

So, how are we to reconcile the caspase-dependent and caspase-independent mechanisms in anoikis? Perhaps the simplest hypothesis is that the caspase-dependent pathways are the default ones in normal cells and that the caspase-independent pathways only become functionally relevant when the caspase-dependent pathways are disabled. Whether there is one caspase-independent pathway or multiple pathways remains to be determined, and the potential relevance of other emerging physiological cell death mechanisms (i.e., autophagy) needs to be evaluated. It is of interest that defects in the control of autophagy may accumulate during breast cancer progression, and it is possible that these defects act in concert with disruption of caspase activation to promote anoikis resistance. There are clearly plenty of unanswered questions in this interesting and highly relevant subspecialty within the field of apoptosis research.

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II Telomeres and Telomerase, Senescence, Genomic Instability, and Tumorigenesis

7 Structure and Function of the Telomere

Jay E. Johnson, PhD, and Dominique Broccoli, PhD

Summary

Telomeres are specialized nucleoprotein structures found at the ends of linear chromosomes that guard against aberrant chromosomal rearrangements and prevent the ends of DNA molecules from being recognized by DNA damage-sensing mechanisms. These structures were initially characterized by Hermann Muller in the 1930s and have subsequently been the subject of intense study. The essential role of the telomere in protecting chromosomes is compromised by the continuous shortening of chromosome ends that accompanies DNA replication. At least two mechanisms have been found that counteract this telomere attrition, and these mechanisms have been implicated in tumorigenesis in that they allow unchecked cellular proliferation. This chapter summarizes our current understanding of the structure and function of the mammalian telomere, its maintenance, and its role in tumor formation.

Key Words: Telomerase; ALT; tumorigenesis; genomic instability; chromosome stability; cancer; aging.

1. INTRODUCTION

Telomeres are found at the ends of DNA molecules and are required for the maintenance of the linear chromosomes present in eukaryotes. These specialized structures protect against chromosome end-to-end fusion events as well as prevent the ends of DNA molecules from being recognized by DNA damage-sensing mechanisms. This essential role is eventually compromised by the progressive shortening of chromosome ends that is caused, at least in part, by DNA replication. Telomere dysfunction typically results in the activation of cellular responses such as programmed cell death (apoptosis) or cell growth arrest (senescence), thereby minimizing the likelihood that an aberrantly growing cell will develop into a tumor. By contrast, telomere stabilization facilitates evasion of such checkpoints by tumor cells and allows uninterrupted proliferation. These observations underline the importance of the telomere in tumor suppression.

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2. HISTORICAL PERSPECTIVE

As early as the 1930s, it was recognized that there was likely a chromosomal "cap" that prevented chromosome ends from fusing with one another. *Drosophila* geneticist, Hermann Muller called this structure a telomere, a term derived from the Greek words meaning "end" (telos) and "part" (meros). In a 1938 lecture entitled "The Remaking of Chromosomes," Muller proposed that

> *. . .the terminal gene must have a special function, that of sealing the ends of the chromosome, so to speak, and that for some reason, a chromosome cannot persist indefinitely without having its ends thusly "sealed." This gene may accordingly be distinguished by a special term, the "telomere" (1).*

Muller's hypothesis that the ends of a chromosome were somehow functionally distinct from the rest of the molecule was based on X-ray-induced chromosomal breakage experiments that he began in the latter half of the 1920s (and for which he would later win a Nobel prize) *(2)*. In these experiments, Muller analyzed the chromosomal rearrangements obtained after inducing double-stranded breaks by X-ray irradiation. Although he obtained many inversions, translocations, and deletions, none of these involved the extreme chromosomal termini, suggesting that the natural chromosome ends were unable to fuse with the newly broken ends.

At about the same time as Muller's studies, Barbara McClintock was performing elegant experiments in maize that would ultimately provide a confirmation of Muller's findings. In these studies, she could generate a dicentric chromosome that would break during mitosis as its centromeres migrated to opposing spindle poles *(3)*. She observed that newly broken ends were highly reactive and would fuse to one another before the next mitosis, producing a new dicentric chromosome. In sharp contrast, she found that normal chromosome ends were highly resistant to such fusion events. These data strongly supported Muller's contention that there was something special about the naturally occurring chromosome end, or telomere, and that this feature was necessary for chromosomal stability.

Further significant insights into telomere biology would unfortunately have to wait until the 1970s, as they required an important change in scientific thought. During the 1960s, many researchers abandoned the idea of the chromosome as a conglomeration of discrete components, beginning instead to consider the implications of the chromosome as a single uninterrupted DNA molecule *(2)*. This led to a switch in the techniques being applied to the study of telomeres, favoring molecular biological approaches over cytogenetics. It was during this time that several groups began sequencing the chromosomal termini in ciliated protozoa and found that, depending on the organism, the termini were composed of tandem repeats of either hexameric or octameric GC-rich sequences *(4–6)*. In pivotal work, Szostak and Blackburn *(7)* demonstrated the functional relevance of these sequences when they showed that the terminal repeats from the ciliate *Tetrahymena thermophila* could provide telomere function in the budding yeast, *Saccharomyces cerevisiae*. As part of these studies, they also demonstrated that the chromosome ends of *Saccharomyces* contained sequence repeats not unlike those observed in ciliates. In rapid succession, simple telomeric repeats were identified and characterized in many higher eukaryotes, including humans.

Attrition of telomeric repeats is caused, at least in part, by the incomplete replication of the chromosome. This "end-replication problem" is a consequence of both the unidirectional action and the primer requirements of conventional DNA polymerases *(8,9)*. Consequently, each time a cell replicates its DNA, telomeric sequences are lost. The mechanism by which these repeats are maintained remained elusive for some time. No known polymerases could be implicated in the maintenance of chromosome ends by the addition of telomere-specific repeats. In 1985, however, Greider and Blackburn *(10)* isolated protein extracts from *Tetrahymena* that possessed just such an activity. The enzyme responsible for this activity was called telomerase and would later be found to be a holoenzyme containing both RNA and protein subunits (see below, Section 6). To assay telomerase activity, Greider and Blackburn *(10)* used a synthetic single-stranded oligonucleotide as a primer and observed that it could be elongated by both dTTP and 32P-dGTP in an extract-dependent fashion. In addition, the reaction products obtained showed a six-nucleotide periodicity, corresponding to the telomeric repeat found in *Tetrahymena*, 5′(TTGGGG)3′. Although a more sensitive polymerase chain reaction (PCR)-based assay would later be devised *(11,12)*, it was the advent of this initial telomerase assay that facilitated much of the work characterizing both telomeres and telomerase over the past two decades.

3. THE PRIMARY STRUCTURE OF TELOMERIC DNA

In vertebrates, telomeres are typically 10–50 kb in length and are composed of tandem repeats of the hexanucleotide, 5′(TTAGGG)3′ (see Fig 1A). The length of the telomere is species specific, being approximately 10 kb in humans and 50 kb in inbred strains of *Mus musculus (13,14)*. Furthermore, telomere length is dependent on both the type and the replication history of the relevant tissue *(15,16)*. In the human germline, for example, the array of telomeric repeats is approximately 15 kb in length, whereas it is shorter in most somatic cells. Lengthening of the telomere is normally accomplished by the sequential addition of telomeric repeats to a pre-existing telomere terminus and requires the action of the large, multi-subunit ribonucleoprotein complex known as telomerase.

An interesting feature of the telomere is the strand asymmetry that results from the G-rich nature of the telomeric repeat. The G-rich strand is oriented in the 5′ to 3′ direction, toward the chromosome end, and in humans, there is a 75–200 nucleotide stretch at the chromosomal terminus where this strand is unpaired *(17–19)*. The presence of the G-rich single-stranded protrusion is correlated with proper telomeric capping *(20)*. Additionally, the G-strand overhang is involved in the generation of secondary structure at the telomere [the telomere loop (t-loop), see below, Section 4], which is thought to further stabilize the chromosome end *(21)*. Although work in human cell culture systems has indicated that telomerase may be involved in the generation of G-strand overhangs *(22)*, other studies have suggested that the enzyme is not specifically required for their maintenance. For example, G-strand overhangs have been shown to be maintained in mouse strains that lack telomerase *(23)*. Thus far, very little is actually known about how these structures are generated and/or maintained.

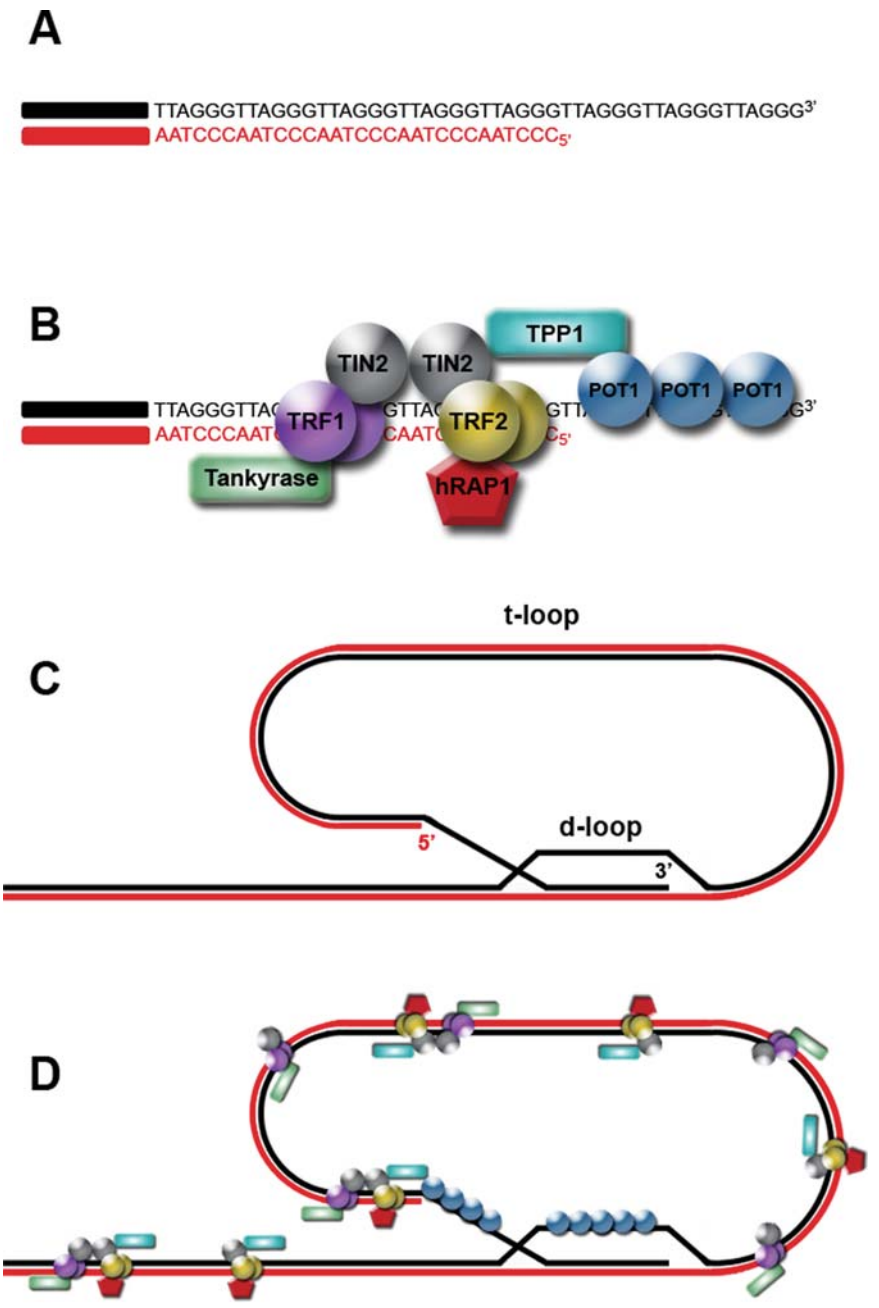

Fig. 1. Structure of the human telomere. **(A)** A typical human telomere is approximately 10–15 kb in length and composed of tandem repeats of the sequence, 5′(TTAGGG)3′. The G-rich strand (shown in black) is oriented in the 5′ to 3′ direction and, in humans, extends 75–200 nucleotides beyond the C-rich strand (shown in red). **(B)** Many proteins localize to the telomere and contribute to its stability and activity. These include TTAGGG repeat binding factor 1 (TRF1) (purple circles) and TRF2 (yellow circles), which bind to duplex telomeric DNA, as well as protection of telomeres 1 (POT1) (blue circles), which interacts with single-stranded telomeric DNA. Additional proteins found at the telomere include TRF1-interacting nuclear protein 2 (TIN2) (gray circles), tankyrases 1 and 2 (green rectangle), hRAP1 (human repressor activator protein 1) (red pentagon), and TPP1

4. THE SECONDARY STRUCTURE OF TELOMERIC DNA

Telomeres allow ongoing cellular proliferation despite the presence of naturally occurring chromosome ends, which resemble double-stranded DNA breaks. This is accomplished by protecting these ends from detection by the genome surveillance machinery, nucleolytic attack, and recombinogenic processing. Consistent with these roles, telomeres directly sequester the ends of the DNA molecule through the formation of a circular structure called a t-loop (see Fig 1C) *(21)*. The t-loop is formed by the invasion of internal double-stranded telomeric repeats by the single-stranded G-rich protrusion. Moreover, the displacement of the Watson strand by the G-strand overhang results in the formation of another structure, the aptly named displacement loop (d-loop). Both these structures have been directly visualized by electron microscopy (EM) *(21)*. Importantly, t-loops have also been observed in the context of isolated telomeric chromatin, providing further evidence that these structures are present *in vivo (24)*.

A less characterized and more controversial telomeric structure is the G-quadruplex. *in vitro* studies have indicated that G-rich telomeric DNA is capable of forming complex four-stranded structures *(25)*. Formation of these structures is mediated by a motif known as a G-quadruplex (sometimes called a G-quartet). Specifically, a G-quadruplex is a relatively stable planar association of four guanines. *In vitro*, G-quadruplexes can stack upon one another, giving rise to four-stranded structures. The carbonyl groups of each guanine can participate in the coordination of a cation, thereby trapping it in between "stacks." Interestingly, this "cationic trapping" results in further stabilization of the stacked structure. Recent work has provided the first evidence that G-quadruplexes actually exist *in vivo*. In this study, a fluorescent G-quadruplex-binding compound, 3,6-bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC), was used to probe chromosomal DNA purified from human cells *(26)*. A fluorescent emission signal specific for G-quadruplexes was detected at telomeric DNA. Furthermore, BMVC fluorescent signal was found to localize adjacent to chromosomal termini on metaphase chromosome preparations, suggesting that G-quadruplexes may be present at human telomeres.

With regard to the physiological relevance of these observations, a number of ideas have been proposed. It has been suggested that G-quadruplexes might interact with the template RNA of telomerase, possibly playing a role in telomere elongation *(27)*. Alternatively, G-quadruplexes might be involved in the formation of highly stable telomeric

Fig. 1. (TIN2-interacting protein 1/POT1- and TIN2-organizing protein/POT1-interacting protein 1) (aqua rectangle). The functions and interacting partners of these factors are summarized in Table 1. **(C)** The G-rich overhang is involved in the generation of two telomeric structural elements that are thought to stabilize the chromosome end. The telomere loop (t-loop) is a circular structure formed by the invasion of internal double-stranded telomeric repeats by the single-stranded G-rich protrusion. The displacement of the Watson strand by the G-rich overhang results in the formation of the displacement loop (d-loop). **(D)** Telomeric proteins are shown associated with the telomere in the context of secondary structural elements such as the t- and d-loops. Shown are many possible associations, including the same grouping of telomeric proteins as depicted in panel B, as well as subsets of this complex that might also exist *in vivo*. Examples of the latter group include complexes of TRF1 and its associated proteins, or TRF2 and its associated proteins. (Note: panels B and D are intended only to demonstrate the association of telomeric proteins with telomeric DNA and are not meant to suggest any specific stoichiometry or order of recruitment).

DNA complexes that provide additional "capping" function at the telomere *(28)*. Several studies have provided evidence that G-quadruplexes may affect the activity of telomerase at chromosome ends. In one study, treatment of cells with the G-quadruplex-interacting compound telomestatin was found to block telomerase activity *(29)*. Interestingly, subsequent work has shown that this compound interacts strongly with the G-rich overhangs found at the end of telomeres and promotes the formation of stable intramolecular G-quadruplex structures *(30,31)*. Taken together, these data suggest that binding of telomestatin to the intramolecular G-quadruplex structures in telomeric DNA abrogates telomerase function through the occlusion of the telomerase holoenzyme. Indeed, there are other telomestatin-like compounds that seem to exert their anti-proliferative effects through interaction with the G-rich portion of the telomere. TMPyP4, a compound that facilitates the formation of intermolecular G-quadruplexes, has an anti-proliferative effect on both telomerase-positive cells and cells that use a telomerase-independent mechanism for telomere maintenance [alternative lengthening of telomeres (ALT), see below, Section 8] *(32)*. In addition, treatment of cells with TMPyP4 results in an increase in the number of chromosome end-to-end fusions observed, whereas no such effect is seen with telomestatin treatment. With respect to the physiological relevance of G-quadruplexes, the above observations are consistent with those of a later study wherein the authors explored the effects of treating cells with guanosine-rich oligodeoxyribonucleotides (GROs) *(33)*. Synthetic GROs based on telomeric DNA sequences formed G-quadruplexes *in vitro* and had profound anti-proliferative effects when added to cultured HeLa cells. Although the current data are insufficient to establish any definitive conclusions, one might imagine that the effect of these G-quadruplex-containing oligodeoxyribonucleotides is elicited by the binding and sequestration of the active cellular pool of telomerase, causing telomere dysfunction and cell morbidity. It is difficult, however, to draw conclusions about how GROs might exert their anti-proliferative effects, as it is not clear from this study that these molecules can even gain access to the nucleus. The data obtained thus far implicate both interaction with the RNA component of telomerase and interactions within and between telomeres as mechanisms by which G-quadruplexes might affect telomerase activity.

5. TELOMERIC PROTEINS AND THE TELOSOME

The chromosomal capping function of telomeres and their ability to evade DNA repair mechanisms are dependent, to a large extent, on the secondary structure of telomeric DNA. This DNA structure, in turn, requires the activity of a number of proteins that localize to the telomere. In mammals, these proteins can be organized into three distinct classes. The first class is that of the telomeric DNA-binding proteins and contains TTAGGG repeat binding factor 1 (TRF1), TRF2, and protection of telomeres 1 (POT1) *(34–37)*. TRF1 and TRF2 bind to duplex telomeric DNA, whereas POT1 interacts with single-stranded DNA (ssDNA), most probably at the d-loop (see Fig 1B, D) *(37,38)*. The specific activities of these factors are summarized in Table 1.

In turn, the telomere-binding proteins interact with a second class of factors and the resulting multi-protein complexes have been implicated in the regulation of telomere metabolism. This includes TRF1-interacting nuclear protein 2 (TIN2), human repressor activator protein 1 (hRAP1), and TIN2-interacting protein 1/POT1- and

Table 1
Protein and RNA Factors Found at the Normal Human Telomere

Factor	*Definition*	*Relevant telomere-interacting partners*	*Known or proposed function*	*References*
TRF1	TTAGGG repeat binding factor 1	Double-stranded telomeric DNA, NBS1, TIN2, tankyrase1/2	Negatively regulates telomere maintenance by telomerase	*(34,35,39,46,49,95)*
TRF2	TTAGGG repeat binding factor 2	Double-stranded telomeric DNA, TIN2, POT1, hRAP1, NBS1	Plays an essential role in telomere end capping; negatively regulates telomere length	*(36,38–40,52)*
POT1	Protection of telomeres 1	Single-stranded telomeric DNA (ssDNA), TRF2, TPP1, PINX1	ssDNA-binding protein that binds to and protects the G-rich strand; negatively regulates telomere length	*(37,38,41–43)*
TIN2	TRF1-interacting nuclear protein 2	TRF1, TRF2, TPP1	Recruits POT1 to duplex telomeric DNA through TPP1; protects TRF1 from tankyrase-mediated destruction	*(39)*
TPP1	TIN2-interacting protein 1/POT1- and TIN2-organizing protein/POT1-interacting protein 1	POT1, TIN2	Regulates telomere length by recruiting POT1 to telomeres	*(41–43)*
hRAP1	Human repressor activator protein 1	TRF2, MRE11/RAD50, Ku	Recruits MRN and Ku to telomeres; negatively regulates telomere length	*(40)*
PINX1	Pin2/TRF1-interacting factor 1	TRF1, hTERT, hTR(hTER)	Negatively regulates telomerase activity by binding to both hTERT and hTR(hTER)	*(45,111)*

tankyrase 1	TRF1-interacting, ankyrin-like poly(ADP-ribose) polymerase (PARP)	TRF1	ADP-ribosylates TRF1, leading to dissociation from the telomere and ubiquitin-dependent degradation	*(46)*
tankyrase 2	TRF1-interacting, ankyrin-like PARP	TRF1	Homolog of tankyrase 1 that can also liberate TRF1 from telomeres	*(47–49)*
hTERT	Human telomerase reverse transcriptase	hTR(hTER), PINX1, Ku, hEST1A/B	A specialized reverse transcriptase that extends telomeric DNA by addition of the sequence, TTAGGG; may also stabilize the telomere by a mechanism independent of DNA elongation	*(45,50,112,79)*
hTR (hTER)	Human telomerase RNA	hTERT, PINX1	Template RNA required for TTAGGG addition to telomeres by hTERT	*(45,68,69,73,111)*
hEST1A/B	Human ever shorter telomeres 1A/B	hTERT	Activates telomerase, possibly by recruitment of hTERT to the telomere	*(79,80)*
Ku70/86 (Ku)	Heterodimer involved in non-homologous end-joining (NHEJ)	hRAP1, hTERT, DNA-PK	NHEJ proteins that may play a role in telomere processing or the recognition of critically short telomeres as DSBs	*(50,51)*
MRE11/RAD50/ NBS1 (MRN)	Meiotic recombination 11/radiation sensitive 50/Nijmegen breakage syndrome 1	TRF2, hRAP1	Like Ku, MRN is involved in DNA double-strand break (DSB) repair and may be involved in maintenance of the telomere, possibly through the nuclease function of MRE11	*(50,52)*

(*Continued*)

Table 1
(Continued)

Factor	*Definition*	*Relevant telomere-interacting partners*	*Known or proposed function*	*References*
DNA-PK	DNA-dependent protein kinase	Ku	Suppresses chromosome end-to-end fusions	*(50)*
WRN	Werner syndrome protein	TRF1, TRF2 (*in vitro*)	RecQ helicase that also has a 3′–5′ exonuclease activity; involved in recombinational repair of stalled replication forks and DSBs; promotes lagging strand synthesis at the telomere, thereby regulating length of the 3′ G-rich overhang; dissociates d-loop *in vitro*	*(54–56,113)*
BLM	Bloom syndrome protein	TRF1, TRF2 (*in vitro*)	Like WRN, RecQ helicase that has been implicated in telomere metabolism and may promote telomere elongation by alternative lengthening of telomeres (ALT); lacks the nuclease domain present in Werner syndrome helicase/exonuclease (WRN)	*(53,54,56)*

TIN2-organizing protein/POT1-interacting protein 1 (TPP1), which together with the telomere-binding proteins make up the so-called shelterin complex *(39–44)*. Additional factors that modulate telomere structure and activity include the Pin2/ TRF1-interacting factor (PINXI) and tankyrases 1 and 2 *(45–49)*. The telomere dysfunction seen upon depletion of TRF1 or TRF2 is likely due to the inability of these factors to localize to and function at the telomere. The effects of these various factors on telomere length, telomere-capping, and overall telomere status are also summarized in Table 1.

The final class of proteins present at the telomere are those that have functions in other cellular processes and include a number of factors involved in the response to DNA damage *(50)*. These include the Ku70/86 heterodimer and the MRE11/RAD50/NBS1 (MRN) complex *(50–52)*. Similar to the telomere-associated factors described above, these complexes do not bind the telomere directly; rather, they require interaction with telomere-specific factors for their efficient localization to the telomere. Other factors, such as the RecQ helicases Werner (WRN) and Bloom (BLM), may be recruited to telomeric sites through association with TRF1 and/or TRF2 where they affect telomere homeostasis *(53–56)*. The functions of these proteins are discussed further in Table 1. Finally, there are a number of proteins that are thought to be involved in sensing telomere dysfunction *(57,58)*. For example, the phosphorylated form of the histone variant H2AX, γ-H2AX, is part of the cellular response to DNA damage and interacts with the MRN complex in DNA damage foci induced by critically short telomeres *(57,59)*. This interaction may contribute to the growth arrest and/or apoptotic responses elicited by non-functional telomeres. Similar to γ-H2AX, the ATM (ataxia telangiectasia-mutated) and ATR (ATM and RAD3-related) kinases, 53BP1 (p53-binding protein 1) and RAD17 (radiation sensitive 17), are DNA damage repair proteins that localize to telomere-associated DNA damage foci and are involved in the activation of telomere-induced senescence and apoptosis *(57,58)*. Interestingly, such factors are not constitutively present at the telomere, rather they are recruited in response to a critically short telomere.

The telomere-binding protein TRF1 plays an important role in telomere length control, whereas TRF2 is required for the end-capping function that prevents chromosome ends from being recognized as double-strand breaks *(20,60)*. There are many proteins, as mentioned above, that associate with each factor and contribute to the overall stability of the telomere. The recent finding that the protein TIN2 interacts with TRF1, TRF2, and POT1 indicates that there is crosstalk between the complexes associated with these factors and provides evidence for the so-called telosome model *(42,61–63)*. Specifically, the telosome is considered to be a high-molecular-weight complex of proteins associated with the telomere *(63)*. As mentioned above, abrogation of any of a number of TRF1- or TRF2-associated factors results in telomere dysfunction. The telosome model suggests that this effect might be the result of an overall decrease in the stability of the telosome, as opposed to the loss of specific functions provided by its components. Moreover, this model hypothesizes that it is the integration or summation of a number of signals from discrete pathways that contribute to the overall stability of the telosome and, consequently, the control of telomere metabolism. This model is consistent with recent work indicating that it is the state of the telomere and not necessarily its length that determines whether cells senesce. In these studies, the telomere-binding protein TRF2 was overexpressed, increasing the rate of telomere erosion without a concomitant acceleration of the onset of senescence *(64)*. The likeliest explanation

for this observation is that in cells overexpressing TRF2, although telomeric DNA is of a shorter length than what would typically activate senescence, the increased levels of TRF2 contribute to the formation of multimeric telomere complexes that are at least as stable as those in cells with normal length telomeres. Therefore, the authors argue, the most relevant checkpoint in telomere-driven senescence would be the state or stability of the telosome and not the length of telomeric DNA. Under physiological conditions, wherein telomeric protein levels are more tightly regulated, telomere length likely determines the quantity of protein bound to the telomere and, therefore, telosome status.

6. TELOMERASE

The human telomerase holoenzyme consists of two essential subunits, a telomere-specific reverse transcriptase (RT) [human telomerase RT (hTERT)] and an RNA template (hTR/hTER), and functions as an interdependent dimer or multimer *(65)*. Whereas these components are necessary and sufficient for telomerase activity *in vitro*, there are a number of accessory proteins that modify the activity and/or assembly of telomerase. Two of these factors are the heat-shock protein 90 (hsp90) and the chaperonin, p23. Both are involved in preserving the folding states of proteins and, consequently, preserving the physiological activities of many enzymes. As both proteins have been shown to bind telomerase directly, it is thought that they enhance telomerase function by facilitating the assembly of the active holoenzyme *(66,67)*. Consistent with this role, an inhibitor of hsp90, geldanamycin, has been shown to reduce telomerase activity *in vitro (66,68)*. Furthermore, recent studies have shown that the use of antisense oligonucleotides to downregulate hsp90 and p23 expression results in decreased telomerase activity both *in vitro* and *in vivo (69)*. Taken together, these results indicate that although hsp90 and p23 may not be required for telomerase activity, their expression has a stimulatory effect on the hTERT-mediated elongation of chromosomal termini. The same holds true for another protein proposed to be part of the telomerase holoenzyme, the nucleolar protein, dyskerin. Cells with mutant alleles of dyskerin show reduced levels of hTR and, not surprisingly, have similarly affected levels of telomerase activity *(70,71)*. Moreover, antisense RNA-mediated downregulation of dyskerin negatively impacts on telomerase function, as described for hsp90 and p23 *(69)*. What is arguably most intriguing about this particular telomerase holoenzyme component is that *dyskerin* point mutants have been shown to be causative agents of the human skin and bone marrow disorder, dyskeratosis congenital (DKC) *(72)*. Dyskerin is an rRNA pseudouridylase and has been proposed to modulate telomerase levels through direct interaction with the H/ACA domain of hTR *(70,71)*. Studies in mice suggest that DKC disease phenotypes are caused by impaired ribosomal biogenesis, as opposed to telomere shortening *(71)*. This is because *dyskerin* knockout mice immediately display symptoms of DKC, although they suffer detectable telomere attrition only in later generations. Despite such nuances, it is clear that dyskerin does play a role in telomere metabolism, although the molecular details remain unclear.

7. REGULATION OF TELOMERASE ACTIVITY

Accessory subunits of the telomerase multi-protein complex such as hsp90 and p23 are expressed at relatively comparable levels in normal and cancerous tissues *(69)*. Similarly, the telomerase template RNA, hTR, is expressed fairly widely and is even

found in many tissues that do not have detectable telomerase activity. By contrast, levels of the catalytic component of the holoenzyme, hTERT, are much more variable. Transcription of hTERT is either low or completely undetectable in the majority of somatic tissues *(11,73,74)*. Interestingly, there are many renewable somatic tissues that represent notable exceptions to this rule as they have been shown to possess moderate levels of telomerase activity *(75)*. These renewable tissues include the lining of the gut, hair follicles, the proliferating keratinocyte layer of the skin, and cells of the hematopoietic system, the last group being the most extensively characterized due to the ease of obtaining such samples. Whereas transcriptional regulation of hTERT represents an efficient means of modulating telomerase activity, many additional regulatory mechanisms exist that affect the levels of active enzyme.

Alternative splicing of the hTERT transcript is a post-transcriptional control that might also play a role in the regulation of telomerase activity, although the relative contribution of this pathway to overall telomerase regulation has yet to be determined. There are, for example, several splice variants of the hTERT message that lack domains crucial for catalytic activity *(76,77)*. Translation of many of these transcripts produces non-functional proteins, whereas the variant known as hTERTα is a dominant-negative inhibitor of normal telomerase activity. This splice variant lacks a conserved RT motif and may exhibit its transdominant effects through sequestration of both DNA and protein factors needed for proper functioning of the active enzyme.

A third mechanism involved in the regulation of telomerase is the human ever-shorter telomeres 1A/B (hEST1A)-mediated enhancement of telomere elongation. hEST1A was identified as the human homolog of the yeast EST1 protein, which is involved in the activation of the telomerase holoenzyme, presumably by recruiting it to the chromosomal terminus *(78)*. The human homolog likely functions similarly to its yeast counterpart, as it has the ability to bind single-stranded telomeric DNA and its overexpression results in telomere elongation *(79,80)*. Somewhat puzzling, however, is the observation that this enhancement of telomerase function is also accompanied by an impairment of the end-protecting capability of the telomere. That is, hEST1A overexpression results in a dramatic increase in the number of chromosomal end-to-end fusions observed. These data are interesting, however, in that they suggest that longer telomeres do not necessarily provide additional chromosomal protection.

Yet another mechanism that contributes to the regulation of telomerase activity is that of protein phosphorylation. A number of potential phosphorylation sites in the telomerase reverse transcriptase have been identified, and many have been implicated in enzyme activation. In one of these studies, phosphorylation of hTERT by the Akt kinase resulted in the upregulation of telomerase activity *(81)*. This post-translational modification of hTERT may play a role in the subcellular targeting of the protein, thereby affecting assembly of a functional holoenzyme. For example, the nucleolar localization of telomerase has also been implicated in regulation of activity *(82,83)*. In one such study, hTERT was exogenously expressed in primary fibroblasts. The exogenous protein was localized to the nucleolus and returned to the nucleoplasm upon entry into S phase. The nucleolar localization of hTERT was abrogated upon transformation, whereas DNA damage induced a transient sequestration of hTERT into the nucleolus *(82)*. The multi-level regulation of telomerase activity may be useful therapeutically, as it provides a variety of potential targets for inhibition of the enzyme.

8. ALTERNATIVE LENGTHENING OF TELOMERES

Telomere stabilization facilitates tumorigenesis. The majority of human tumors utilize telomerase to offset the critical shortening of telomeres that would otherwise occur under conditions of rapid cell division. A subset of tumors and cell lines are capable of maintaining chromosome ends in the absence of telomerase, however *(84,85)*. Such cells are said to use ALT for telomere maintenance. Although the specific genetic determinants required for ALT are unknown, there are several key differences between ALT-positive and telomerase-positive cells.

In contrast to the fairly homogenous telomeres found in telomerase-positive cells, in most ALT-positive cells telomeres are quite heterogenous, ranging from 4 kb to >25 kb in size *(84)*. However, a single telomerase-negative immortal cell line, AG11395, in which the terminal DNA array is composed of telomeric repeats interspersed with SV40 sequences, has been described *(86,87)*. Moreover, ALT cells contain extrachromosomal DNA molecules that contain telomeric repeats *(88,89)*. These circular molecules are readily detected by both pulsed-field gel electrophoresis (PFGE) and EM and their existence, combined with studies in *Kluyveromyces lactis (90)*, suggest that telomeres may be maintained in ALT-positive cells by a mechanism involving rolling-circle replication and homologous recombination *(89)*. ALT-positive cells also differ from telomerase-positive cells in that a subpopulation of cells in an ALT-positive cell line often show co-localization of telomeric DNA, the telomeric-binding proteins TRF1 and TRF2, and the promyelocytic leukemia (PML) nuclear body in a complex called the ALT-associated PML body (APB) *(91)*. The PML nuclear body is a large multi-protein structure that has been implicated in various cellular processes, including growth control, differentiation, and apoptosis *(92,93)*. It is possible that the association of TRF1 and TRF2 with the PML body generates signals that mediate transcriptional changes in response to telomere status. An alternative possibility is that they are sites of telomere elongation through recombination. As discussed above (see Section 5) and in Table 1, a number of factors involved in the DNA damage response have been detected at telomeres. DNA repair factors such as the MRN and Ku70/86 complexes might be involved in recombination-based telomere maintenance at APBs. Finally, these structures may represent the accumulation of byproducts of the telomere elongation reaction. Interestingly, cell synchrony experiments show that the number of APB-positive cells is increased significantly when cultures are enriched for late S or G2/M phase cells *(94,95)*. This observation suggests that the formation of the APB complex, whatever its function, is a cell cycle-dependent event. In the AG11395 cell line, telomeric components do not co-localize with the PML nuclear body, although these cells contain a multiprotein complex wherein recombination factors, telomeric factors, and many other APB components are present *(86,87)*. Whatever their role, APBs are tightly linked with ALT and have served as a diagnostic marker for cells that use this mechanism for telomere maintenance.

Another significant feature of ALT-positive cells is an increased frequency of chromosome end-to-end fusions *(96)*. This condition is evidenced quite clearly by the presence of anaphase chromosome bridges, structures that result from the bi-directional migration of the two halves of a dicentric chromosome. Conveniently, these structures serve as simple visual indicators of the chromosome fusion-breakage cycle that occurs in cells with elevated levels of telomere dysfunction. As mentioned above, it is thought that the mechanism by which telomeres are maintained in ALT-positive human cells involves

recombination between chromosome ends. Such a mechanism is strongly suggested by a number of studies in *S. cerevisiae* examining both the genetic requirements for telomerase-independent telomere maintenance and the structure of the telomere itself *(78,97,98)*. In human cells, rapid changes in the length of the telomere repeat array were observed rather than the gradual changes in size more commonly associated with telomerase activity or the telomere loss that accompanies cell division *(99)*. Subsequently, it was demonstrated that a unique tag embedded in a single telomere will spread to other telomeres in ALT cell lines *(100)*. However, this only occurs when the tag is flanked by telomeric sequences, suggesting that the recombination event occurs within the TTAGGG repeat array. By contrast, characterization of telomeric structure in mouse embryonic stem (ES) cells deficient in telomerase raised the possibility that recombination may occur within subtelomeric repeats *(101)*. The idea that ALT functions in human cells by a recombination-based mechanism is further supported by the finding that the ALT pathway becomes activated upon telomerase inhibition in cells deficient in DNA mismatch repair ($MSH6^{-/-}$) *(102)*. In addition, it was recently shown that sister chromatid exchange at telomeres is elevated in ALT cells *(103,104)*. Although such studies implicate DNA recombination in the maintenance of telomeres by ALT, the specific molecular details of this mechanism remain undetermined.

9. CELLULAR IMPLICATIONS OF TELOMERE DYSFUNCTION

As mentioned previously, telomeres provide an essential cellular role by facilitating evasion of DNA damage-sensing mechanisms, thereby preventing the chromosomal termini from being recognized as double-stranded breaks. Telomeres achieve this by providing an end-capping function, guarding against potentially deleterious chromosomal rearrangements. Loss of telomere function by either the critical shortening of telomeric DNA or the perturbation of telomeric proteins involved in end-capping ultimately provokes cellular responses such as programmed cell death (apoptosis) or cell cycle arrest (senescence).

In most cells, the rate of telomere elongation, if any, is insufficient to offset the progressive shortening of telomeres that is associated with DNA replication. Recent work has shown that critically short telomeres directly engage DNA damage repair mechanisms *(57,58)*. That is, upstream sensor kinases typically undergo autophosphorylation and initiate a phosphorelay that eventually signals downstream effectors, such as protein kinases and transcription factors, to change the cellular expression profile. Although this induced profile is marked by upregulation of factors necessary to repair DNA damage, there can also be a concomitant growth arrest or an induction of apoptosis. The specific response triggered is largely dependent on the background of the cell *(20,105)*. In mammals, the upstream kinases, ATM and ATR, initiate signaling cascades that act through signal transducers such as γ-H2AX, a histone protein involved in the alteration of chromatin structure at the site of DNA damage *(106)*. Upon telomere attrition, γ-H2AX and a number of other DNA damage response proteins directly engage the chromosomal terminus, as shown by chromatin immunoprecipitation and whole genome-scanning techniques *(58)*. Notably, these studies provided the first direct evidence that DNA damage repair checkpoints recognize dysfunctional telomeres as double-stranded DNA breaks. Intriguingly, these checkpoints are activated in response to both truncated telomeric DNA and dysfunctional telomeres that result

from the depletion or mutation of telomeric-binding proteins such as TRF2 *(105,106)*. Such results imply that in the case of critically short telomeres, DNA damage repair mechanisms directly sense DNA ends, which have been generated and/or de-protected because of an insufficiently high concentration of the telomeric proteins that ordinarily cap chromosomal termini. One possibility is that the inability of telomeric proteins to associate with the telomere might de-stabilize t-loop structures, in turn allowing telomeres to be recognized as DNA damage. To date, however, no conclusive evidence has been obtained to address such speculation.

With respect to telomere-induced senescence or apoptosis, mutations arise that abrogate these normal cellular responses and allow continued proliferation despite critically short or otherwise dysfunctional telomeres. Ablation or impairment of any number of signaling components, from the upstream sensor kinases to the downstream effector proteins, can allow a cell to evade these surveillance mechanisms. Indeed, the circumvention of both senescence and apoptosis are crucial steps in cellular immortalization and tumor progression.

10. THE ROLES OF TELOMERASE ACTIVATION IN GENETIC INSTABILITY AND TUMOR PROGRESSION

As discussed previously, the majority of human tumors have overcome telomere crisis by the reactivation of the telomerase holoenzyme. It has been suggested, however, that dysfunctional telomeres actually play a large part in promoting tumorigenesis. In mice, ablation of the RNA component of telomerase results in telomere shortening, increased genomic instability, and a decreased proliferative capacity in high-renewal tissues *(107)*. The cellular senescence and apoptosis responsible for this decrease in proliferation are dependent on the action of the tumor-suppressor protein, p53 *(106)*. Interestingly, coincident telomere attrition and p53 deficiency result in increased tumor formation. This increased tumorigenesis is likely because dysfunctional telomeres cause a global chromosomal instability that allows for the accumulation of growth-promoting mutations. Although this hypermutational state may facilitate the progression of early tumors, it would be disastrous to more advanced tumors. In addition to those genetic changes that promote cell growth, mutations might also be produced that decrease the rate of proliferation or reduce overall cell viability. In particular, these mutations might even re-activate pathways involved in senescence or apoptosis, re-establishing anti-proliferative processes that were likely bypassed in the earliest stages of tumor formation.

It had been reasoned that the possible accumulation of growth-arresting mutations might be circumvented by the upregulation of telomerase and the consequent stabilization of the genome *(108)*. According to this model, tumor cells that were newly telomerase-positive would be selected and amplified as their viability is increased with respect to the remainder of the tumor population. Indeed, this hypothesis has been supported by studies in many human cell culture systems. In one study, telomerase was added exogenously to human mammary epithelial cells and genomic instability assayed by spectral karyotyping *(109)*. It was found that the re-introduction of telomerase, the activity of which was confirmed by telomerase holoenzyme assays, produced an extremely stable karyotype compared with that of control cells. These data are consistent with those of another study, wherein the authors established a cell culture

system to model the role of telomere dysfunction on both genomic instability and tumor progression *(110)*. Briefly, the authors used fluorescence in situ hybridization (FISH) to assay genomic instability, also in human mammary epithelial cell cultures, while monitoring telomere lengths and telomerase activity. Following these cells through telomere crisis and immortalization, they observed that the frequency of chromosomal rearrangements, as assayed by comparative genome hybridization, correlated with telomere attrition, becoming more prominent as telomeres shortened. Following telomere crisis, cultures were enriched for cells that had reactivated telomerase. These cells, not surprisingly, showed a marked decrease in chromosomal instability with respect to earlier populations. These findings were then confirmed in breast carcinomas of varying stages, providing further evidence that the genomic instability resulting from telomere attrition promotes cell immortalization and tumorigenesis.

11. CONCLUDING REMARKS

It can be said that our cells walk a "tight-rope" of telomere length, where a fall to one side results in growth arrest or apoptosis and a fall to the other allows unchecked cell growth and, potentially, tumorigenesis. Under the latter conditions, where telomeres are abnormally stable, efficient tumor formation is still not guaranteed, however. Just as with normal cells, there is likely a narrow range of telomere dysfunction that is optimal for tumor cells. Although an initial decrease in telomere length allows for chromosome end-to-end fusions and many other genetic rearrangements that can produce growth-enhancing alterations, continued growth of the tumor requires the subsequent rescue of this telomere dysfunction. Therefore, efficient tumor progression likely involves the initial shortening of telomeres, as well as their eventual stabilization. As it is unlikely that screens for telomeric proteins have been saturated, it is possible that future work will reveal additional factors that might be exploited in an attempt to exogenously manipulate telomeres and perturb tumor progression.

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8 Overview of Senescence

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Summary

Cellular senescence refers to the limited capacity of normal somatic cells to proliferate indefinitely *in vitro*. There are at least two types of senescence that prevent infinite proliferation: replicative senescence and stress-induced senescence (SIS). In replicative senescence, failure of the DNA polymerase to faithfully duplicate the chromosomal ends leads to progressive telomeric DNA sequence loss with each subsequent cell division and signaling for growth arrest. There are, at least, two mechanisms that prevent further telomere shortening and as a consequence bypass replicative senescence: upregulation/reactivation of endogenous telomerase and alternative lengthening for telomeres (ALT) pathway. In SIS, however, several stressors have been found to signal for growth arrest, such as DNA damage, damage to chromatin structure, oxidative stress, and oncogene activation. The ability to induce growth arrest as a consequence of telomere shortening or stress is thus the essential feature distinguishing the mortality of normal cells (presumably to provide a barrier against the accumulation of mutations and the formation of cancer) and the immortality of tumor cells.

Key Words: Cellular senescence; aging; telomeres; telomerase; alternative lengthening for telomeres; end replication problem.

1. CELLULAR SENESCENCE

Cellular senescence has been defined as the limited capacity to proliferate indefinitely *in vitro*. The ability to proliferate indefinitely in tissue culture is one of the many differences between normal human cells and established human cell lines. For nearly 40 years *(1)*, the contrast between the immortality of cancer cells and the senescence of normal cells has been controversial. The major source of skepticism rests on the knowledge that *in vitro* culture conditions represent a departure from the *in vivo* environment and that the aneuploidy and altered growth controls accumulated by tumor cells might simply allow them to divide under conditions inadequate to support the long-term proliferation of normal cells.

Presently, two distinct processes to elicit cellular senescence are recognized: senescence due to cell replication (replicative senescence) and senescence due to other causes

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[stress-induced senescence (SIS)]. Replicative senescence is a specific type of cellular senescence that results from an intrinsic mechanism: loss of telomere sequences with each round of cell division. SIS, on the contrary, results from a number of stressors, such as DNA damage, damage to chromatin structure, oxidative stress, and increased oncogene activity.

Cellular senescence is a viable, metabolic active, and non-dividing *in vitro* state, in which the phenotype of senescent cells involves changes in cell morphology (cells become enlarged and flattened in shape), dramatic changes in chromatin structure, changes in gene expression, and lack of response to mitogenic stimuli *(2,3,4)*. It has been suggested that cellular senescence is a fundamental cellular program involved in aging and is a potential tumor suppressive mechanism to limit the replicative capacity of potential cancer cells. Thus, efforts have lately been directed to develop chemopreventive agents that turn immortal tumor cells back to mortality.

2. REPLICATIVE SENESCENCE

Replicative (from the Latin words *replicare*, meaning to fold back, to repeat, to duplicate) senescence is a specific type of cellular senescence referring to limited number of *in vitro* cell divisions. Hayflick *(1,5)* postulated that the limited proliferative capacity of somatic cells was pre-programmed. Today, the "Hayflick limit" refers to the "preset" number of doublings that a particular cell type is able to achieve. Currently, the best understood mechanism for replicative senescence involves shortening of the chromosomal ends (telomeres), which results from a problem in completely duplicating the telomeric ends. Consequently, the only known way to retard replicative aging and to exceed the Hayflick limit in intact normal cells is to prevent telomere shortening through either the expression of telomerase or activation of the alternative lengthening of telomeres (ALT) pathway. Cellular immortalization through the ectopic expression of the catalytic component of telomerase [human telomerase reverse transcriptase (hTERT)] *(6–10)* or through activation of ALT pathway *(11–14)* has been reported in a number of articles. These results suggest that the number of cell divisions is limited, not by chronological or metabolic age but rather by the process of telomere shortening, which acts as a genetic, mitotic clock.

2.1. DNA Duplication

2.1.1. DNA Polymerase Cannot Initiate DNA Synthesis De Novo

During the process of cell division, genomic DNA must be faithfully replicated. To this end, the cell must engage in the enormous task of copying the 3.2 billion base pairs comprising the human genome into a new DNA strand. To ensure proper duplication of each base pair without errors and in a timely manner, we must meticulously regulate and monitor replication.

DNA replication is a very intricate mechanism, which involves several proteins just in the process of replication initiation *(15,16)*. Prior to the S phase, initiation of replication starts by assembling a multiprotein structure called the pre-replicative complex (pre-RC), which is then converted to a replication fork during the G1–S transition. For replication initiation to continue, origin unwinding, stabilization of single stranded DNA, and loading of DNA polymerases are required. There are essentially three polymerases for replication: DNA pol α, DNA pol δ, and DNA pol ε. After the

origin of replication becomes unwound, DNA pol α is recruited to the origin where the enzyme mediates the production of short RNA primers used for the initiation of leading and lagging strand synthesis. Following RNA primer synthesis, DNA pol α is replaced by DNA pol δ and/or DNA pol ε. The exchange of DNA polymerases produces an increase in DNA synthesis processivity and adds proofreading exonuclease activity during the process of DNA replication through the S phase. It has been thought that both polymerases perform mutually exclusive essential functions *(17,18)*. Previous reports have shown that DNA pol δ displays greater processivity of DNA synthesis of both leading and lagging strand in the absence of pol ε, whereas DNA pol ε fails to significantly elongate DNA strands in the absence of DNA pol δ, suggesting that the main DNA polymerase is DNA pol δ *(17)*.

DNA polymerases mediate the synthesis of a strand of DNA by using a complementary DNA strand as template. Sequences of DNA nucleotides are essentially copied by base-pairing into complementary new nucleotide sequences. The DNA replication is semiconservative, because each of the two daughter cells receives from their progenitor a DNA double helix containing one old and one new strand. Furthermore, the DNA replication fork is asymmetrical. One strand is synthesized continuously and is known as the leading strand, whereas the other strand is synthesized discontinuously and is known as the lagging strand. The leading strand requires only the RNA primer at the start of the replication. However, the lagging strand requires constantly synthesized RNA primers to generate the Okazaki fragments throughout the strand. When the DNA polymerase from the previous Okazaki fragment runs into the RNA primer of the downstream Okazaki fragment, DNA polymerase's exonuclease activity removes the ribonucleotides and DNA polymerase replaces them with deoxynucleotides. DNA ligase then joins the 3′-end of the new DNA fragment to the 5′-end of the previous one to complete the process. Thus, the complete duplication of the entire genome requires a complementary DNA strand for annealing an RNA primer and initiation of strand synthesis by DNA polymerase. This poses a problem for DNA sequences at the end of linear chromosomes (explained below).

2.2. The End Replication Problem

2.2.1. DNA Polymerase Cannot Faithfully Duplicate the Ends of Chromosomes

After the DNA structure was discovered and the duplication mechanisms during S phase of the cell cycle were elucidated, it was apparent first to Olovnikov *(19,20)* and later to Watson *(21)* that in the absence of special mechanisms, replication of linear chromosomes would be incomplete. Because of the requirement for an RNA primer to initiate DNA synthesis, it has been postulated that lack of telomeric end synthesis is due to inability to position RNA primers at the very end of telomeres (Fig. 1).

DNA polymerases, the enzymes responsible for duplicating the DNA strands during the S phase of the cell cycle, can only replicate DNA in the 5′-to-3′ direction and cannot initiate synthesis of the lagging DNA chain without the assistance of RNA primer templates. One result of these restrictions is that there is no template downstream of the 3′ telomeric end that DNA polymerase can use to synthesize the rest of the lagging strand (Fig. 1). The end replication problem mandates that the 5′-end of the daughter strands will shorten with each round of cell division. Thus, in actively proliferating

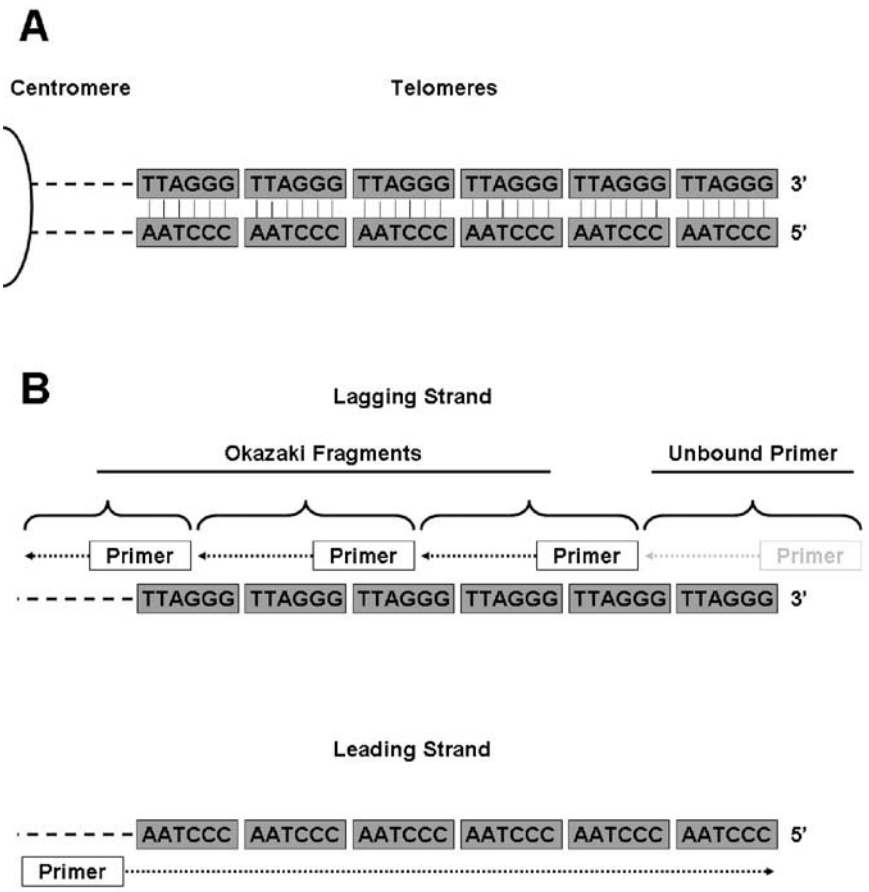

Fig. 1. (A) The chromosomal ends are called telomeres and consist of tandem repeats of the DNA sequence TTAGGG in vertebrates. **(B)** Upon unwinding of DNA strands, RNA primers are synthesized to initiate DNA replication. The lagging strand is copied by DNA polymerase synthesizing the new strand discontinuously; thus RNA primers are positioned at different intervals along the DNA template. Following elongation of the RNA primers by DNA polymerase, the Okazaki fragments are formed. The lack of DNA sequences further downstream of the 3′ telomeric end prevents binding of RNA primers and precludes complete DNA replication, resulting in shorter chromosomal ends at each round of cell division. By contrast, the leading strand is copied by DNA polymerase synthesizing the new strand continuously; thus RNA primers are positioned only at the start of the replication.

cells, chromosomes shorten progressively from both ends *(20)*, and in the absence of any compensating mechanisms, the ends of the chromosomes are not replicated completely, resulting in a net loss of terminal chromosomal DNA with each cell division. It has been proposed that the erosion of telomeres during cell division is the mitotic clock that limits the replicative capacity of normal somatic cells *(22,23)* and that the reaching of a critical telomere length signals replicative senescence or aging *(19,20,24)*.

The inability to position RNA primers at the very end of telomeres is not the only hypothesis accounting for telomere shortening. Two additional extensively studied

hypotheses are (i) unrepaired oxidative damage to telomeres *(25)* and (ii) nucleolytic degradation of one or both strands *(26)*.

2.3. Telomere Theory

2.3.1. Telomeres Are Tandem Repeats at the End of Chromosomes and Protect Against Genome Instability

Telomeres (from the Greek words *telos*, meaning end, and *meros*, meaning part) are the terminal ends of linear chromosomes. For nearly 65 years, it has been known that the chromosomal ends behave differently than the internal free ends. For example, X-ray ionizing radiation can cause internal inversions far more readily than terminal inversions *(27,28)*. Compared with the telomeric free ends of linear chromosomes, the internal free ends of broken chromosomes are more reactive and easily undergo end-to-end fusion reactions with internal free ends from other broken chromosomes. In the absence of a repair system, the high reactivity of internal double-strand breaks could eventually lead to gross karyotypic changes. To prevent the development of abnormal karyotypes, organisms have developed monitoring systems that arrest cell division in response to DNA damage, allowing time for DNA repair before proceeding into mitosis. The *p53* gene product functions as a monitoring system for genomic damage *(29,30–33)*.

Telomeres are composed of tandem repeats of the sequence TTAGGG in vertebrates *(34,35)* and are thought to prevent the degradation or aberrant recombination of the chromosomal ends *(36–38)*. In addition, telomeres also contain specific proteins, such as the TTAGGG repeat binding factor 1 (TRF1) *(39,40)*, TRF2 *(41,42)*, tankyrase 1 (TANK1) *(43,44)*, tankyrase 2 (TANK2) *(45)*, TRF1-interacting nuclear protein 2 (TIN2) *(46)*, PIN2-interacting protien 1 (PINX1) *(47)*, and protection of telomeres 1 (POT1) *(48)*. Among vertebrates, the size of telomeres is variable *(49,50–53)* and their precise length can only be estimated. Capping the chromosomal ends to prevent signaling for cell cycle arrest and helping in maintaining chromosomal stability are among the different biological roles that have been described for telomeres *(54)*. They protect chromosomes from degradation and fusion *(53)*, contribute to the architecture of the nucleus *(55, 56)*, and play a role in gene expression through the phenomenon of telomere silencing *(57,58)*.

In the last 10 years, a dual role in chromosomal homeostasis has been described for telomeres: they buffer the loss of terminal sequences resulting from incomplete DNA replication by DNA polymerases due to the end replication problem *(19–21)*, and telomeres provide sites for the de novo elongation of the chromosome by telomerase, thus compensating for the DNA sequence loss. Overall, telomeres are special protein/DNA structures essential for chromosomal stability.

2.4. Telomerase

2.4.1. Telomerase is a Reverse Transcriptase that Elongates the 3′-End of Telomeres

In most immortal eukaryotic cells, telomerase functions as the compensating mechanism that maintains telomere length. Telomerase is a ribonucleoprotein enzyme

that mediates the synthesis of the telomeric repeat sequence de novo using its RNA *(59)* component [human telomerase RNA (hTR)] as a template and the telomerase catalytic protein subunit (hTERT) *(60–62)* for the addition of telomeric repeats to the 3′-ends of the chromosomes. Telomerase activity was first identified in *Tetrahymena* and later in other species, including ciliates, yeasts, mice, and humans *(63–67)*. Because most normal somatic cells in vertebrates express no or very low levels of telomerase activity, it is thought that their telomeres progressively shorten, undergo genomic instability, and cease proliferating once a critical length is reached *(24)*. It has been suggested that upregulation or reactivation of telomerase may result in chromosomal stability leading to continuous cell proliferation *(23)*. Thus, telomerase is thought to partially or completely compensate for the progressive telomere erosion that would occur in its absence.

Although telomerase is not expressed in most normal somatic cells, telomerase activity is found in the vast majority of primary tumors *(68,69)*. This observation suggests that telomerase activity plays a role in preventing senescence in malignant infinitely dividing cells. It also seems reasonable to assume that cells with extreme proliferative requirements, such as those in renewal tissues (e.g., the blood, skin, and intestine), should also be capable of expressing telomerase. In support of this concept, recent reports have demonstrated the presence of telomerase activity in hematopoietic progenitor cells and activated lymphocytes *(70–72)*, the epidermis *(73,74)*, and the intestine *(75)*. Nevertheless, the telomeres of cells from these renewal tissues also shorten with age *(76–78)*, suggesting that the level of expression of telomerase in cells of renewal tissues is sufficient to slow, but not prevent, telomere erosion. This low level of telomerase expression appears to extend the life span of these cells but does not confer immortality.

2.5. ALT Mechanism

2.5.1. Telomerase-Independent Telomere Maintenance Mechanism

Even though most immortal cell lines and human tumors bypass cellular senescence by compensating for the telomere loss through reactivation of telomerase *(79)*, a telomerase-independent mechanism of telomere elongation has also been described *(11,80,81)*. The ALT pathway has been studied for nearly 12 years *(11,80,81)*. It has been determined, at least in some cases, that elongation of telomeres in immortal cell lines expressing no telomerase activity is mediated by homologous recombination of telomeric DNA sequences *(12,13)*. To date, all the human ALT cell lines analyzed have several characteristics in common: (i) cells lack significant levels of telomerase activity *(80)*, (ii) telomeres are highly heterogenous in length, ranging from < 2Kb to > 20–80 Kb *(80–82)*, (iii) ALT cells' telomeres show rapid telomeric lengthening or shortening events *(13)*, and (iv) the presence of ALT-associated promyelocytic leukemia (PML) bodies (APB) is detected in ALT cells *(83)*.

3. STRESS-INDUCED SENESCENCE

Another type of cellular senescence is SIS resulting from a number of stressors, such as DNA damage, damage to chromatin structure, oxidative stress, and increased oncogene activity. This variety of cellular senescence suggests that multiple stresses can trigger a common cellular response. Senescence is activated once a cell has

reached a critical level of damage, regardless of the type of damage. Common features with replicative senescence are morphology (enlarged cell shape), senescence-associated β-galactosidase activity, cell cycle regulation, gene expression, and telomere shortening.

3.1. DNA Damage

3.1.1. Direct Genomic DNA Damage Induces Senescence

It has been known for years that direct damaging of DNA, either by irradiation of cells *(84)* or by treatment with reactive chemicals *(85)*, can induce cells to undergo senescence *(86,87)*, which has been usually interpreted as an arrest to allow time for repair. Prolonged arrest due to ionizing radiation has often been reported to be dependent on p53 *(84–88)*. In mammalian cells, both homologous and non-homologous mechanisms seem to be involved in repairing double-strand breaks *(89)*. Other types of DNA damage can also induce senescence. For example, absence of Ku86 (a protein essential for repair of DNA breaks through non-homologous end joining) results in a high frequency of end-to-end chromosomal fusions despite having long telomeric segments, and induction of senescence *(90)*. Generation of chromosomal fusions has also been reported as a consequence of inactivating the function of the telomere-binding protein TRF2 *(91)*.

In line with this notion that DNA damaging induces senescence (and/or apoptosis), telomere shortening can also be interpreted as loss of DNA material and hence trigger senescence as well. It has been reported that unprotected chromosomes can induce DNA damage checkpoint cascades and ultimately lead to senescence both in mice and humans in a p53-dependent manner *(91–94)*. Thus, initial telomere length setting determines the proliferative capacity of each cell.

3.2. Damage to Chromatin Structure

3.2.1. Epigenetic Changes in Microenvironment of Heterochromatin Induce Senescence

Major epigenetic mechanisms include methylation modification of DNA and acetylation/packaging of DNA by histone proteins into chromatin structure. It has been known that formation of heterochromatin-like chromatin structures is implicated in epigenetic silencing *(95–98)*. In fact, functional chromosomal domains have been linked to silencing in yeast (reviewed in *97*) and Drosophila (reviewed in *96*). In addition, heterochromatin-like regions in mammalian cells have also been reported to be underacetylated at selected lysine residues in histone H4 *(99)*. Thus, proper function of histone deacetylase has been assumed to be required for the reformation of these regions during the latter part of each cell cycle *(100)*. Accordingly, histone acetylation has already been related to senescence *(101)*. Interestingly, telomere length in mice has also been reported recently to be directly regulated by histone methylation *(102)*. Therefore, besides increased oxidative stress *(103,104)* and telomere shortening *(20,22,76,105)*, alteration of epigenetic maintenance mechanisms can also induce cellular senescence.

3.3. Oxidative Stress

3.3.1. Stress-Induced Premature Senescence is Related to Oxidative Stress

Premature growth arrest in response to stress has previously been described in both fibroblasts and melanocytes as stress-induced premature senescence (SIPS) (reviewed in *106*). SIPS has been demonstrated for a variety of experimental conditions, including exposure to hydrogen peroxide (H_2O_2), tert-butylhydroperoxide (t-BHP), hyperoxia, UV light, and radioactivity. Oxidative stress is the most well understood inducer of SIPS (reviewed in *106*). Because growth arrest occurs at G_1 in human dermal fibroblasts post-H_2O_2 treatment, it has been speculated that cyclin-dependent kinase inhibitors are involved. In support of this, mRNA levels of $p21^{WAF1}$ and $p16^{INK4a}$ increased 72 h post-H_2O_2 stress followed by a decrease in hyperphosphorylated Rb which last several weeks *(25,107)*. It was also reported that there is a decrease in hyperphosphorylated Rb 5 days post-chronic t-BHP treatment *(108)*. Thus, p16/Rb pathway seems to function as a stress sensor mechanism to prevent further DNA damage due to exposure to oxidative damage.

3.4. Oncogene Activity

3.4.1. Overexpression of Oncogenes Induces Senescence in Normal Cells

Activated oncogenes have been known to trigger tumor suppressor mechanisms in primary cells to prevent propagation of potentially transformed cells. For example, *c-myc* has been shown to cause apoptosis *(109–112)*, activated *ras* has been shown to elicit senescence *(113,114)*, and β-catenin has been shown to induce p53-dependent apoptosis *(115)*. Tumorigenesis is an evolutionary process that selects for genetic and epigenetic changes to escape normal anti-proliferative and cell-death-inducing mechanisms and allows clonal expansion of malignantly transformed cells *(116)*. Thus, induction of apoptosis and/or senescence upon increased oncogenic signaling is crucial for eliminating or preventing dissemination of cells with damaged DNA or deregulated proliferation. Several mechanisms to restrain oncogenesis have been proposed, including hypoxia *(117)*, telomere attrition *(118)*, and induced expression of the Arf tumor suppressor *(119)*. However, the mechanism representing the major force guarding against genetic instability and tumorigenesis is actually unknown.

4. CONCLUSION

It is generally believed that unlimited replicative capacity is a required attribute for most malignant tumors to sustain long-term growth. As a result, cellular senescence has been thought as a potential tumor suppressor mechanism preventing cells from replicating indefinitely. However, direct experimental evidence that cellular senescence is a tumor suppressor pathway has remained elusive. Because telomere shortening regulates the onset of cellular senescence, one should undoubtedly conclude that loss of the growth controls without inducing a compensatory mechanism on telomere dynamics would lead to formation of tumors with an extended, but not infinite, life span because of continued telomere shortening. That is, if tumor cells lack a telomere maintenance mechanism, these cells would have a finite life span and consequently would not

produce life-threatening tumors. Clearly, dysfunction of cellular senescence must be among the changes required for perpetuation of malignant tumors.

In the past 15 years, two pathways that bypass cellular senescence have been extensively investigated: a telomerase-dependent (upregulation/reactivation of telomerase) pathway and a telomerase-independent (ALT) pathway. Because both pathways seem to abrogate the same basic mechanism that mediates cellular senescence, e.g., loss of telomeric sequences, targeting telomere maintenance mechanisms has become an attractive anti-neoplastic strategy. Continued progress in the study of cellular senescence, telomeres, and their role in cancer progression should help in the development of novel therapeutic strategies against cancer.

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9 Contributions of Telomerase to Tumorigenesis

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Summary

In eukaryotic cells, the telomere acts as a molecular cap, protecting the chromosome ends and preventing end-to-end fusions. In addition to this end protection function, dysfunctional telomeres limit replicative potential by inducing a nonproliferative state termed replicative senescence. In premalignant cells, telomere loss results in genomic instability, which promotes the formation and selection of protumorigenic mutations. Telomeres are maintained by the reverse transcriptase telomerase, an enzyme whose activity is tightly controlled by limiting the expression of the catalytic subunit, telomerase reverse transcriptase (TERT). The majority of human tumors exhibit abundant telomerase activity, which protects the chromosome ends to prevent additional genomic instability and confers unlimited replicative potential. This unique biochemical activity is vital to tumorigenesis and presents an ideal target for potential pharmacologic and immunologic therapies.

Key Words: Telomerase reverse transcriptase; telomere; tumorigenesis; transformation; cancer.

1. INTRODUCTION

Over the past decade, several lines of investigation have established the critical role of the biology of telomeres and telomerase in cancer development. Early work in the field defined the essential role of telomeres in chromosome end protection. However, later studies demonstrated a correlation between telomere length and replicative lifespan in cultured human fibroblasts. Specifically, telomere attrition occurs with successive cell divisions, suggesting that telomere structure specifies the number of cell divisions permitted in cultured diploid human fibroblasts *(1,2)*. Telomeres are maintained by telomerase, a reverse transcriptase that synthesizes de novo telomeric repeats *(3)*. Most human cancers express abundant telomerase activity, and genetic and cell biological

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studies have confirmed that telomerase expression facilitates cell immortalization, suggesting one function for telomerase activation in cell transformation. In addition to its role in cell immortalization, recent work suggests that telomerase may play additional roles during cancer development and tumor progression. This chapter will review our current understanding of the roles of telomerase and telomere biology in malignant transformation.

2. TELOMERE STRUCTURE

Eukaryotic chromosomes terminate in large arrays composed of tandem repeated guanine-rich sequences termed telomeres. Although the sequence and length of telomeres vary among eukaryotic organisms, in mammals the telomeric repeat is TTAGGG *(4,5)*. The extreme terminus of the telomere is composed of a single stranded overhang of variable length that invades distal telomeric sequences forming a lariat structure, or t-loop, which is thought to protect the chromosome terminus while masking it from cellular DNA repair machinery *(6)*. A number of proteins bind specifically to telomeres and regulate telomere function and structure. In mammals, telomere repeat-binding factors (TRF1 and TRF2) localize to the telomere through their ability to bind to double-stranded telomeric DNA *(6)*. TRF1 has been shown to negatively regulate telomere length whereas TRF2 protects telomere integrity, preventing telomeric end-to-end chromosome fusions *(7,8)*. Protection of telomeres 1 (POT1) binds to the 3′ single-stranded telomeric overhang, protecting the chromosome end *(9)* and regulating access to the telomere *(10)*.

These telomere-binding proteins are a foundation for the formation of large protein complexes at telomeres. Indeed, recent work indicates that TRF1 is linked to TRF2 through interactions with the TRF1 interacting nuclear factor 2 (TIN2) and POT1 and TIN2 organizing protein (PTOP1) *(11)*. These telomere-specific complexes play critical roles in regulating telomere maintenance. In addition, a number of proteins implicated as members of DNA repair complexes are found at the telomere. The homologous recombination repair Mre11 complex, which consists of Mre11, Rad50, and Nbs1, is recruited to the telomere by TRF2 *(12)*. Also present at the telomere is the non-homologous end-joining DNA-dependent protein kinase (DNA-PK) complex, consisting of Ku70 and Ku86 and the catalytic subunit DNA-PKcs *(13,14)* Through yet undefined mechanisms, these numerous proteins participate in the maintenance of telomeres.

Telomeres are maintained by telomerase, a tetramer composed of two subunits of the telomerase reverse transcriptase (TERT) catalytic subunit and two copies of the teleomerase RNA component (TERC) as well as an accessory protein known as Dyskerin *(3,15,16)*. Telomerase adds telomeric repeats to the telomere using a template provided by the RNA subunit *TERC (17)*. *TERC* is a member of the H/ACA family of small nucleolar RNAs and binds TERT through interactions involving the pseudoknot/templating domain and two conserved loop structures *(18,19)*. Co-expression of *in vitro* translated TERT and *in vitro* transcribed TERC reconstitutes telomerase activity, providing evidence that these two components form the minimal enzymatic core *(20)*. Although accessory proteins such as TEP1 have been found associated with the telomerase holoenzyme, the functions of this and other proteins remain undefined *(21,22)*. Both p23 and Hsp 90 are associated with telomerase and play a role in the assembly of the telomerase holoenzyme *(22)*.

3. TELOMERES AND CELL MORTALITY

In normal human cells, hTERT expression closely correlates with telomerase activity, suggesting that hTERT is the rate-limiting telomerase subunit *(23)*. High levels of telomerase activity have been identified in cells with greater replicative potential, such as cancer cell lines derived from tumors and immortal cell lines derived from the transformation of human cells with viral oncoproteins *(24)*. By contrast, mortal cell lines express telomerase only transiently and at low levels insufficient to maintain telomere length *(25)*. When such cells are cultured *in vitro*, they are only capable of undergoing a finite number of cellular divisions *(26,27)*. Preliminary studies concluded that the number of cellular divisions a somatic cell is able to undergo correlates with telomere length *(1,28)*, and there is a weak correlation between the age of an individual and the average length of the telomeres in their somatic cells *(2)*. Whether such cells would similarly stop dividing *in vivo* remains undefined; nevertheless, these observations suggest that the telomere serves as a molecular clock that can limit cell lifespan by counting cell divisions.

Human cells that are cultured for extended time *in vitro* cease dividing and enter a metabolically active, but non-proliferative state termed mortality stage 1 (M1) or replicative senescence *(27,29)*. The observation that telomere length shortens with each division *(1,28)* has led some groups to postulate that cells enter replicative senescence due to production of critically short telomeres *(30)*. Whereas some evidence suggests that a single short telomere may induce the senescence program *(31)*, other studies suggest that telomere state rather than a specific telomere length triggers senescence *(32,33)*. Functional ablation of the retinoblastoma (Rb) and p53 tumor suppressor pathways, by expression of the SV40 large T antigen or human papillomavirus E6 and E7 oncoproteins for example, permits cells to bypass the senescence state induced by each of these stimuli, suggesting that these tumor suppressor pathways play important roles in triggering the senescence program *(34)*.

Cells that bypass replicative senescence through the inactivation of these tumor suppressor pathways continue to proliferate until they reach another barrier, termed mortality stage 2 (M2) or crisis *(34)*. Crisis is characterized by widespread apoptotic cell death, and cells in crisis exhibit short telomeres yet continue to replicate their DNA, suggesting that cells entering M2 have lost the ability to halt cell cycle progression *(35)*. At a low frequency (approximately one in 10^7), cells survive crisis and achieve immortality, resulting in a line of immortal descendants *(36)*. Surviving cells harbor stable telomere lengths and the majority of such cells express detectable levels of telomerase *(37)*.

The notion that telomere length and replicative potential are intimately intertwined is supported by the evidence that exogenous expression of hTERT in post-senescent cells results in immortalization without crisis. In pre-senescent cells such as fibroblasts, endothelial cells, and mesothelial cells, expression of hTERT induces constitutive activation of telomerase and confers telomere length stability and immortality *(38–40)*. However, in other cells including many epithelial cell types, expression of hTERT fails to immortalize without the simultaneous inactivation of the p53 and Rb pathways *(41–44)*. Moreover, several other stimuli, including exposure to genotoxic agents, increased hypoxic stress, and oncogene activation, also induce a state that shares functional and morphologic similarity to replicative senescence *(45–47)*. The senescent state caused by these stimuli cannot be reversed by the expression of hTERT *(48)*, and such cells are senescent despite having normal telomere lengths. These stimuli

likely do not involve alteration of the telomere structure, suggesting that telomere dysfunction is not necessary to induce this state. Indeed, it is not yet clear whether short telomeres suffice to induce replicative senescence, or simply a trait of many of the early characterized senescent cells: whereas some groups have been able to determine the precise number of short telomeres needed to trigger senescence *(49)*, others have demonstrated robust cell viability in cells with critically short telomeres *(50)*. These observations have led to the postulate that there are different types of senescent states, triggered by excessive or accumulated stresses. Nevertheless, it remains unclear as to how telomerase expression and telomere length contribute to each of these types of senescence.

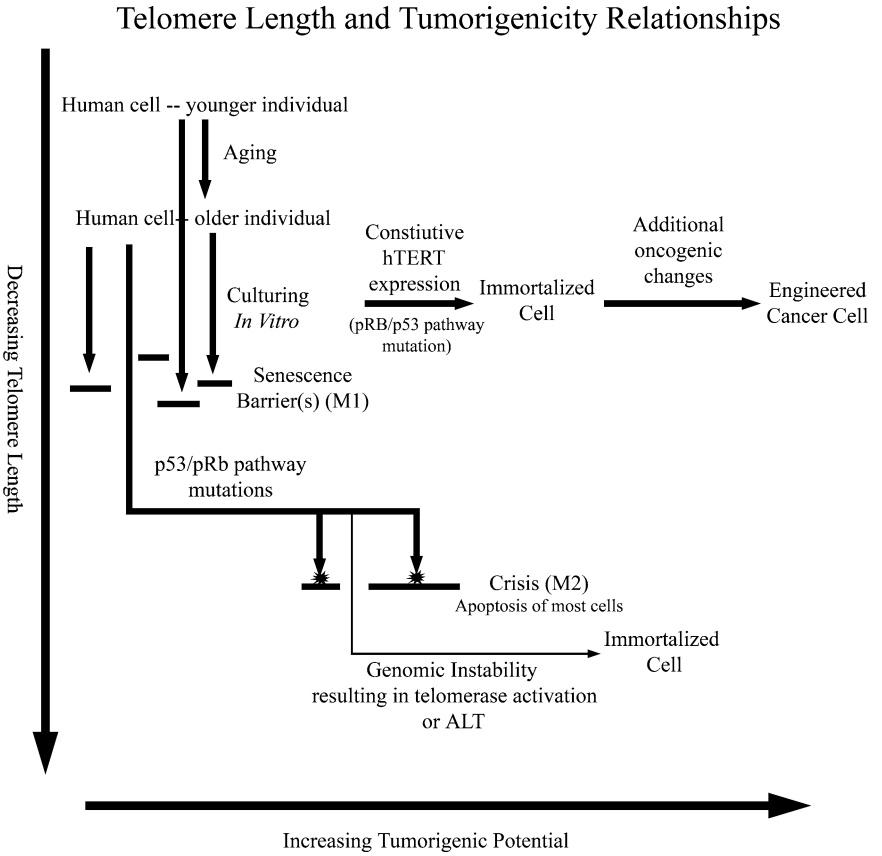

Fig. 1. Relationships between telomere length and tumorigenicity in human cells. Telomere lengths decrease with cell proliferation in cell types that suppress telomerase activity. Culturing of such cells *in vitro* leads to continued shortening followed by senescence (possibly linked to overall telomere length). Cells containing pRb and p53 pathway mutations continue dividing until cells enter crisis, triggered by critically short telomere lengths. Crisis is characterized by genomic instability resulting in apoptosis except in rare cases where telomerase or the alternative lengthening of telomeres (ALT) pathway is activated. The resulting immortal cells are not necessarily tumorigenic but can be transformed with additional oncogenes. Introduction of human telomerase reverse transcriptase (hTERT) (and loss of pRB or p53 pathway function) in pre-senescent cultured cells results in immortalized cells with longer telomeres than post-crisis cells. Additional oncogenic changes can render these cells tumorigenic.

Confounding these observations, there exists a minor yet important class of cells that is immortal but does not express telomerase and is believed to operate under a second pathway for telomere maintenance termed alternative lengthening of telomeres (ALT) *(51,52)*. Such cells are capable of maintaining their telomere lengths without elevated levels of hTERT expression or telomerase activity. There is some evidence suggesting that this pathway may involve telomeric recombination, but overall this alternative mechanism of telomere maintenance remains poorly understood *(53)*. Nevertheless, approximately 10–15% of human cancers display the ALT phenotype, suggesting that ALT may not contribute to transformation as efficiently as telomerase. A visual summary of the preceding observations relating to telomere length and tumorigenicity are included (Fig. 1).

Several lines of evidence have suggested that the structure of the telomere and not its actual length is the more important determinant of the immortalized versus senescent phenotype. A host of telomere-associated proteins binds the complex, high-order structure of the t-loop, among them TRF2 *(54)*. De Lange and colleagues have demonstrated that loss of TRF2 from the telomeres leads to destabilization of the t-loop, chromosomal fusions, and cell death *(8)*. TRF2 and the other proteins associated with the telomere are thought to protect the telomere by preventing its recognition as a double-strand break. However, at present, precisely how telomere-binding proteins and telomerase cap and protect chromosomes remains undefined *(55)*.

4. TELOMERES, DNA DAMAGE, AND REPLICATIVE LIFESPAN

One prediction that follows from these observations is that uncapped or dysfunctional telomeres should be recognized as DNA double-strand breaks. The core histone, H2AX, is phosphorylated in response to chemical and physical agents that damage DNA at sites surrounding DNA breaks *(56)*. These collections of phosphorylated H2AX mark DNA damage foci and serve to recruit other DNA repair factors to the site of damage *(57)*. In senescent cells, these DNA damage foci accumulate over time, suggesting that the senescent state can also be characterized by an ongoing DNA-damage response *(58)*. Conversely, in cells from patients afflicted with ataxia telangiectasia, Bloom's syndrome, or Werner's syndrome, whose DNA repair response is impaired, the onset of senescence is more rapid *(59,60)*. Furthermore, the chronic treatment of normal cells with DNA-damaging agents such as ionizing radiation or hydrogen peroxide also leads to an accelerated onset of senescence in the absence of telomere shortening *(61,62)*. Such observations have raised the question of whether shortened telomeres are triggering the senescent state or are simply correlative with cell division, which itself is correlated with DNA damage. Another possible interpretation of these data lies in observations by several groups that telomeres are either damaged by or particularly sensitive to DNA-damaging agents *(63–66)*. Damage to the telomere without causing simultaneous telomere shortening may promote telomere uncapping, leading to the onset of senescence. Nevertheless, the several reports of senescence-associated DNA-damage foci do not agree on whether such foci form preferentially or only coincidentally at the telomeres *(58,67)*.

Thus, the interplay between DNA damage, the telomere, and senescence remains intriguing yet poorly understood. Indeed, it is possible that this accumulated damage arises primarily at telomeres, thus making telomeres a prime stimulus for initiating

senescence. However, because a wide range of cellular stresses can trigger senescence, it is clear that other mechanisms including changes in chromatin state and oxidative damage may also contribute to this process.

5. THE TELOMERE AND CANCER

The current paradigm for the role of telomere biology in cancer is twofold. In normal cells, the telomere limits replicative potential while transient expression of telomerase maintains telomeres. In the malignant state, telomere loss results in genomic instability, scrambling the genomes of a population of premalignant cells and driving the selection of pro-tumorigenic mutations while expression of telomerase or maintenance of telomeres by the ALT pathway facilitates immortalization.

5.1. Potential Tumor-Suppressing Functions of the Telomere

Although the role that the telomere plays in senescence is not entirely understood, many of the observations mentioned previously are predictive of an *in vivo* cell-growth-limiting function of the telomere. In this model of tumor suppression, most cells are expected to divide a limited number of times in a single individual. The presence of the telomere, a molecular clock that counts these cell divisions, and telomerase, which potentially preserves genomic stability through maintenance of the telomeric cap, serves as a mechanism to limit replication, forcing cells that somehow step outside of their allotted number of divisions into apoptosis. Any cell division in excess of this amount required for most cell types may be the result of a necessary biological process (e.g., regeneration of the digestive tract and immunologic responses), and such cells often exhibit active telomere maintenance through expression of telomerase and potentially other factors. However, excessive cell division is clearly detrimental, and thus, activation of the p53 and Rb pathways in response to telomere shortening or some correlating phenomenon acts to ensure that any cells that have gained the ability to grow in the absence of proliferation signals or that have become insensitive to antiproliferative signals do not become immortal. Furthermore, the phenomenon of telomere uncapping, manifested as crisis *in vitro*, may play an additional role in eliminating cells that have somehow been able to become post-senescent by escaping p53 and Rb activities.

5.2. Tumor-Promoting Functions of the Telomere

In addition, the telomere contributes to cancer progression through an indirect mechanism involving genomic instability. As mentioned previously in Section 3 , cells in culture that have undergone a critical number of cell divisions can reach a proliferative barrier termed crisis. This cellular state is characterized by rampant genomic instability resulting from critical telomere shortening. The few surviving cells that are able to stably maintain their telomere lengths through expression of hTERT or the ALT pathway exhibit aneuploidy, a hallmark of many epithelial malignancies *(68)*. This genomic instability may facilitate the accumulation of the additional genetic changes necessary for transformation. An example of this phenomenon is seen in murine embryo fibroblasts (MEFs) derived from *mTerc* and *p53* null mice *(69)*. Mice lacking *mTerc* are viable despite being unable to maintain telomere lengths with each successive

division; however, with each passing generation, telomere lengths become critically short such that late generation mice become increasingly less viable. These late generation *mTerc* null mice with extremely short telomeres are refractory to transformation by pairs of oncogenes compared with early generation mice with longer telomeres, suggesting that limiting the lifespan of murine cells by artificially beginning with short telomeres inhibits transformation. Furthermore, as the senescent state induced by telomere shortening is in part due to the activity of p53, *mTerc*-deficient mice become prone to tumor formation in a p53 heterozygous background. Karyotype analysis of cells derived from the tumors of these mice identified several reciprocal translocations, a rare phenomenon in murine tumors, but reminiscent of structures seen in human cancer *(70)*. These observations implicate critical telomere shortening as a major mechanism that drives genomic instability in human tumorigenesis and demonstrate that whereas short telomeres may be able to limit replicative potential and therefore be antineoplastic, cells that are capable of escaping the chaos generated by loss of telomeric sequences are more prone to transformation.

5.3. Telomerase and Cancer

Although most human cancer cell lines and tumors exhibit constitutively high levels of telomerase activity, most normal human cells express little or low levels of telomerase. These observations suggest that telomerase activation in premalignant cells results in an immortalized state. In support of this hypothesis, overexpression of telomerase facilitates cell immortalization *(38)*, whereas inhibition of telomerase in already immortal cancer cell lines results in loss of telomeric sequences, a loss of proliferative capacity, and the onset of crisis *(71–73)*.

Consistent with the view that telomerase activation plays an important role in facilitating human cell transformation, co-expression of hTERT with cooperating oncogenes such as the rat sarcoma viral oncogene homolog (RAS) and the early region of Simian Virus 40 (SV40) generates cells with a transformed phenotype, capable of forming colonies in semi-solid medium or tumors when injected subcutaneously into immunodeficient mice *(74)*. Such systems have been used to transform a variety of cell types and investigate members of contributing pathways by substitution experiments. As an example, introduction of an oncogenic allele of Harvey Ras (H-RAS), the SV40 large T and small t antigens, and hTERT into human fibroblasts is sufficient to transform these cells; however, this same combination of genes without hTERT fails to permit transformation. In this setting, hTERT contributes to cell immortalization but may also play other, yet undefined roles in cell transformation.

6. ALTERNATIVE FUNCTIONS OF TELOMERASE

Indeed, somewhat unexpectedly, emerging evidence suggests that telomerase has an additional role in tumorigenesis distinct from its role in maintaining telomere structure. For example, ALT phenotype cells harboring the SV40 ER and H-RAS fail to form tumors unless hTERT is co-expressed *(75)*. Moreover, ALT phenotype cells derived from mTerc null mice exhibit an attenuated metastatic phenotype compared with cells in which telomerase was restored *(76)*. Consistent with these observations, several groups have noted that hTERT expression and telomerase activity are present in a number of precancerous lesions. The presence of hTERT expression before sufficient

telomere shortening has occurred to make telomerase activity requisite for continued replication suggests that hTERT may contribute to transformation beyond its effects on telomere length. Indeed, mice engineered to overexpress mTERT show an increased ability to form skin or breast tumors, although murine telomeres are already extremely long *(77,78)*. Indeed, analysis of murine tumors has found a small increase in telomerase activity *(79,80)*. As an initial hint to these alternative functions, some reports suggest that hTERT-expressing cells are relatively resistant to apoptosis *(48,81,82)*. Moreover, ectopic expression of mTERT in the mouse epithelium leads to an overgrowth of hair in these mice due to the increased proliferation and mobilization of hair follicle stem cells *(83,84)*. Finally, hTERT appears to play a role in setting chromatin state *(85)*. Taken together, these results suggest that a catalytically active telomerase enzyme may have a function beyond telomere maintenance, although the mechanisms behind these alterative functions have yet to be fully uncovered.

7. TELOMERE-BASED THERAPIES AND THERAPEUTIC CONSIDERATIONS

Given the strength of the connection between malignancy and telomere maintenance, several groups are engaged in the development of pharmacologic inhibitors of telomerase to abrogate telomerase activity in patients. Using several different methodologies, several groups have chosen to target hTERT. Genetic studies demonstrate that disrupting functional telomerase complexes with dominantly interfering mutants of hTERT leads to cell death by apoptosis dependent on telomere length in many but not all cell lines *(71,86)*. In addition, Damm et al. have reported the characterization of BIBR3132, a small molecule inhibitor of hTERT *(87)* which appears to inhibit telomerase processivity *(88)*. Cancer cell lines, when treated with these molecules, undergo rapid telomere shortening and eventually enter a non-proliferative state similar to senescence. Although effective, such drugs have a reversible mode of action, resulting in telomere lengthening and resumed proliferation once the drug is removed. This observation suggests that therapies involving telomerase inhibitors of this class would likely need to be administered for extended periods of time.

Several other groups have addressed the challenge of targeting telomerase activity by focusing on the telomerase RNA component. Suppression of telomerase RNA subunit by antisense constructs selectively suppresses tumor cell growth *(89,90)*. Expression of hTERC mutants expected to incorporate mutant telomere sequences has been shown to lead to apoptosis of cancer cells, suggesting that the telomere itself may be a potential antitumor target *(91,92)*. Furthermore, ablation of hTERC expression by RNAi has also proven effective alone and in combination with mutant RNA expression to inhibit the growth of transformed cells *(93)*. Another class of telomerase drugs is telomeric mimic sequences, targeted to the hTERC template and expected to form duplexes with hTERC, requestering telomerase away from telomeres *(94)*. Such drugs have shown efficacy in animal models but have limitations similar to those mentioned above.

The constitutive expression of hTERT in the majority of cancer cell lines has provided two additional classes of telomere-specific anticancer therapy involving the telomerase promoter and using telomerase as a target of immunotherapy. Many studies have shown that the fusion of proapoptotic genes such as *bax* or toxic genes such as thymidine kinase to the telomerase promoter can promote selective cell death of cancer

cells when these constructs are expressed *(95,96)*. Moreover, telomerase-expressing cells have been successfully targeted by cytotoxic T lymphocytes that recognize hTERT peptides *(97–100)*.

Despite the success of generating methodologies and drugs to exploit telomerase activity and expression in cancer cells, any successful therapy will need to be mindful of a multitude of factors specific to telomerase. These considerations range from cell types that normally exhibit telomerase activity as well as any alternative functions of telomerase and long-term effects of telomerase inhibition. Given that many tumors have activated telomerase, telomere lengths vary in different tumors. Thus, even given complete pharmacological ablation of telomerase activity, each cell in the tumor may have tens or hundreds of population doubling left before the onset of crisis. One may therefore expect the use of antitelomerase therapy in conjunction with other therapies that typically cause complete but temporary regression of a tumor, as such situations require the selection and excessive proliferation of a resistant clone. Another promising aspect of antitelomerase therapy not addressed in the above example is the phenomenon of oncogene addiction seen often in cancer cell lines in which the removal of a transforming factor leads to immediate cell death and regression of the tumor. Such a phenomenon has been observed in cancer cell lines in which telomerase has been inhibited, leading to cell death in the absence of critically short telomeres, perhaps due to the aforementioned capping (or alternative) activity of telomerase *(101,102)*.

As has been seen with other antineoplastic agents, one can expect clones to arise which are resistant to the antitelomerase therapy employed. Prediction of the mechanism of such resistance may help to design proper therapies involving telomerase. Given the already existent ALT phenotype, one might expect that resistant clones may maintain telomere lengths by this mechanism. However, genetic or antisense inhibition of telomerase has not to date resulted in a resistant clone with the ALT phenotype.

Ultimately, the ability to effectively implement an integrated antineoplastic therapy involving targeting of the telomere or telomerase will depend intimately on the elucidation of the mechanisms by which these biologic factors contribute to replicative potential. Answering such questions may also depend on determining the biological relevance of senescence and whether such a phenotype has an *in vivo* equivalent. It remains to be seen whether telomere length, structure, or some combination of the two is a determining factor of replicative potential, or if telomere uncapping or shortening is simply one (albeit a sometimes critical) destabilizing event in a series of stresses placed upon the cell.

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10 The Role of Telomeres in Genomic Instability

John P. Murnane, PhD

Summary

Genomic instability is now considered to play an important role in promoting the genetic changes necessary for cancer. It has been reported that exposure of cells to ionizing radiation or chemical agents can promote genomic instability, although the mechanisms involved are not understood. Knowledge concerning mechanisms of genomic instability has been derived from studies in yeast and human genetic disease, where mutations in a variety of genes involved in DNA replication, repair, and cell cycle regulation have been found to promote genomic instability. However, the identity of the mechanisms responsible for the genomic instability common to many cancer cells has yet to be determined, although increasing evidence demonstrates that loss of telomere function is important in this process. Telomeres are nucleoprotein structures that serve as caps that protect the ends of chromosomes and are maintained by the enzyme telomerase. Telomeres are maintained in the germ line but shorten with age in most somatic cells due to the lack of sufficient telomerase. This telomere shortening serves as a signal for replicative cell senescence, which protects against the unlimited cell division required for advanced forms of cancer. As a result, malignant cancer cells invariably maintain their telomeres, which is necessary to prevent massive chromosome fusion and cell death. However, telomere loss can occur during "crisis," in which cells that fail to senesce undergo critical telomere shortening prior to re-establishing the ability to maintain telomeres. In addition, it is now clear that even tumor cells that maintain telomeres continue to show a high rate of telomere loss, suggesting a fundamental defect in telomere maintenance in many cancer cells. Telomere loss results in sister chromatid fusion that initiates prolonged breakage/fusion/bridge (B/F/B) cycles, in which chromosomes repeatedly fuse and break for many cell generations. This prolonged instability results in various chromosomal rearrangements that are commonly associated with cancer, including amplification, deletions, nonreciprocal translocations, and duplications of whole chromosome arms. This instability is not confined to the chromosome that originally lost its telomere but can be transferred to other chromosomes as well. Thus, the loss of a single telomere can result in a large population of cells with a variety of changes in many different chromosomes. Understanding the mechanisms of telomere

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loss and the factors that control chromosome instability resulting from B/F/B cycles is therefore an important goal in cancer research.

Key Words: Amplification; chromosome fusion; deletion; duplication; genomic instability; isochromosome; telomere; translocation.

1. GENOMIC INSTABILITY AND CANCER

Genomic instability has been proposed to play an important role in cancer by accelerating the accumulation of genetic changes responsible for cancer cell evolution *(1,2)*. Genomic instability can occur through a variety of different mechanisms, including a defective DNA damage response, defects in DNA replication, and defects in chromosome segregation. Most cancer cells do not show an increased rate of mutation at the nucleotide level, suggesting that increased misincorporation during replication or a failure to correct mismatched or damaged nucleotides is not a common mechanism for instability in human cancer *(3)*. Instead, most genomic instabilities associated with human cancer involve chromosome instability (CIN), which includes gross chromosome rearrangements and/or alterations in chromosome number. Studies in yeast have identified a large number of genes that can lead to CIN when inactivated, including genes involved in spindle formation, cell cycle checkpoints, recombination pathways, and telomere maintenance *(4)*. In mammals, defects leading to CIN can arise through either inherited mutations associated with genetic diseases or mutations arising in somatic cells. A number of human genetic diseases demonstrate increased CIN and cancer predisposition. Many of the proteins responsible for these diseases, including ATM in ataxia telangiectasia, NBS1 in Nijmegen breakage syndrome, FANC genes in Fanconi anemia, and BRCA1 and BRCA2 in hereditary breast cancer, are critical for cell pathways signaling the presence of DNA damage and are necessary for proper cell cycle regulation and repair *(5)*. However, unlike the well-defined genetics of these inherited syndromes, the identity of the genetic defects leading to CIN in sporadic human tumors is much less well defined. Alterations in the MAD2 protein involved in the mitotic checkpoint *(6)* and the *hCDC4* gene involved in the G1/S checkpoint *(7)* have been found to be associated with CIN in a small number of human cancers. Inactivation of the Rb protein can also promote CIN by uncoupling cell cycle progression from mitotic control *(8)*. However, the identity of specific genes responsible for CIN in many tumors has yet to be determined, although increasing evidence now points to loss of telomere function as a major contributor to CIN in human cancer (see Section 5).

2. STRESS-INDUCED GENOMIC INSTABILITY

Genomic instability can also result from exposure to ionizing radiation or chemical agents *(9–11)*. Unlike mutations or chromosomal rearrangements occurring at the time of exposure, these alterations continue to occur for many cell generations. Like the genomic instability associated with genetic disease or cancer, this stress-induced genomic instability can take different forms, ranging from small mutations to gross chromosomal rearrangements. The high percentage of cells in the population that demonstrate increased genomic instability following exposure to ionizing radiation strongly suggests that this stress-induced genomic instability is not due to mutations in one or a few genes. Instead, it has been proposed that stress-induced genomic

instability is due to epigenetic changes leading to alterations in gene expression that result in increased DNA damage or reduced repair. These epigenetic changes could result from long-term activation of signal transduction pathways or prolonged release of cytokines in response to cell damage. In fact, exposure to ionizing radiation has been demonstrated to result in changes in expression of a large number of genes *(12)*, and irradiated cells have been demonstrated to generate factors that can promote instability in unirradiated cells *(13,14)*. In addition, radiation-induced genomic instability has been found to correlate with increased levels of reactive oxygen species that can lead to a higher rate of spontaneous DNA damage *(14,15)*. However, the exact mechanisms by which ionizing radiation can promote long-term genomic instability through *trans*-acting mechanisms have yet to be established.

An alternative mechanism by which radiation or chemicals can induce genomic instability in a high percentage of cells is through *cis*-acting mechanisms involving the formation of unstable chromosomes. One mechanism by which unstable chromosomes could be created is through the juxtaposition of incompatible chromatin structures as a result of the DNA damage-induced chromosome rearrangements. The possibility of unstable chromatin structures resulting from chromosome rearrangements was first proposed from data demonstrating variability in the stability of integration sites of transfected DNA *(16)*. This model proposes that unstable chromosome regions would be more likely to undergo subsequent rearrangements, leading to a cascade of chromosome rearrangements involving multiple chromosomes. In support of this hypothesis, subsequent studies found that chromosome rearrangements involving centromeric heterochromatin showed a high rate of subsequent rearrangements *(17,18)*. Thus, DNA damage occurring near centromeres might be more likely to initiate CIN. Similarly, DNA damage occurring near the ends of chromosomes, called telomeres, might also promote CIN. In fact, there is increasing evidence that the loss of telomeres can lead to CIN and that telomere loss is a mechanism for CIN in cancer (see Section 5).

3. TELOMERE STRUCTURE AND FUNCTION

Telomeres are DNA–protein complexes that contain short repeat sequences added on to the ends of chromosomes by the enzyme telomerase *(19,20)*. Telomeres serve multiple functions, including protecting the ends of chromosomes *(21)* and preventing chromosome fusion *(22)*. Telomeres are maintained in germ line cells but shorten with age in most somatic cells due to the lack of sufficient telomerase activity to compensate for the loss of DNA from the ends of chromosomes in dividing cells *(22)*. Telomere shortening in somatic cells is a signal for replicative cell senescence *(22)*, which results from the inability of the telomere to form a cap that protects the end of the chromosome *(23)*. Primary human fibroblasts that have lost the ability to senesce continue to show telomere shortening and eventually enter "crisis," which involves extensive chromosome fusion, aneuploidy, and cell death *(24,25)*. Similarly, human epithelial cells that fail to senesce can enter "agonescence," which like crisis involves increased chromosome fusion *(26,27)*. Thus, it has been proposed that most somatic cells must first regain the ability to maintain telomeres to undergo the large number of cell divisions required for cancer *(24,28)*. Consistent with this hypothesis, the introduction of telomerase into normal human fibroblasts can result in a greatly extended capacity to divide in culture *(29)*. In addition to telomerase, immortal human cells have

also been found to maintain telomeres through a telomerase-independent mechanism, termed alternative lengthening of telomeres (ALT) *(30,31)*, in which telomeres are maintained through recombination *(30,32)*. Studies found that most tumors demonstrate telomerase activity *(33,34)*, although tumors maintaining telomeres through the ALT mechanism have also been observed *(35)*.

A large number of different proteins have now been identified that are involved in telomere structure and function *(36)*. Two of the initial proteins that were identified included TRF1, which is involved in the regulation of telomere length *(37,38)*, and TRF2, which is involved in the formation of capping structures that prevent chromosome fusion *(39)*. Subsequent studies have identified a myriad of other telomere-associated proteins, many of which have other functions in DNA repair and cell cycle regulation *(36)*. This overlap in proteins involved in the cellular response to DNA double-strand breaks and telomere maintenance points to the requirement for processing and sequestering the ends of chromosomes to prevent telomeres from being recognized as double-strand breaks and to prevent chromosome fusion. This relationship between DNA repair and telomere maintenance is further illustrated by increasing evidence that telomere function influences the cellular response to DNA damage *(40–43)*. Furthermore, mice deficient in telomerase are sensitive to ionizing radiation *(42,43)* and alkylating agents *(44)*. Because radiosensitivity is not seen in early generations of these mice, it is attributed to the cumulative affects of loss of telomere function due to gradual shortening rather than to the absence of telomerase. A slower rate of repair of double-strand breaks in cells from telomerase-deficient mice suggests that a deficit in repair pathway is involved. Consistent with this conclusion, another study reported that expression of telomerase in human fibroblasts can affect transcription of proteins involved in DNA repair *(41)*. In addition, it has been reported that ionizing radiation increases telomerase activity in some cells, although the mechanism and significance of this observation are not clear *(40)*.

4. MECHANISMS AND CONSEQUENCES OF TELOMERE LOSS

Telomere loss can initiate CIN through chromosome fusion. Although as discussed in section 3, the loss of telomere function can serve as a signal for cell senescence, chromosome fusion is common in cells that are deficient in the senescence pathway. The importance of telomeres in preventing chromosome fusion is demonstrated by the extensive chromosome fusion observed in cells in crisis or agonescence when telomeres become critically short *(24–27)*. Similarly, the loss of telomere function brought about by expression of dominant-negative TRF2 also results in extensive chromosome fusion and cell death *(39)*. The type of fusions resulting from telomere loss depends on which chromosome ends lack telomeres. Loss of telomeres from both ends of the same chromosome can result in the formation of ring chromosome, whereas the loss of telomeres from the ends of two different chromosomes can result in the formation of dicentric chromosomes. Both these types of chromosome aberrations are visible at metaphase and are common in cells in which telomere function has been severely compromised, such as in yeast defective in telomerase *(45)* or in mammalian cells deficient in TRF2 *(39)*.

Unlike the extensive chromosome fusion observed in TRF2-deficient cells or cells in crisis that have severe telomere dysfunction, the loss of a single telomere is not

necessarily lethal in cells that are deficient in the senescence pathway. Although fusion between two different chromosomes will not occur, following DNA replication, the loss of a single telomere can result in fusion of sister chromatids *(46–49)*. This type of chromosome rearrangement is more difficult to monitor than ring or dicentric chromosomes, because sister chromatid fusions are not detectable by routine cytogenetic analysis. As first described by McClintock *(50)*, sister chromatid fusion can result in prolonged CIN involving breakage/fusion/bridge (B/F/B) cycles. B/F/B cycles occur when the fused sister chromatids form a bridge during anaphase and then break when the sister chromatids are pulled toward the daughter cells during cytokinesis (Fig. 1). This process is then repeated in the next cell cycle, as the chromosome will again be without a telomere in each of the daughter cells. Because of unequal breakage of the fused sister chromatids, repeated B/F/B cycles result in amplification and deletion events on the end of the chromosome. These B/F/B cycles can continue until the chromosome acquires a new telomere and again becomes stable.

There are multiple mechanisms by which a chromosome can lose a telomere. As mentioned above, telomere loss may occur from exposure to exogenous sources of DNA damage such as ionizing radiation or chemical mutagens. The likelihood of the loss of a telomere due to double-strand breaks occurring near the end of a chromosome may be increased by a deficiency in DNA repair in subtelomeric or telomeric regions. Studies

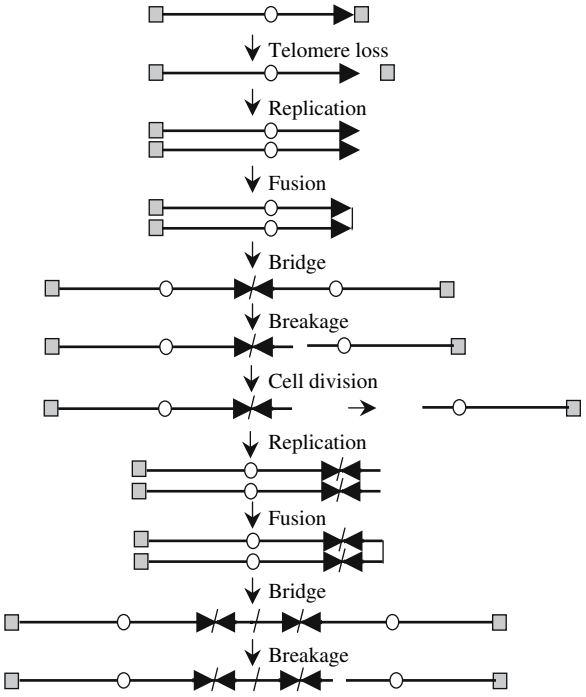

Fig. 1. Chromosome instability resulting from breakage/fusion/bridge (B/F/B) cycles initiated by telomere loss. B/F/B cycles are initiated when sister chromatids fuse following the loss of a telomere. The sister chromatids then form a bridge during anaphase when their centromeres are pulled in opposite directions. Breakage occurs at locations other than the site of fusion, resulting in amplification of sequences in one daughter cell and deletions in the other. The telomeres (squares), centromeres (circles), and orientation of the subtelomeric sequences (horizontal arrows) are shown.

in yeast have shown that regions around telomeres are deficient in nonhomologous end joining *(51)*, a primary mechanism for double-strand break repair in mammalian cells. Studies in human cells have also found a reduced rate of repair of single-strand breaks or ultraviolet light-induced DNA damage within telomeric repeat sequences *(52,53)*. A deficiency in DNA repair in regions near telomeres in mammalian cells would explain why the spectrum of rearrangements resulting from the introduction of double-strand breaks with the I-*Sce*I endonuclease near telomeres is much different than that observed at interstitial sites. Double-strand breaks at interstitial sites most often result in small deletions *(54,55)*, whereas double-strand breaks near telomeres most often result in sister chromatid fusions or direct telomere addition at double-strand breaks near telomeres *(46–48,56)*. DNA damage occurring at or near telomeres may therefore be much more likely to result in telomere loss and complex chromosome rearrangements. In fact, a role for radiation-induced telomere loss in cancer has been suggested by studies in radiation-induced breast cancer in BALB/c mice. The increased radiation-induced breast cancer in these mice has been associated with a deficiency in the DNA-PKcs (DNA-dependent protein kinase active site) gene involved in double-strand break repair *(57)*. However, cells deficient in DNA-PKcs also show increased end-to-end chromosome fusion, demonstrating that DNA-PKcs also has a role in telomere maintenance *(58–60)*. This combined deficiency in double-strand break repair and telomere maintenance may together contribute to the predisposition of BALB/c mice to cancer, because the dysfunctional telomeres can recombine with misrepaired double-strand breaks in the cells from these mice following ionizing radiation *(61)*.

Telomere loss can also occur through spontaneous events resulting from alterations in proteins involved in telomere maintenance. This type of telomere loss can involve gradual telomere shortening due a failure to compensate for telomere attrition, or sudden loss of telomere function due to rapid deletion of telomeric repeat sequences or failure of capping structures. Gradual loss of telomeres in cancer cells has been shown to result from a periodic reduction in expression of telomerase *(62,63)*. Cancer cells can also show rapid fluctuations in telomere length due to an inability to properly regulate telomere length *(64)*. These increases and decreases in telomere length are likely to be due to changes in expression of proteins involved in the regulation of telomere length. In fact, defects in a large number of proteins involved in telomere maintenance have been shown to promote chromosome fusion, including Ku and DNA-PK *(58–60)*, the Mre11/Rad50/Nbs1 complex *(65–67)*, and the Werner ryndrome nelicase (WRN) helicase *(68)*. Breaks at telomeres could also result when replication forks become stalled at telomeres, as has been demonstrated in yeast *(69,70)*. Consistent with this possibility, reduced function of proteins involved in the resolution of stalled replication forks, such as Nbs1 or WRN, can influence telomere loss *(67,68)*. Deletions due to recombination within telomeric repeat sequences can also result in rapid loss of telomeres, as has been observed in cells expressing TRF2 protein with a deletion in the basic amino acid domain at its N terminus *(71)*. Finally, a failure to maintain the capping structure that protects the ends of chromosomes could result from altered function of a number of different proteins known to be involved in cap formation, including telomerase itself *(72,73)* and TRF2 *(39)*.

5. TELOMERE LOSS AND CANCER

Spontaneous telomere loss is likely to be an important source of CIN in cancer. As mentioned above, loss of telomere function can occur when cells escape senescence and therefore enter crisis or agonescence *(24–27)*. The chromosome fusion due to loss of telomere function at crisis has been proposed to be an important mechanism for the chromosome rearrangements leading to cancer *(74)*. Consistent with this conclusion, *in vivo* studies in humans demonstrated a dramatic increase in CIN during the transition from ductal hyperplasia to ductal carcinoma in situ when breast epithelial cells appear to progress through crisis and become immortal through expression of telomerase *(75)*. However, this study also found that more advanced cancers continued to show a high rate of telomere instability even after expression of telomerase. This observation is consistent with studies demonstrating a high rate of telomere instability and anaphase bridges in early passage tumor cells in culture *(76)* and the high rate of loss of individual telomeres in a human tumor cell line *(47,48)*. Therefore, despite the expression of telomerase, cancer cells commonly have problems maintaining telomeres, suggesting that transformation is associated with a fundamental defect in telomere function. A fundamental change in telomere function in cancer cells is also indicated by the fact that despite the expression of telomerase, most cancer cells have telomeres shorter than those found in normal cells *(77,78)*.

Additional proof of the importance of telomere loss as a mechanism for CIN can be found in the increasing evidence demonstrating that B/F/B cycles are a common mechanism for chromosome rearrangements in human cancer. Despite B/F/B cycles being described in maize more than 50 years ago *(50)*, until recently there has been little evidence for a role of B/F/B cycles in CIN in human cells. Several studies had demonstrated that high-copy gene amplification occurs through B/F/B cycles in hamster cells *(79–82)*. However, highly amplified genes in human cells are found on double-minute chromosomes or as homogenously staining regions cells *(83)*. More recently, structures consistent with B/F/B cycles have been observed in low-copy gene amplification in some human tumor cell lines *(84,85)*. Although not initially apparent, B/F/B cycles may also be an early step in high-copy gene amplification in human cells. Most clones of a human tumor cell line selected in culture for gene amplification contained double minute (DM) chromosomes; however, one clone showed amplification consistent with B/F/B cycles *(86)*. When placed under more stringent selection conditions, the amplified region in this latter clone converted to DM chromosomes, leading to the conclusion that B/F/B cycles can be an initial step in the formation of DM chromosomes. Consistent with this conclusion, the regions amplified by B/F/B cycles in hamster cells have also been shown to form DM chromosomes *(81,87,88)*. In fact, telomere instability and anaphase bridges have been observed in many early passage tumor cells *(76)*. In addition, in histologic tumor sections, anaphase bridges show a correlation with abnormal karyotypes in sarcomas and pancreatic and colon carcinomas *(89)*. This initiation of gene amplification by B/F/B cycles would explain why inverted repeats are commonly observed in amplified regions in human cancer cells *(90)*.

Cell cycle regulation plays an important role in preventing CIN through B/F/B cycles. Cells involved in B/F/B cycles enter the next cell cycle with a chromosome lacking a telomere, which would signal a cell cycle arrest. Studies on replicative senescence resulting from telomere shortening *(91–94)* or loss of telomere function due to overexpression of dominant-negative TRF2 *(94)* demonstrate that the presence

of double-strand break repair foci associated with telomeres is a signal for replicative cell senescence *(92)*. The importance of a similar mechanism in preventing CIN through B/F/B cycles is demonstrated by studies in telomerase-deficient mice. The progressive telomere shortening in telomerase-deficient mice results in only a modest increase in cancer *(95)* because in addition to promoting chromosome fusion, telomere shortening can also serve as a tumor suppressor mechanism *(96)*. However, mice that are deficient in both telomerase and p53 show a dramatic increase in the frequency of carcinomas that are similar to those seen in humans *(97,98)*. Cytogenetic analysis of these tumors demonstrated chromosome rearrangements consistent with B/F/B cycles, suggesting that telomere loss and B/F/B cycles play an important role in human cancer. Similarly, lymphomas in mice with knockouts in p53 and various proteins involved nonhomologous end joining were also shown to contain chromosome rearrangements consistent with B/F/B cycles *(99–102)*.

6. MECHANISMS OF RESTORATION OF LOST TELOMERES

There are multiple mechanisms by which a chromosome can acquire a new telomere and either prevent or terminate B/F/B cycles. The most straightforward mechanism is through de novo addition of a telomere by telomerase, which has been observed in *Tetrahymena* and yeast *(103,104)*. Direct telomere addition at the end of a broken chromosome has also been observed in mouse embryonic stem (ES) cells and human tumor cells, as well as at the ends of chromosomes terminal deletions associated with various human genetic diseases *(105–107)*. Extracts from HeLa cells have demonstrated that human telomerase is capable of de novo addition of telomeres to DNA ends using only a few base pairs of homology *(108)*. Despite this evidence, there is little data to suggest that de novo addition of telomeres is a common occurrence at double-strand breaks resulting from ionizing radiation. In fact, as mentioned earlier, mammalian cells have been found to demonstrate similar types of chromosome rearrangements in response to ionizing radiation regardless of whether they express telomerase *(109,110)*. Why direct telomere addition to the ends of broken chromosomes should be a rare event in mammalian cells expressing telomerase is not known. One possibility is that the small amount of telomerase in mammalian cells is not capable of competing with the ubiquitous double-strand break repair proteins. Alternatively, de novo addition of telomeres may be actively suppressed. In yeast, de novo telomere addition is inhibited by the PIF1 helicase (Petite integration frequency), which has been proposed to prevent de novo telomere addition from competing with double-strand break repair and generating terminal deletions *(111)*.

In addition to direct telomere addition, telomeres can also be acquired by either break-induced replication (BIR) or translocation of the ends of other chromosomes. BIR has been observed in yeast *(45,112)* and occurs when the broken end of a chromosome invades a region of homology near another telomere and initiates replication, thereby duplicating the end of that chromosome. Like de novo addition by telomerase, BIR results in the net gain of a telomere and thereby stabilizes the genome. Telomeres can also be acquired through telomere capture, involving the translocation of the ends of other chromosomes, as has been observed in human cancer cells *(48,113)* and in mouse ES cells *(46)*. However, unlike de novo synthesis or BIR, telomere acquisition through translocation can have important consequences for the stability of the genome,

as translocations can lead to genetic imbalances or transfer of instability to other chromosomes (see below).

7. THE USE OF SELECTABLE MARKER GENES TO MONITOR THE CONSEQUENCES OF TELOMERE LOSS

To more fully understand the consequences of telomere loss for CIN, mouse ES and human tumor cell lines were developed that contain an Herpes simplex virus-thymidine kinase (*HSV-tk*) selectable marker gene located adjacent to a telomere *(46–49,56)*. Using ganciclovir to select for loss of the *HSV-tk* gene, cell clones were isolated that had lost the telomere on this marker chromosome. Because of the presence of an I-*Sce*I recognition sequence that is also present at this telomere, double-strand breaks can also be introduced at this location following transfection with expression vectors containing the I-*Sce*I gene. I-*Sce*I has been used extensively to introduce double-strand breaks in mammalian cells to study recombination and DNA repair processes *(54,55,114–117)*. This approach has the advantage over previous studies in that selecting for telomere loss instead of later downstream events such as amplification provides the method to study the process from the beginning and therefore identify the early events following telomere loss. Several important observations have been made through the analysis of the consequences of telomere loss on individual chromosomes. The first important observation is the high rate of spontaneous loss of the telomeric *HSV-tk* gene in the human tumor cell line relative to the mouse ES cells. In the human tumor cell line, the mutation rate in telomeric *HSV-tk* genes was 10^{-4} events/cell/generation, which was 100-fold higher than the mutation rate at interstitial *HSV-tk* genes in this cell line *(48)*. Most of the mutations involving the telomeric *HSV-tk* gene were due to telomere loss, whereas those at the interstitial *HSV-tk* gene were due to point mutations. By contrast, the rate of spontaneous mutation of the telomeric *HSV-tk* gene in the mouse ES cell line was less than 10^{-6} and was never observed to result from telomere loss *(56)*. Thus, the human tumor cell line has a high rate of spontaneous telomere loss, which is consistent with the telomere instability, anaphase bridges, and CIN that have been observed in human tumor cells both in culture and in vivo *(75,76)*.

A second important observation made from these studies is that the types of events resulting from telomere loss are much different than those observed at double-strand breaks at interstitial sites. Two major types of events were observed following spontaneous or I-*Sce*I-induced telomere loss: direct addition of telomeres to the end of the broken chromosome and inverted repeats resulting from sister chromatid fusion *(46–49,56)*. By contrast, mutations resulting from I-*Sce*I-induced double-strand breaks at interstitial sites primarily involve small deletions *(54,55)*. Although other types of events have also been observed, including large deletions and translocations, direct telomere addition or inverted repeats have not been reported. This observation suggests that double-strand breaks occurring near telomeres are processed differently than those occurring at other locations. In fact, studies in yeast have demonstrated that double-strand breaks generated with the I-*Sce*I endonuclease are repaired by nonhomologous end joining much more efficiently at interstitial sites than near telomeres *(51)*.

A third important observation derived from these studies is the prolonged period that B/F/B cycles can continue once initiated in human tumor cells *(46–49)*. This is a critical observation, as the prolonged B/F/B cycles result in a large clonal expansion

of cells containing an unstable chromosome without a telomere. Unlike the mouse ES cells where B/F/B cycles lasted only a few generations *(46)*, chromosomes that had lost a telomere in the human tumor cell line were still involved in B/F/B cycles in 20% of the cells in the population even after more than 20 generations *(47)*. The presence of B/F/B cycles was evident from the absence of a telomere and the presence of anaphase bridges specific to the marker chromosome that had lost its telomere. Because of unequal breakage of the fused sister chromatids at anaphase, these prolonged B/F/B cycles resulted in extensive amplification of DNA on the end of the marker chromosome even without selection. The use of cosmid probes containing DNA from different locations on the chromosome demonstrated that the fused sister chromatids most often broke within 1 Mb of the site of fusion. Thus, the region near the site of fusion is prone to breakage, possibly due to the presence of the large inverted repeat that can serve as a hotspot for recombination *(118–120)*. However, breaks also occurred at a lower frequency at other locations, as demonstrated by the presence of large duplications on the end of the chromosome in some cells. Studies in hamster cells have shown that the breakage at other sites tends to occur at fragile sites, and is favored when there is selection for amplification of specific genes *(82,85)*. Because each amplification/duplication is accompanied by a corresponding deletion on the other sister chromatid, B/F/B cycles are highly efficient in generating large deletions, consistent with the presence of both amplified DNA and deletions in tumors from telomerase-deficient mice *(121)*. Studies in yeast have found that much of the instability resulting from telomere loss is due to deletions resulting from degradation and not B/F/B cycles *(122)*. However, the extent of degradation in mouse ES cells following telomere loss was relatively small, consistent with the small deletions observed at I-*Sce*I-induced double-strand breaks at interstitial sites in mammalian cells *(54,55)*. By contrast, the deletions resulting from B/F/B cycles can be much larger, some consisting of large portions of the chromosome.

A fourth important observation made in the course of these studies is that although chromosomes involved in B/F/B cycles can be stabilized through the acquisition of a telomere, this does not commonly occur by direct telomere addition by telomerase. An extensive analysis of a tumor cell subclone in which the marker chromosome was actively involved in B/F/B cycles demonstrated that most of the acquired telomeres originated by translocation or duplication of existing telomeres *(49)*. Only approximately 16% of the telomeres that were acquired during B/F/B cycles did not contain detectable translocations or duplications. In fact, direct telomere addition may be completely absent, because it is possible that these chromosomes also have duplications or translocations that are too small to be detected. This low level of direct telomere addition is consistent with studies demonstrating that the expression of telomerase has little effect on the cellular response to ionizing radiation *(109,110)*. Why direct telomere addition should be a rare event during B/F/B cycles even in human tumor cells that express telomerase suggests that direct telomere addition is actively suppressed, as proposed in yeast *(111)*. In this regard, it is interesting to note that translocations were also a common event for telomere acquisition during B/F/B cycles in mouse ES cells *(46)*, even though direct telomere addition was by far the most common event at double-strand breaks occurring near telomeres *(56)*. One possible explanation for this difference is that direct telomere addition involving de novo synthesis of telomeres by telomerase preferentially occurs near existing telomeres.

A fifth important observation from these studies is that the mechanism by which telomeres are acquired during B/F/B cycles has important ramifications for the stability of the genome as a whole. Multiple mechanisms of telomere acquisition were observed, all of which resulted in the stabilization of the marker chromosome (Fig. 2). One mechanism for telomere acquisition that was observed in about 8% of the cells involved small duplications of the other end of the marker chromosome *(49)*. The mechanism for these small duplications is not known but may occur through BIR, as BIR was found to be involved in a similar duplication of the other end of a chromosome that had lost a telomere in yeast *(112)*. This mechanism of telomere acquisition stabilizes both the chromosome undergoing B/F/B cycles and the genome as a whole, because it involves the net gain of a telomere. Another mechanism for telomere acquisition involved translocations resulting from large duplications of the arms of other chromosomes,

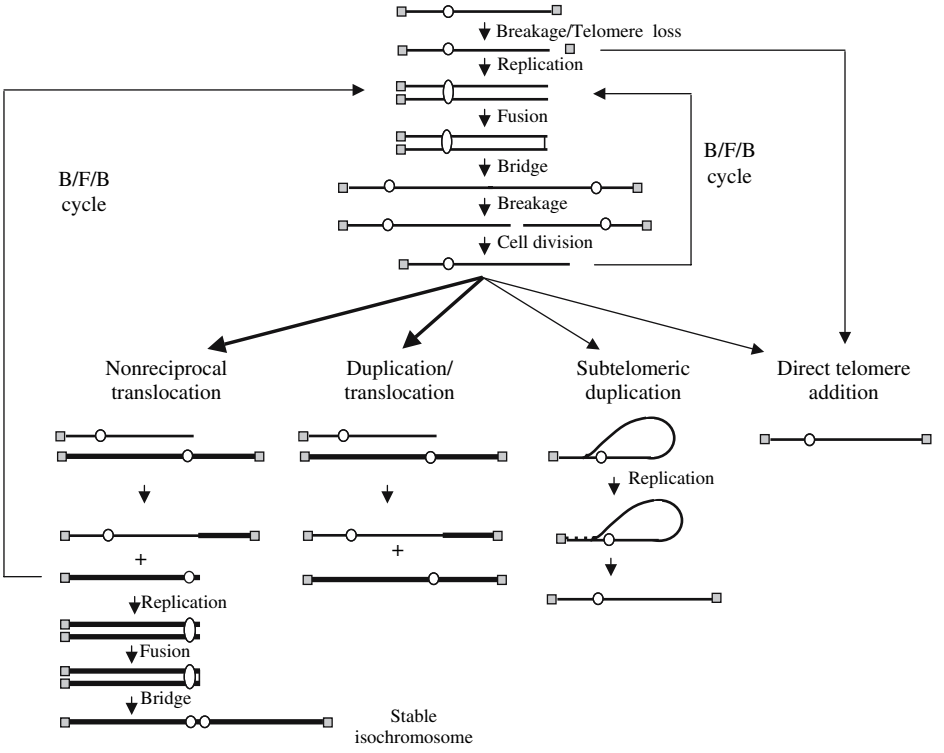

Fig. 2. Mechanisms of telomere acquisition during breakage/fusion/bridge (B/F/B) cycles and their consequences for stability of the genome. The loss of a telomere, either as a result of a double-strand break or failure to properly maintain telomeres, results in either direct addition of telomeres or sister chromatid fusion and B/F/B cycles. B/F/B cycles continue until the chromosome acquires a new telomere by (i) nonreciprocal translocation (NRT), (ii) translocation involving duplications of large portions of other chromosomes, (iii) small duplications of a telomeric region on the other end of the same chromosome, or (iv) direct addition of a telomere, possibly involving de novo synthesis by telomerase. The chromosome donating the NRT can enter the B/FB cycle due to the loss of its telomere, or when breakage occurs near the centromere, the fused sister chromatids can form a stable dicentric isochromosome. Telomere acquisition by NRT can therefore transfer instability from one chromosome to another, while duplications and direct addition of telomeres result in stabilization of the genome, although they can contribute to the clonal karyotypic evolution of the cancer cell.

which accounted for one-fourth of the acquired telomeres *(49)*. However, unlike the small duplications of the other end of the marker chromosome, these large duplications of chromosome arms had a significant impact on the genome as a whole, because they resulted in large gene imbalances. Finally, telomeres were also acquired through nonreciprocal translocation (NRT), which involved the transfer of large portions of the arms of other chromosomes. Like large duplications, NRTs also accounted for one-fourth of the telomeres acquired during B/F/B cycles *(49)*. However, these NRTs were unique in that they resulted in the loss of the telomere from the donor chromosome. As a result, the chromosomes donating these NRTs became unstable and underwent subsequent rearrangements, including NRTs, duplications, and isochromosome formation. Thus, in addition to gene amplification and deletions, telomere loss is an efficient mechanism for generating chromosome duplications and NRTs, both of which are commonly associated with human cancer *(113,123)*. The mechanism by which these translocations occur is not known. However, the presence of duplications at the sites of translocations on chromosomes that have undergone B/F/B cycles suggests that it may be an active process that is initiated when the chromosome end without a telomere invades another chromosome and initiates DNA replication, similar to BIR *(124)*. Combined with the clonal expansion of the cell containing a chromosome without a telomere due to the prolonged B/F/B cycles, these results demonstrate that the loss of a single telomere can rapidly result in a population of cells with extensive karyotypic heterogeneity involving multiple chromosomes. Telomere loss is therefore likely to be a major mechanism for CIN in cancer cells, not only during crisis but also during later stages of tumor cell progression. Understanding the mechanisms of telomere loss and the factors that control CIN associated with B/F/B cycles is therefore an important goal in cancer research.

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III DNA Damage Response, Signaling Pathways, and Tumorigenesis

11 Overview of the DNA Damage Checkpoint

ATM and ATR

Aude Dupré, PhD, and Jean Gautier, PhD

Summary

Maintenance of genome stability in response to DNA damage is essential for cell survival. DNA damage occurs following external insults such as ultraviolet light and ionizing radiations or as a consequence of DNA replication or physiological DNA rearrangements. Cells respond to DNA damage by the coordinated induction of cell-cycle arrest and DNA repair or trigger apoptosis if the damage is too extensive. The DNA damage response is induced by two prominent intermediates: double-strand breaks and single-strand DNA. These pathways are well conserved from yeast to mammals and comprise a network of proteins acting together to monitor, sense, and repair DNA damage. In this review, we will focus on the central role played by two protein kinases, Ataxia-Telangiectasia mutated (ATM) and ATM related (ATR), during S-phase checkpoints and how these proteins are regulated by other components of the DNA damage response such as BRCA1, the Mre11-Rad50-Nbs1 complex, Claspin, and ATR-interacting protein (ATRIP). All these proteins, which are involved in sensing the damage (ATM, Mre11, Nbs1), in mediating (BRCA1) or transducing (Chk2) the signal, are essential for maintaining genome stability. Notably, mutations in genes encoding these proteins lead to increased risk for cancer.

Key Words: ATM; ATR; checkpoints; replication; double-strand break DNA; single-stranded DNA.

1. INTRODUCTION

Cells are continuously subjected to DNA damage that arises as a consequence of external insults such as ultraviolet (UV) light, ionizing radiation (IR), oxidative stress, and DNA-damaging agents or as a consequence of DNA replication, meiotic and V(D)J recombination. DNA damage may lead to mutations as well as chromosomal rearrangement or chromosomal loss. To maintain genome stability, cells have evolved a protective mechanism that we refer as the DNA damage response. Critical components

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of the DNA damage response are checkpoint pathways. These pathways regulate cell-cycle arrest, which allows DNA repair and cell survival, or apoptosis if the damage is too extensive (Fig. 1). Much progress has been made in our understanding of these pathways that are rapidly activated following damage. Cells lacking a functional DNA damage response display genome instability, faulty DNA replication, or aberrant chromosome segregation, resulting in an acceleration of the mutation phenotype *(1)*.

The DNA damage response can be functionally separated into three steps using different sets of proteins that act in concert to transduce the signal from the lesion to the physiological output. Proteins of the first group are "sensor" proteins, which recognize aberrant DNA structures or subsequent chromosome alterations and initiate signaling (Fig. 2). Once the damage is detected, the signal is relayed to "transducers," typically protein kinases, which convey and amplify the DNA damage signal to downstream effectors. These effectors are then modified and activated to evoke the physiological response (Fig. 2). Because some of these transducers are also involved in the assembly of DNA–repair complexes at the site of damage, DNA repair and checkpoint responses are concomitant and functionally linked. Finally, a class of proteins, called "mediators" or "adaptors," may promote functional interactions between sensors and effectors.

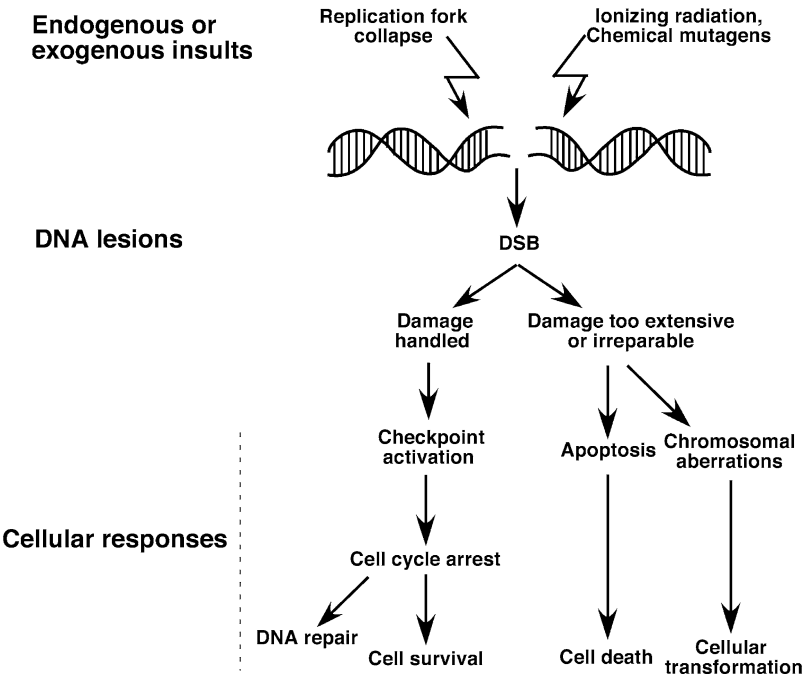

Fig. 1. Schematic representation of cellular responses to double-strand breaks (DSBs). DSBs are induced in response to external insults such as ionizing radiations (IR) and chemical mutagens or occur during normal cellular physiology, that is, when the replication fork collapses. The cellular response to DSBs leads to the activation of cell-cycle checkpoints that allow the coordination of cell-cycle arrest with DNA repair, ensuring cell survival and genome integrity. If DNA damage is too extensive or irreparable, cell can also induce apoptosis. Failure to detect or to repair DSBs can result in mutations, deletions, and rearrangements, which may cause cellular transformation and genomic instability.

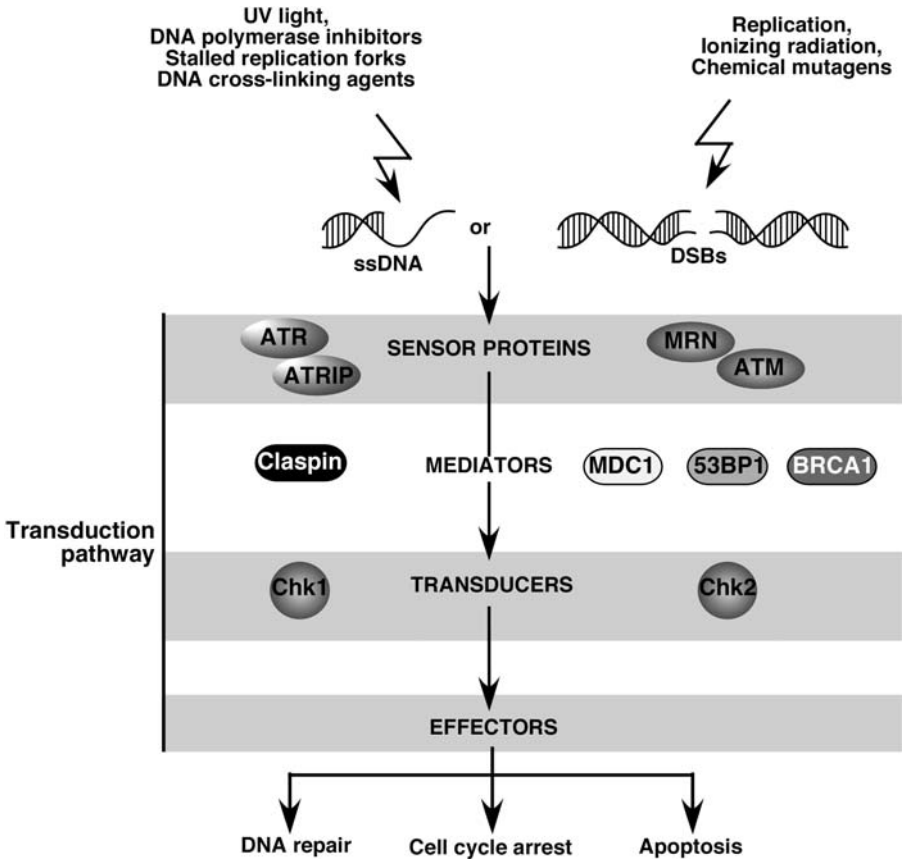

Fig. 2. Overview of the molecular components of the DNA damage response. The cellular response to single-strand DNA (ssDNA) or double-strand breaks (DSBs) can be divided into three different steps that induce cell-cycle arrest, DNA repair, or apoptosis. ssDNA or DSBs are detected by "sensor" proteins, ATR-ATRIP or ATM-MRN respectively. ATM and ATR are then activated and phosphorylate transducers such as Chk1 or Chk2, with the help of "mediator" proteins. Transducers are in turn activated and regulate effectors responsible for cell-cycle arrest at the G1/S and G2/M transitions or during the S phase.

1.1. DNA Lesions and Aberrant DNA Structures

DNA damage includes covalent modifications and non-covalent abnormal structures such as mismatches, loops, and bubbles arising from a string of mismatches *(2)*. Covalent changes are induced by several intrinsic and extrinsic insults and include (i) structures generated at the replication fork: Holliday junctions, stalled or collapsed replication forks; (ii) base damages such as thymine glycols, oxidized or fragmented bases in response to reactive oxygen species, IR, and UV light; (iii) backbone damages including abasic sites, single- and double-strand DNA breaks; (iv) DNA interstrand crosslinks formed by crosslinking agents such as cisplatin, mitomycin C (MMC), and psoralen. It is currently thought that not all aberrant structures are recognized as such and directly trigger a response. Instead, they are converted into a limited number of signaling intermediates. This enables cells to share a limited number of pathways in response to a large number of problems. The most prominent intermediates are

double-strand breaks (DSBs) and single-stranded DNA (ssDNA) that are generated in response to IR, UV light, and radiomimetic agents. During DNA replication, nicked DNA produces DSBs, whereas treatments with hydroxyurea (HU), aphidicholin, or UV create ssDNA. Finally, DSBs could be converted into ssDNA by processing and signaling from these two DNA structures therefore overlaps functionally.

1.2. Cell-Cycle Checkpoint

DNA damage checkpoints are signal transduction pathways that induce cell-cycle arrest or delay in response to DNA damage. Cell-cycle progression is tightly coordinated with repair during the DNA damage response. Here, we will focus on the Ataxia-Telangiectasia mutated (ATM)- and ATM related (ATR)-dependent checkpoints that regulate S-phase progression (Fig. 3). These checkpoints regulate early steps of DNA replication: the assembly or the activation of the pre-Replicative Complex (pre-RC). These checkpoints can be classified in two groups whether it requires or not DNA replication and fork progression.

Checkpoints that are independent of active DNA replication operate during G1 or S phases and are induced by DSBs or ssDNA. During G1, the G1/S checkpoint recognizes the presence of damaged DNA and delays the subsequent S phase by inhibiting origin firing. Similarly, the intra-S-phase checkpoint blocks DNA replication in S phase. Following damage sensing outside of replication forks, ATM and ATR are activated, leading to the phosphorylation and activation of Chk2 and Chk1, respectively. Ultimately, these pathways downregulate Cdk2 and Cdc7 protein kinase activities, which are both required for Cdc45 loading and subsequent replication origin activation (see also Chapter 3.2.1.2.).

During S phase, two DNA replication-dependent checkpoints are active. The first one is called the "Replication" checkpoint and is initiated when progression of replication forks is stalled in response to genotoxic stresses such as deoxynucleotides depletion, chemical inhibition of DNA polymerases, or as a consequence of aberrant structures or DNA damage (Fig. 3). This checkpoint inhibits unfired origins through the activity of ATM and Chk1. In addition, ATM and ATR are involved in the recovery of stalled replication forks. The second checkpoint is the S/M checkpoint that monitors completion of DNA replication before entry into mitosis (Fig. 3). The downstream target of this checkpoint is the inactivation of Cyclin B–cdk1 complexes, thereby preventing mitosis entry. Once activated, Chk2 phosphorylates and inhibits Cdc25 phosphatase activity, leading to the inactivation of Cyclin B–Cdk11 complexes. Other ATM substrates such as Nbs1 or BRCA1 have been involved in the S/M checkpoint (Fig. 3).

Finally, some recent data also support the idea that the signaling pathways regulating these checkpoints are functional even in the absence of DNA damage and help to coordinate progression through the cell cycle *(3)*.

1.3. Genome Instability Syndromes

Several hereditary cancer-prone syndromes exhibit defects in the DNA damage response (Table 1). These disorders include ATM, Ataxia-telangiectasia (A-T)-like disorder (ATLD) (Mre11), Nijmegen Breakage Syndrome (NBS), Werner syndrome (WRN), Fanconi anemia (FA), and familial forms of breast and cervical cancers with

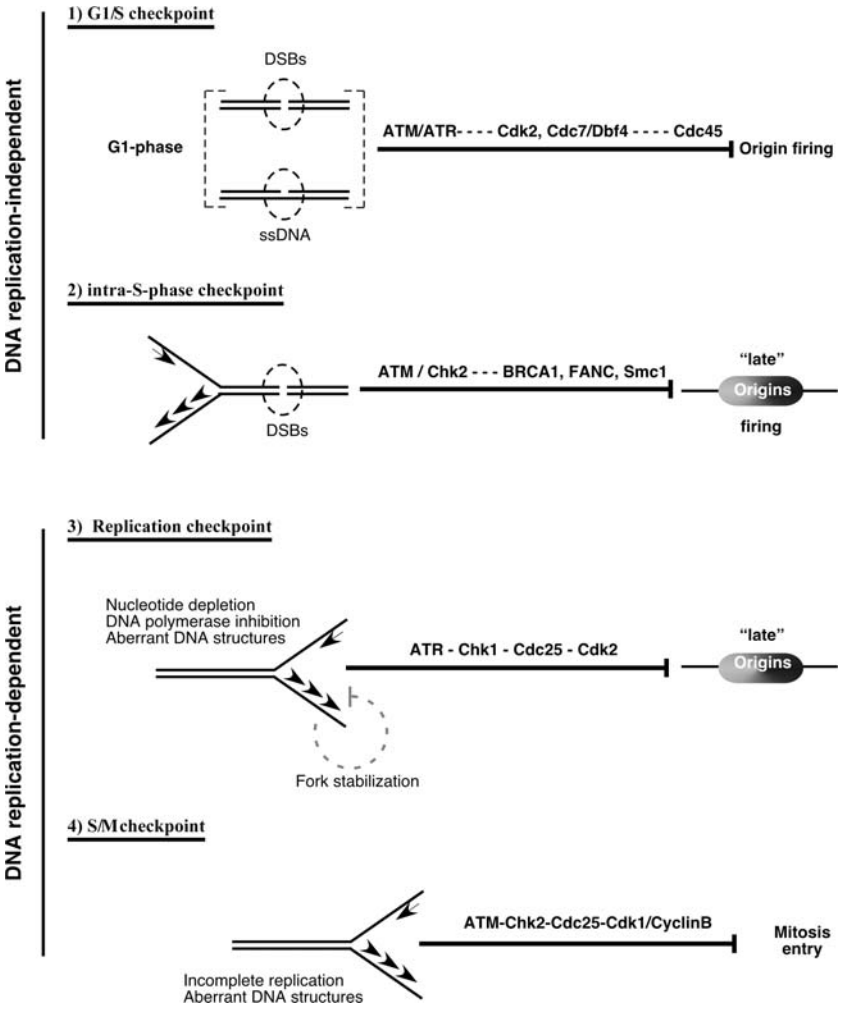

Fig. 3. Checkpoints that influence S phase. Checkpoints that regulate S phase can be divided in two groups whether it requires or not DNA replication and fork progression. The first group is independent of replication forks and includes the G1/S and the intra-S-phase checkpoints triggered by the presence of DSBs or ssDNA. **(A)** The G1/S inhibits S-phase entry by preventing the loading of Cdc45 onto replication origins and origin firing. This mechanism is mediated by the inhibition of two major S-phase kinases, Cdk2 and Cdc7, in response to ATM and ATR activation. **(B)** The intra-S-phase checkpoint blocks DNA replication in S-phase by inhibiting the firing of late replication origins. In response to DSBs, ATM is activated and phosphorylates proteins involved in the DNA damage response including Chk2, the Mre11-Rad50-Nbs1 complex (MRN), BRCA1, Fanconi Anemia (FANC) proteins, and the SMC protein 1 (Smc1). Abrogation of these phosphorylations events following exposure to IR results in a radio-resistant DNA synthesis (RDS). The second group of checkpoints influencing S phase depends on DNA replication. It contains **(C)** the "replication" checkpoint that is induced in response to stalled replication forks, nucleotides depletion or caused by aberrant DNA structures. This checkpoint inhibits unfired origins by activating the ATR/Chk1 pathway, which inactivates the activity of the Cdk2 kinase. **(D)** The S/M checkpoint is activated when DNA replication is incomplete or in the presence of aberrant DNA structures. This checkpoint is mediated by the activation of ATM and Chk2 pathway, which in turn inactivates by phosphorylation the activity of Cdc25 phosphatase. The major M-phase kinase, Cdk1, is inactivated, and mitosis entry is prevented.

Table 1
Clinical presentation associated Genome Instability syndromes

	A-T	*NBS*	*ATLD*	*FA*	*WRN*	*BRCA*
Clinical symptoms						
Telangiectasia	+	−	−	−		
Ataxia	+	−	+	−		
Microcephaly	−	+	−	+		
Immunodeficiency	+	+	−	ND		
Malignancy	+	+	ND	+		
Cellular phenotypes						
Radiation sensitivity	+	+	+	+	+	+
Chromosomal instability	+	+	+	+	+	+
RDS phenotype	+	+	+	ND	ND	+

ND, not described.

mutations in the *BRCA1* or *BRCA2* genes. In addition, a splicing mutation in the *atr* gene is responsible for Seckel syndrome.

1.3.1. Ataxia-Telangiectasia: ATM

ATM protein is the product of the gene that is mutated, lost, or inactivated, in the human autosomal recessive genetic disorder A-T *(4,5)*. Ataxia or the inability to coordinate and control motor movement is a result of cerebellar degeneration. A-T is also characterized by immunodeficiency, genome instability (a markedly increased rate of chromosome breakage in cell culture), thymic and gonadal atrophy, and sensitivity to IR and DSB-inducing agents *(6,7)*. A-T patients commonly develop cancer during their second or third decade of life, particularly Hodgkin's and non-Hodgkin's lymphomas as well as various lymphoid leukemias *(8)*. Some patients with A-T harbor mutations that cause frame-shifting, premature termination of translation, or exon-skipping during mRNA processing *(9,10)*. As these mutations result in the truncation and complete destabilization of ATM, they behave like null mutations. Less severe forms of the disease also occur because of missense mutations in the ATM gene *(9)*.

1.3.2. Seckel Syndrome: ATR

Seckel Syndrome is an extremely rare recessive inherited disorder characterized by intrauterine growth retardation, dwarfism, microcephaly, varying degrees of mental retardation, and/or unusual characteristic facial features *(11)*. The gene responsible for this disease has been mapped to chromosome 3 and encodes the ATR protein *(12)*. The mutation identified to date results in a splicing defect leading to reduce levels of wild-type ATR protein.

1.3.3. Other Chromosome Instability Syndromes

Two rare inherited diseases have phenotypes similar to A-T. ATLD is a very rare disorder caused by hypomorphic mutations in the *Mre11* gene, which lead to C-terminal truncations or missense mutations. ATLD patients show a phenotype very similar to that of A-T with regard to cerebellar ataxia. In contrast to A-T, these patients do not

develop telangiectasia, and the progression of the disease is very slow. None of the ATLD patients being described to date have developed cancer, and mice carrying a mutation analogous to the ATLD mutation do not develop lymphomas *(13)*. NBS is a recessive autosomal syndrome caused by hypomorphic mutations in the gene encoding the Nbs1 protein. Immunodeficiency, small head and growth failure, mental deficiency, genome instability, and an increased risk of malignancy characterize NBS patients.

Several other chromosomal instability and cancer predisposition syndromes have been linked to ATM and ATR signaling pathways. Bloom syndrome (BLM) is a very rare disorder caused by mutation in the *blm* gene. The BLM protein is a member of the RecQ helicase family *(14)*. Importantly, Bloom patients are predisposed to various cancers such as lymphomas, carcinomas, and leukemias. Cells from Bloom patients are characterized by spontaneous sister-chromatid exchanges, chromosomal breakages, and rearrangements. WNS is caused by mutations in the WRN protein, another RecQ homolog. The most characteristic manifestation of WNS is premature aging. Cells from WNS patients exhibit chromosomal translocations and breakage and are hypersensitive to camptothecin, an inhibitor of DNA topoisomerases I, and some DNA crosslinking agents. FA is a rare recessive disorder that is characterized by bone-marrow depletion leading to aplastic anemia. Many patients eventually develop acute myelogenous leukemia (AML) and carcinomas at a very early age. Cells from FA patients show chromosomal alterations and are sensitive to DNA-crosslinking agents such as MMC but not to IR *(15,16)*. This disease is heterogeneous with mutations affecting at least 12 different genes. Finally, two major genes have been identified that are associated with familial breast and ovarian cancers: *BRCA1* and *BRCA2* (breast cancer susceptibility genes). BRCA1 mutations account for approximately 40% of inherited cases of breast cancer. Bi-allelic inactivation of BRCA1 is also observed in sporadic cases of breast cancer. Inherited mutations affect both germline alleles. BRCA1 and BRCA2-deficient cells exhibit hypersensitivity to IR and chromosomal abnormalities.

All of these cancer-prone syndromes were originally thought to be unrelated and to lead to cancer predisposition by affecting distinct pathways. However, recent advances in the understanding of the molecular connections between the DNA damage response and the maintenance of genome stability have unraveled an increasing number of connections between all these pathways affected in these diseases. ATM and ATR protein kinases are critical for the maintenance of genome stability. In the following sections, we attempt to highlight the biochemical and functional connections between these protein kinases and other proteins that are inactivated in disease affecting the maintenance of genome stability.

2. BIOCHEMISTRY OF ATM AND ATR

2.1. ATM and ATR Genes

The gene mutated in A-T (ATM) is localized at the q22–23 locus of chromosome 11 *(17)*. The 150 kb gene encodes a 13 kb transcript with 66 exons *(18)*. ATM is ubiquitously expressed at all stages of development and its level does not fluctuate during the cell cycle or in response to DNA damage *(19–22)*. In most cells, ATM is localized in the nucleus with a small fraction associated with cytoplasmic endosomes or peroxisomes *(22–24)*. In highly differentiated cells, such as purkinje cells of the

cerebellum, ATM is exclusively cytoplasmic, but the mechanisms controlling the subcellular distribution of ATM have not been yet determined *(24)*.

The *ATR* (ATM and Rad3 related) gene was identified by homology with Rad3 and Mec1p in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively, and mTor in human cells *(25,26)*. This gene maps to the 3q22–24 locus of chromosome 3 and is highly expressed in testis, skeletal muscle, and pancreas *(25)*. Unlike ATM, disruption of the *ATR* gene in mice is embryonic lethal, suggesting that ATR plays an essential role in early development or in cell growth *(27,28)*.

2.2. Structural Properties

ATM and ATR genes encode for two related serine/threonine protein kinases of 3056 and 2644 amino acids, respectively (Fig. 4). These proteins are conserved in all eukaryotes and belong to the phosphoinositide 3-kinase-like kinase (PIKK) protein family based on sequence homology in the C-terminal catalytic domain *(29)*. Other mammalian members of this family include DNA-dependent protein kinase (DNA-PK), mTor, and SMG1. No lipid kinase activity has been described for these proteins. ATM and ATR contain also two FAT (FRAP/ATM/TRRAP) domains, commonly found in FRAP proteins: one directly N-terminal to the PI3K domain and the other localized at the C-termini of both proteins called FAT-C *(30)*. The N-terminal portion domain of both proteins has no similarity with other proteins and is composed of large HEAT repeats that form superhelical scaffolding matrices to interact with other macromolecules *(31)*. ATM contains also a Proline-rich motif (PXPXPPX2P) that could be involved in specific protein–protein interactions *(29)*. ATM and ATR phosphorylate serines or threonines followed by a glutamine, that is, (S/T)-Q motif *(32–34)*. This (S/T)-Q motif is present in all known ATM and ATR substrates including p53, Chk2, BLM, BRCA1, FANCD2, Nbs1, Mre11, TRF1, Smc1, Histone H2AX, and c-Abl.

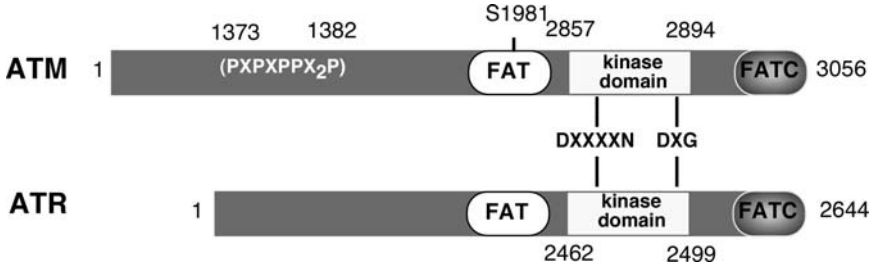

Fig. 4. Structural organization of ATM and ATR. ATM and ATR belong to the phosphoinositol kinase-like kinase (PIKK) family of protein kinases. ATM and ATR share three different conserved motifs within their C-terminal sections: the FAT (FRAP/ATM/TRAAP) domain, the FAT-C (FAT-C terminal) domain, and the kinase domain that contains conserved catalytic residues DXXXXN and DXG. The catalytic domain of ATM has about 30% amino acid identity with the PI3K and is the signature motif for members of the PIKK proteins. ATM and ATR are serine/threonine protein kinase activity and no lipid kinase activity has been described for those proteins. Serine 1981 is within the FAT domain of ATM. This residue is important for ATM autophosphorylation and activation in response to IR (see text). The N-terminal region of ATM has no significant amino acid similarity to other proteins. This region is composed of HEAT repeats and contains a proline-rich motif, both probably involved in protein interactions.

2.3. Mechanisms of ATM and ATR Activation

As mentioned earlier, two prominent types of DNA damage intermediates are DSBs and ssDNA coated with replication protein A (ssDNA-RPA). Although ATM- and ATR-dependent responses partially overlap, ATM is central to the DSB-induced response, whereas ATR is primarily involved in responding to ssDNA.

2.3.1. Activation of ATM

2.3.1.1. ATM Autophosphorylation. ATM activity is stimulated 2–3-fold following IR or neocarzinostatin (NCS) treatments *(35,36)*. Following IR, ^{32}P is incorporated into ATM supporting a mechanism for ATM activation by autophosphorylation *(37,38)* (Fig. 5). In undamaged cells, ATM exists as an inactive protein dimer; the catalytic domain of one ATM molecule is blocked by the FAT domain of the other ATM molecule. Following DNA damage, each ATM molecule within a dimer trans-phosphorylates the other one at serine 1981 within the FAT domain, leading to the dissociation of the dimer and to the generation of active monomers (Fig. 5). ATM activation could be triggered by chromatin alterations as well as by direct interactions between ATM and the broken DNA ends *(37)*. It is important to emphasize that, even though ATM autophosphorylation is a critical process for its activation, additional phosphorylation steps and/or mechanisms are likely to be required to reach full ATM activation. ATM activity is then assayed by phosphorylation of ATM substrates such as Smc1, Chk2, or H2AX that is one of the earliest ATM susbtrates phosphorylated in response to IR.

2.3.1.2. Co-Factors Required for ATM Activation: MRN Complex. The MRN complex is composed of three proteins: Mre11, Rad50, and Nbs1 (Xrs2 in yeast). Mre11 contains four N-terminal phosphoesterase motifs, required for its 3′–5′ exonuclease and endonuclease activities and two DNA-binding domains *(39)* (Fig. 6). Rad50 belongs to the structural maintenance chromosome (SMC) protein family and contains a Walker A and B motifs brought together by an intramolecular coiled-coil formation, resulting in an active ATPase domain. The central region, also called "hook," can chelate Zn^{2+} and thus allows intermolecular interactions between Rad50 molecules *(39)*. Nbs1 is composed of two N-terminal domains: Forkhead-associated (FHA) and BRCT (BRCA1-C terminal) phosphopeptide-binding motifs, predominantly found in proteins participating in the DNA damage response and acting to recruit and activate downstream protein kinases *(40)*. The C-terminal region of Nbs1 contains the Mre11-binding domain, the ATM-binding site and three NLS *(41–44)*. In response to DNA damage, Nbs1 and Mre11 are phosphorylated at "SQ" sites (serine 343 and 278 for Nbs1, whereas the "SQ" sites for Mre11 have not been yet identified) leading to the hypothesis that MRN is an effector of the DNA damage response, positioned downstream of ATM *(45,46)*. However, more recent work established that MRN also acts upstream of ATM, as a direct activator *(47)*.

In yeast, Mre11 is one of the first proteins recruited to the sites of DSBs in response to IR *(48,49)*. ATLD and NBS cells are partially deficient for ATM-dependent phosphorylation of p53, Chk2, Smc1, and FANCD2 following irradiation, indicating that full ATM activation requires a functional MRN complex *(49–56)*. *In vitro*, addition of baculovirus-expressed MRN complex to purified ATM recruits ATM to damaged DNA and dissociates ATM into monomers *(57)*. MRN also stimulates

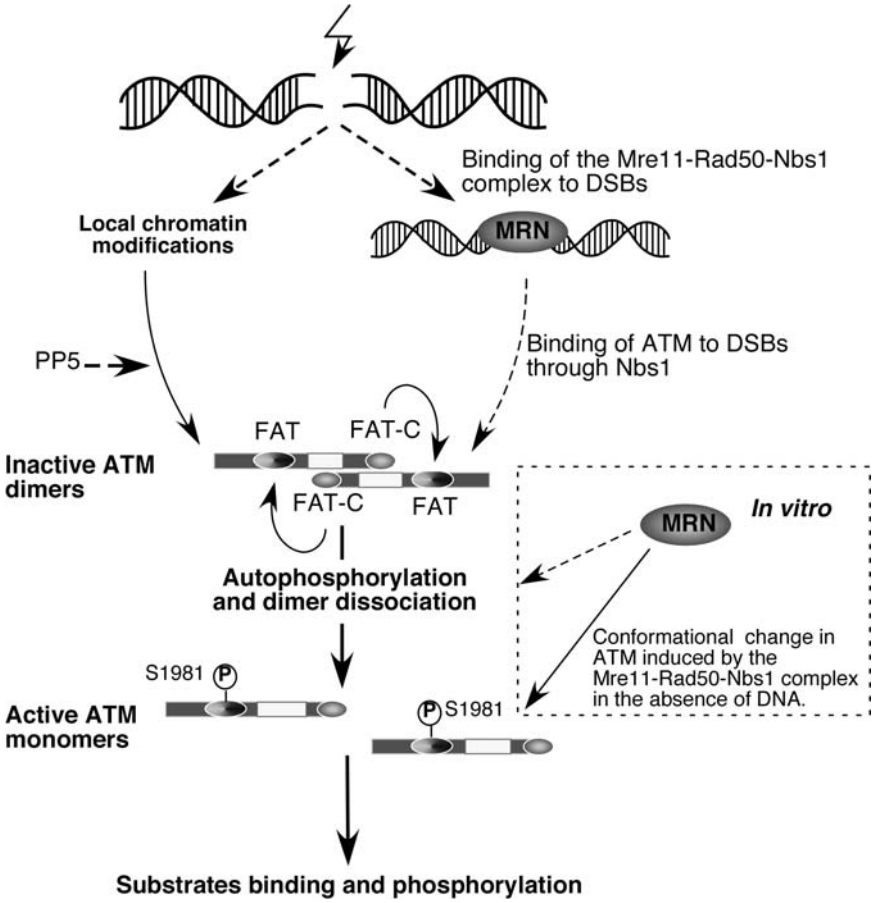

Fig. 5. ATM activation. In untreated cells, ATM is present as inactive dimers. The catalytic domain of both ATM monomers is then blocked by the FAT domain of the other ATM molecule. In the presence of double-strand breaks (DSBs) or following changes in chromatin structure, ATM becomes autophosphorylated on serine 1981 and dissociates into active monomers. In mammalian cells, the Mre11-Rad50-Nbs1 (MRN) complex acts as a direct activator of ATM by promoting ATM's recruitment to damaged DNA in response to IR. *In vitro*, MRN is required for the monomerization of ATM in response to DNA broken molecules. Surprisingly, MRN also increases ATM affinity for its substrates in the absence of DNA, suggesting that MRN may induce a conformational change in ATM increasing its substrate's affinity.

ATM-dependent phosphorylations of p53 and Chk2 in the absence of free DNA ends *(58)*, arguing that MRN may induce a conformational change in ATM, increasing its affinity for substrates (Fig. 5). ATM autophosphorylation was shown to be impaired in cells expressing adenoviral proteins that degrade MRN further supporting the hypothesis that MRN is a direct ATM activator *(56)*. Finally, biochemical evidence supporting this idea came from experiments using *Xenopus* cell-free extracts. Addition of linear double-strand DNA fragments to these extracts activates ATM in an MRN-dependent manner *(43,59,60)*. Active Mre11 and ATM are recovered in a high molecular weight protein/DNA complex associated with DSB-containing DNA. This result establishes that the MRN complex is required for ATM activation and that the

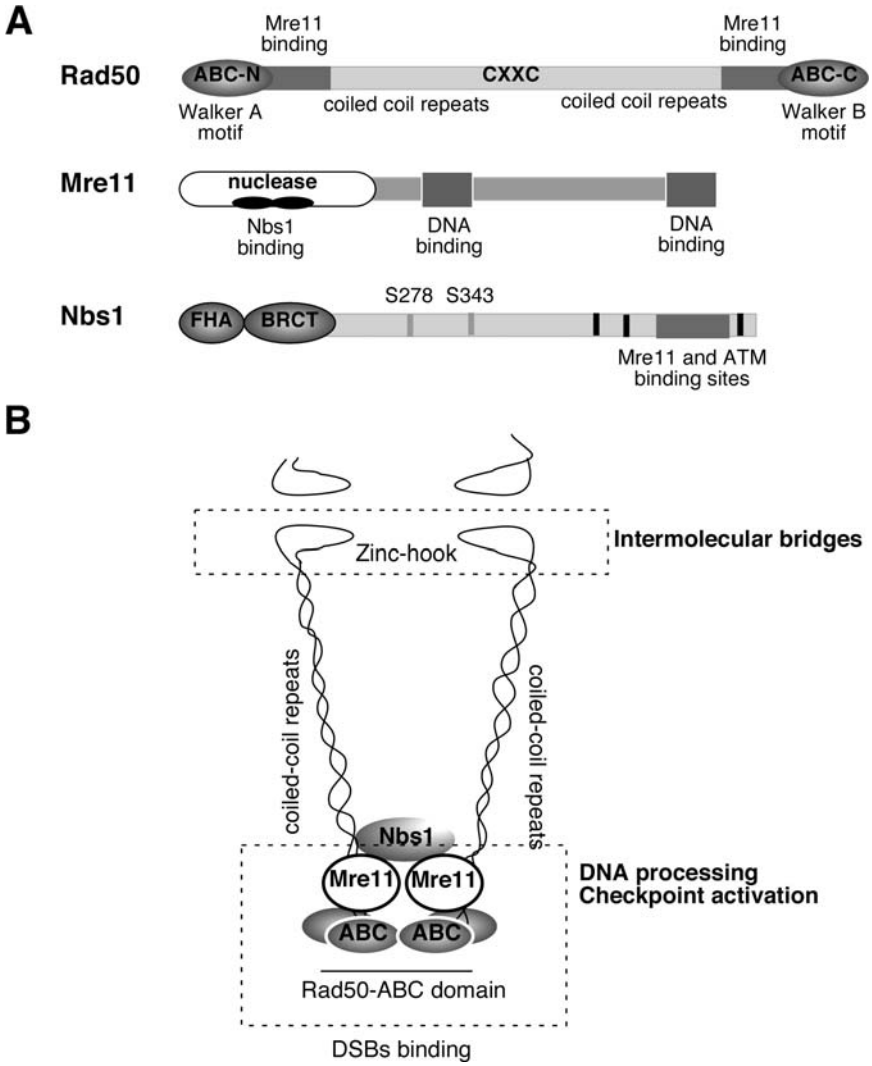

Fig. 6. Structure and spatial organization of the Mre11-Rad50-Nbs1 (MRN) complex. MRN is composed of three different proteins: Mre11, Rad 50, and Nbs1. **(A)** Domain organization of Rad50, Mre11, and Nbs1. Rad50 belongs to the SMC proteins and contains two N- and C- terminus Walker motif (A and B) that binds to ATP through ABC domains. Rad50 has two Mre11-binding domains and two coiled-coil repeats separated by a sequence containing two cystein residues also called the "Hook" domain. Mre11 is a nuclease containing DNA-binding sites. Finally, Nbs1 is composed of two N-terminus phosphopeptide-binding domains: a Forkhead-associated (FHA) and a BRCA1 C-terminus (BRCT) domains. The C-terminal region of Nbs1 contains an Mre11- and an ATM-binding sites that mediate the recruitment of ATM to damaged DNA and ATM activation. The central region of Nbs1 contains several serine/glutamine motifs phosphorylated by ATM and ATR. In particular, the serine residues 278 and 343 are phosphorylated by ATM in response to IR, and these phosphorylation events are essential for the intra-S-phase checkpoint and the telomere maintenance. **(B)** Spatial organization of the MRN complex. The MRN complex binds to DNA ends through Mre11 and the Walker domains of Rad50. Intramolecular coiled-coil interactions within Rad50 bring together its Walker A and B domains generating an active ATPase. In addition, chelation of Zn^{2+} through the CXXC (hook) sequence at the base of the coiled-coil repeats of Rad50 allows intermolecular cohesion between MRN complexes. The stoichiometry of the complex is thought to be 2Mre11/2Rad50/1Nbs1.

association of MRN with the DNA is required to transduce ATM-dependent pathways in response to DNA damage *(59)*.

Taken together, these results support a model, in which MRN complex binds directly to DSBs, promoting or facilitating the processing of DNA ends. ATM is recruited to DNA by MRN and activated. ATM activation involves ATM autophosphorylation and its monomerization. ATM is then released from the DNA lesion and acts to phosphorylate/activate other "soluble" substrates, probably to amplify and propagate the checkpoint response *(49,61)*. It remains to be determined whether other proteins participate in DNA–ATM–MRN interactions *in vivo* and to clarify how ATM activation is triggered by its binding to DSBs and/or by the MRN complex.

2.3.1.3. Other Proteins Involved in ATM Activation. Several studies have implicated "mediator" proteins in ATM-dependent pathways. Among them are MDC1 (for mediator of DNA damage checkpoint protein, also called NFBD1) *(62)*, 53BP1 *(63)*, BRCA1, and protein phosphatases.

MDC1 contains an FHA and a BRCT domain and is found in a high molecular weight complex containing ATM, BRCA1, MRN, FANCD2, Smc1, BARD1, and 53BP1 *(64)*. A number of studies have suggested that MDC1 is important for the MRN complex-dependent activation of ATM at high doses of IR. First, MDC1 is phosphorylated in an ATM- and Nbs1-dependent manner *(64)*. MDC1 forms foci in a manner that is dependent of the phosphorylated form of H2AX, also called γH2AX. Finally, MDC1 is required for the accumulation of active ATM, MRN, and BRCA1 into IR-induced foci *(61,64–66)*. This suggests that MDC1 could act as a signal amplifier of the ATM pathway by controlling the stable recruitment of the MRN complex and other checkpoint/DNA-repair proteins in the vicinity of DSBs. In addition, MDC1 may also have a role upstream of ATM, because ATM autophosphorylation as well as Chk1 and Chk2 phosphorylation is deficient in MDC1-downregulated cells in response to DNA damage *(64)*.

53BP1 is a nuclear protein that interacts with p53 through two C-terminal BRCT domains *(67–71)*. In response to IR, etoposide, and NCS, 53BP1 forms discrete foci and colocalizes with γH2AX and MRN at DSBs sites *(72–77)*. The suppression of 53BP1 expression by siRNA decreases the phosphorylation of various ATM substrates and knockout mice for 53BP1 and ATM have similar phenotypes including growth retardation, immunodeficiencies, and radiation sensitivity *(78)*. Three different mechanisms for the requirement of 53BP1 in ATM activation have been proposed *(78)*. 53BP1 could either facilitate the ability of ATM to phosphorylate its substrates or could be a direct activator of ATM. Because 53BP1 also interacts with ATM in irradiated cells, this could reflect the 53BP1-dependent recruitment of ATM to DSBs *(79)*. In turn, ATM phosphorylates 53BP1 in response to DNA damage but the functional significance of this event is not known *(73–75,80,81)*.

Another interesting candidate to mediate ATM activation is the BRCA1 protein that is also a substrate for ATM, ATR, and Chk2 *(82)*. BRCA1 is a nuclear protein of 1863 amino acids composed of a N-terminal RING domain involved in protein–protein interactions, such as BARD1 protein, and two C-terminal BRCT domains. BRCA1 is required for the phosphorylation of several ATM substrates in response to IR *(83,84)*. Although BRCA1 does not directly activate ATM following immunoprecipitation, BRCA1 has been detected in a high molecular weight complex called

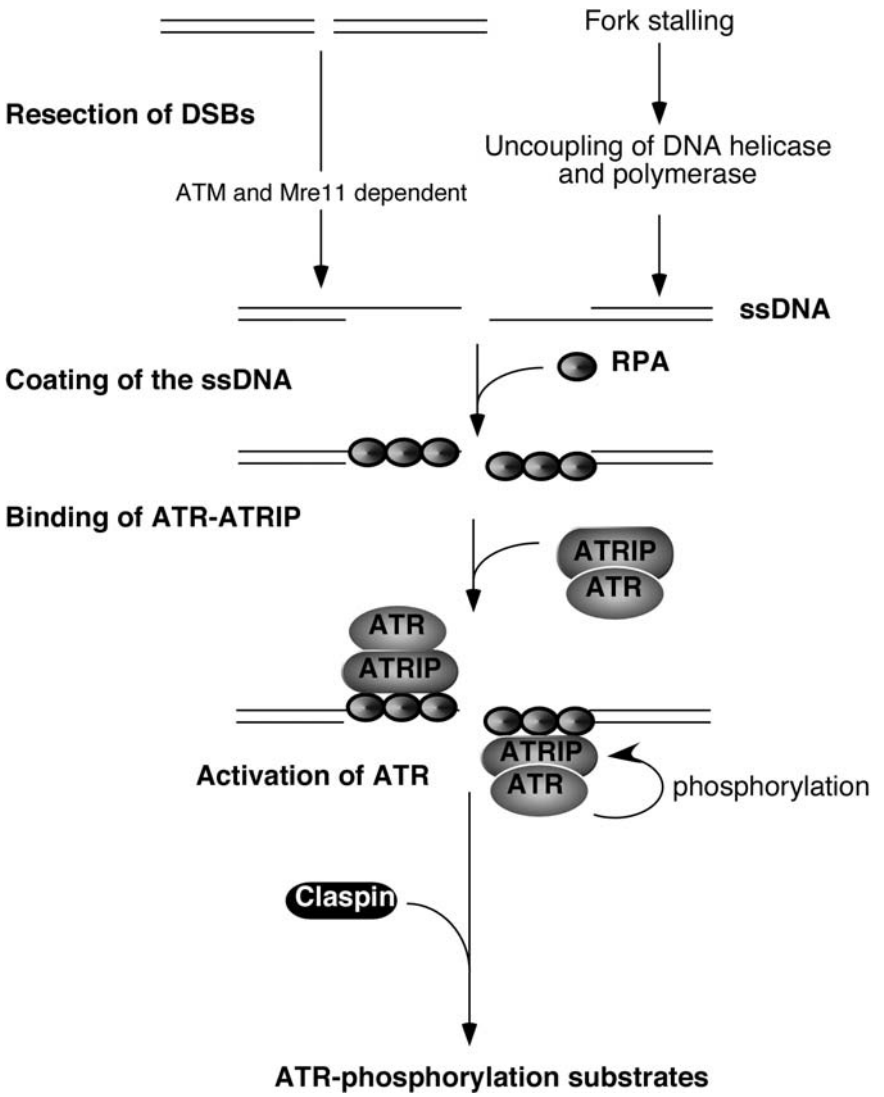

Fig. 7. ATR activation. ATR is directly activated in response to bulky DNA lesions, DNA polymerase inhibitors, stalled replication forks, or hypoxia, which all generate ssDNA as a result of uncoupling between the MCM DNA helicase and the stalled DNA polymerases. In human cells, ATR exists in a stable complex with ATRIP protein, a potential regulatory partner. In the presence of ssDNA, the RPA binds to ssDNA, which induces the recruitment of ATR to DNA damages site through ATRIP–RPA interactions. Following IR treatment, ATR is also activated. This process depends on ATM and the MRN complex that are both required for the processing of the DSB, thus generating ssDNA. ATR is then recruited to ssDNA.

BRCA1-associated genome surveillance complex (BASC), which contains ATM, MRN, and BLM *(85)*. BRCA1 could act as a scaffolding protein, increasing the accessibility of substrates to ATM *(83)*.

Finally, protein phosphatases PP5 and PP2A have been shown to participate in ATM activation *(86,87)*. PP5 interacts with ATM after treatment with NCS. Downregulation

of PP5 by siRNA or by overexpressing a catalytically inactive mutant reduces ATM autophosphorylation and ATM-dependent phosphorylation of p53 in response to NCS resulting in an S-phase checkpoint defect *(86)*. This suggests that PP5 might remove inhibitory phosphorylation on ATM in response to IR. In undamaged cells, ATM interacts constitutively with PP2A and treatment with okadaic acid, an inhibitor of PP2A, induces ATM autophosphorylation. Because ATM and PP2A interactions are suppressed by IR through a mechanism involving phosphorylation, PP2A could modulate ATM activation by catalyzing the dephosphorylation of ATM on serine 1981 or by promoting the dissociation of ATM into active monomers *(87)*.

2.3.2. ATR Activation

2.3.2.1. ssDNA-RPA Intermediates and ATRIP. ATR is a nuclear protein kinase that can directly bind to DNA *in vitro (88)*. In *Xenopus* cell-free extracts, ATR activity is increased following DNA binding *(89)*. ATR is activated primarily in response to bulky DNA lesions, DNA polymerase inhibitors, stalled replication forks, hypoxia, and also in response to DSBs *(90–92)*. Similar to ATM, ATR forms nuclear foci in response to IR *(93)*. Unlike ATM, ATR activation requires processing and generation of ssDNA from DSBs *(37,49,94,95)* (Fig. 7). ssDNA is rapidly coated by RPA, thus creating a platform for the recruitment of ATR to the damage sites *(96–99)*. ATR's recruitment to ssDNA/RPA intermediates requires the C-terminus end of ATRIP a homolog of yeast Rad26/DDC2 *(42,97)* (Fig. 7). ATRIP and ATR interact *in vivo* and colocalize to DNA damage sites or stalled DNA replication forks. Although ATRIP is phosphorylated by ATR, this event is not necessary for ATR's recruitment and the initial ATR-dependent response *(100,101)*. ATRIP or ATR protein levels are co-regulated, and downregulation of either protein causes a defect in the DNA damage S-phase checkpoint, suggesting that ATR–ATRIP interactions are essential for their stability and function *(97)*. Once ATR is recruited to ssDNA-RPA, ATR interacts with and phosphorylates its substrates, such as BRCA1 and H2AX.

2.3.2.2. ATR Activation in Response to IR. During G1, S, and G2 phases, ATR is activated following exposure to IR and recruited to damaged DNA *(102–104)*. This process depends on ATM and the MRN complex, which are both required for the processing of DSBs, thus generating a DNA structure able to recruit ATR through RPA and ATRIPs *(102,104)*.

3. FUNCTIONS OF ATM AND ATR

3.1. Roles as Damage Sensors

A DNA damage sensor must physically interact with DNA before activation of the DNA damage response, and this association should be independent of activating modifications of the sensor protein. Mutations in this sensor should affect both DNA–protein interactions and the cellular responses to DNA damage. ATM fulfills in part these criteria. ATM is one of the first proteins detected at DSBs sites in response to IR *(48)*. Purified ATM binds preferentially DNA ends rather than ssDNA or double-strand DNA, an event that can be correlated with an increase of ATM activity *(34,105,106)*. In *Xenopus* extracts, active ATM is preferentially precipitated with DSB-containing DNA and not with circular DNA *(59,60)*. Furthermore, ATM acts at DSB sites to modify transducers of the DSB-signaling pathways such as Nbs1 and Chk2, thus

supporting the hypothesis that ATM could be a DNA damage sensor *(49)*. Finally, active ATM is found in two fractions following the generation of DSBs, a chromatin-bound fraction, possibly signaling locally, and a soluble nuclear fraction, free to move throughout the nucleus, that could propagate the damage response *(107)*. However, ATM activation, as monitored by autophosphorylation, can take place in the absence of DSBs presumably as a sole consequence of changes in chromatin structure. Therefore, DSB recognition might not be essential for ATM-dependent signaling. In summary, the exact requirement for ATM–DNA interactions and their role in ATM activation needs to be further investigated, especially in light of recent data showing DNA-independent ATM activation *(58)*.

ATR is able to bind chromatin even in the absence of external genotoxic stress and associates with ssDNA cellulose *in vitro (95,96)*. However, in contrast to ATM, ATR is not able to directly interact with damaged DNA by itself and requires RPA and ATRIP to generate a signal from ssDNA *(95,108)* (see also Section 2.3.2.1.). Therefore, there is no strong evidence implicating ATR as a DNA damage sensor.

3.2. Signaling to Cell-Cycle Arrest

3.2.1. G1/S Checkpoint

DNA replication is initiated at origins of replication, defined by the stepwise assembly of a pre-RC containing the origin recognition complex (ORC), Cdc6, Cdt1, and mini-chromosome maintenance proteins (MCMs) (Fig. 8). The activity of the S-phase kinases Cdk2 and Cdc7 is then required sequentially to activate origins, to promote the loading of Cdc45 onto chromatin and for subsequent DNA unwinding by MCMs and elongation *(109,110)*. Cellular exposure to IR or radiomimetic agents before S-phase triggers a G1/S checkpoint that prevents origin firing and subsequent DNA replication until the damage is repaired. Several pathways have been described that lead to the inhibition of two essential protein kinases for DNA replication initiation, Cdc7 and Cdk2. An important characteristic of these checkpoint pathways is that they do not require an active replication fork to function.

3.2.1.1. p53-Dependent Mechanisms. The dominant checkpoint response to DNA damage during the G1 phase is the ATM/ATR-Chk2/Chk2-p53/MDM2-p21 pathway which triggers G1 arrest *(2)*. In the absence of stress, p53 protein is unstable and a poor activator of transcription. Following DNA damage, p53 is stabilized and activates transcriptional targets involved in the DNA damage response. The activation of p53 is mediated by ATM- and ATR-dependent phosphorylations that are reinforced by Chk1/Chk2, leading to the acetylation, sumoylation, stabilization and nuclear translocation of p53 *(2)*. A critical transcriptional target of p53 for cell-cycle arrest is p21Cip1/Waf1, which inhibits Cdk2 kinase activity and thereby causes G1 arrest. This process inhibits not only the initiation of DNA replication but also prevents the inactivation of the E2F pathway required for a sustained G1 arrest *(111)*.

3.2.1.2. p53-Independent Mechanisms. The p53-dependent response to DNA damage is slow, as it requires transcription and protein synthesis following p53 stabilization. However, cells have developed rapid mechanisms to respond to DNA damage that rely only on post-translational protein modifications. Two distinct, p53-independent checkpoint pathways coexist in G1 and are activated by ATM and ATR, respectively (Fig. 8).

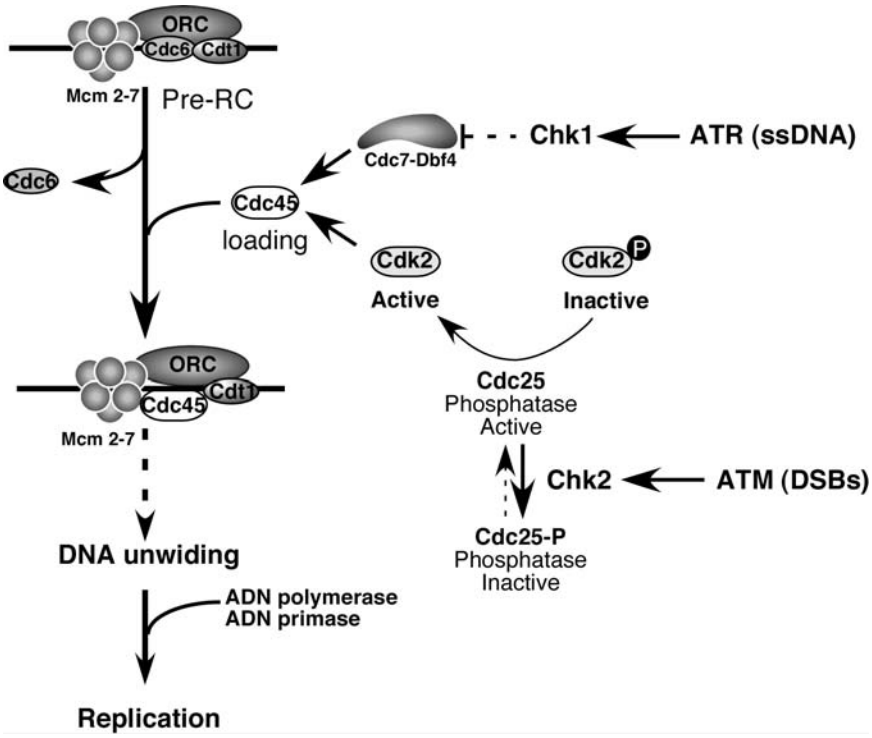

Fig. 8. pre-Replicative Complex (pre-RC) activation. The pre-RC is formed through the stepwise assembly of the origin recognition complex (ORC) and the Cdc6 and Cdt1-dependent loading of MCM helicase. At the onset of S phase, the pre-RC is activated by the action of Cdk2 and Cdc7. Cdk2 and Cdc7 then triggers the loading of the protein Cdc45 onto origins, leading to the activation of MCM proteins and initiation replication. These kinases are the downstream effectors of checkpoint signaling pathways. In the presence of DSBs, ATM is activated and phosphorylates Chk2. In turn, Chk2 is activated and phosphorylates the Cdc25 protein phosphatase leading to its degradation. Cdk2 is then inactivated, which prevents Cdc45 loading onto chromatin and the initiation of DNA replication. In the presence of ssDNA, ATR and Chk1 are activated which inhibits the Cdc7 kinase, probably by dissociating its interaction with Dbf4. Cdc45 loading onto origins is then prevented.

Addition of DBS-containing DNA to *Xenopus* extracts triggers an ATM-dependent checkpoint independently of transcription and protein synthesis *(112)*. This signaling pathway ultimately results in phosphorylation of tyrosine 15 of Cdk2, thus inhibiting its activity. Cdc25A carries out this inhibitory phosphorylation. Inhibition of Cdk2 activity prevents the loading of Cdc45 onto chromatin and blocks subsequent origin activation. A similar pathway was also identified in mammalian cells *(113,114)*.

A second checkpoint pathway that inhibits initiation of DNA replication has been described in *Xenopus* cell-free extracts. This checkpoint is triggered by ssDNA-RPA intermediates generated in G1. These aberrant DNA structures can be generated experimentally by treatment with exonuclease III or by addition of etoposide, an inhibitor of DNA topoisomerase II *(99)*. The signaling is dependent on ATR and RPA. Activation of ATR ultimately results in the inhibition of Cdc7/Dbf4 protein kinase activity. Cdc7 is a conserved serine/threonine kinase, which is required for origin firing with Dbf4, its regulatory subunit *(115)*. The essential targets of Cdc7 have not yet been

unambiguously identified. However, MCM2, a member of the MCM family and a component of the putative replicative helicase, is a potential substrate *(116–119)*. In conjunction with Cdk2, Cdc7 is also required for the loading of Cdc45 onto the chromatin *(115)*. In response to ssDNA/RPA intermediates, Cdc7 activity is downregulated, possibly by dissociation of the Dbf4 subunit, which leads to the inhibition of Cdc45 chromatin loading and a failure to activate the pre-RC *(99)*. This signaling appears to be conserved in mammalian cells as some checkpoint-defective lymphoma cell lines exhibit ATR downregulation *(120,121)*.

3.2.2. S-Phase Checkpoints

In contrast with the G1/S checkpoints, these pathways usually require active replication to be elicited and are under the control of ATR. ATR and its direct target Chk1 are activated following UV, IR, and HU treatments. ATR phosphorylates Chk1 on serine residues 317 and 345 *(122–124)*. In turn, Chk1 phosphorylates Cdc25A on serine 123 that is then targeted for ubiquitin-dependent degradation, leading to the inhibition of Cdk2-Cyclin E complexes *(125)*. This pathway requires an active replication fork and leads to the inhibition of late-firing origins *(126,127)*. In addition, ATR and Chk1 have been involved in the maintenance of genome integrity during DNA replication and stabilize stalled replication forks *(128–130)*.

3.2.2.1. DNA Damage and Replication Fork Progression. Whether signaling in response to DNA damage can directly influence fork progression is still controversial. However, recent studies show that ATM and ATR can directly phosphorylate the MCM protein complex, suggesting that these protein kinases could regulate the activity of the replicative helicase following DNA damage *(116,131,132)*.

3.2.2.2. Other Regulators of the S-Phase Checkpoint.

Claspin/Mrc1: *Xenopus* claspin or the yeast homolog MRC1 (mediator of replication checkpoint) mediates ATR function during DNA replication. Claspin/Mrc1 interacts with DNA structures that are generated by active replication forks and contributes to ATR-dependent activation of Chk1 *(108,133–136)*. Claspin depletion abolishes ATR-dependent Chk1 phosphorylation and abrogates the cell-cycle arrest triggered by DNA replication inhibition, causing a radio-resistant synthesis (RDS) phenotype *(133)*. In human cells, the phosphorylated form of claspin associates with Chk1 in response to replicative stresses or DNA damage. Downregulation of claspin by SiRNA decreases Chk1 activation in response to HU *(137,138)*. The protein kinase responsible for claspin phosphorylation in response to DNA damage has not been identified, but ATM and ATR are good candidates. Claspin has 12 SQ/TQ motifs in its sequence, coimmunoprecipiates with ATR, and its phosphorylation is caffeine sensitive *(137–139)*. Moreover, phosphorylation of serine 864 is abolished following ATR depletion in *Xenopus* egg extracts *(140)*. Similarly, in yeast, Mrc1 is hyperphosphorylated in response to replication blocks, and this phosphorylation is dependent on the ATR homolog Mec1 *(135)*.

MRN Complex: ATLD and NBS cells with hypomorphic mutations in Mre11 and Nbs1, respectively, are defective in S-phase checkpoint signaling, demonstrating the role of the MRN complex in checkpoint response. ATM phosphorylates Nbs1 on several

sites in response to IR, including serine 343. This phosphorylation is required for proper S-phase checkpoint and takes place at DSBs sites, suggesting that Nbs1 is recruited to DNA damage sites *(49,61,141–143)*. Although Nbs1 retention to DSBs is dependent on γH2AX, its recruitment to damage sites involves its FHA and BRCT domain *(144,145)*. This result suggests that Nbs1 could act to recruit MRN at DSBs sites.

A second branch of the intra-S-phase checkpoint, reflecting the impact of the ATM-dependent phosphorylations of Nbs1 and Smc1, is thought to be important for the transient arrest of DNA replication in response to IR. Smc1 is phosphorylated on serine 957 and 966 in an ATM- and Nbs1-dependent manner *(51,146)*. Cells derived from knockin mice in which Smc1 cannot be phosphorylated or surexpression of a non-phosphorylable mutant of Smc1 suppresses the intra-S-phase checkpoint *(51,146,147)*. The phosphorylation of Smc1 is also observed in response to UV or HU treatment, is independent of ATM under these conditions, and thus, is probably under the control of ATR *(148)*. However, a better understanding of the ATM-Nbs1-SMC1 pathway, which also depends on BRCA1 and FANCD2, needs to be developed, specially because the hypersensitivity to radiation/damage in cells lacking Nbs1 or Smc1 seems to be due to the inability of ATM to phosphorylate Smc1 *(62,146,147,149)*.

BRCA1: BRCA1-deficient cells are defective in the S-phase checkpoint and display a RDS phenotype *(150)*. BRCA1 is hyperphosphorylated following exposure to DNA damaging agents. In response to IR, ATM phosphorylates a cluster of serines 1387, 1423, and 1524 that stabilize its interactions with BRCA1 *(150,151)*. ATM-dependent phosphorylations of BRCA1 are essential for the DNA damage response because non-phosphorylable mutants of BRCA1 do not rescue the radiosensitivity of BRCA1-deficient cancer cells *(152)*.

BRCA1 is also regulated by ATR. Both proteins coimmunoprecipitate and colocalize to nuclear foci in S-phase and after cellular exposure to IR or UV *(122)*. ATR phosphorylates BRCA1 at serines 1143, 1280, 1387, 1423, 1457, and threonine 1394 *(122)*. In response to IR, BRCA1 is phosphorylated by ATM, followed by ATR to maintain these events, suggesting a model in which ATM and ATR functions overlap in response to IR *(153)*. BRCA1 could help integrating the DNA damage response by regulating ATR-dependent Chk1 activation *(84)*.

FANC Proteins: Eleven complementation groups have been described for FA based on somatic cell fusion studies: A, B, C, D1, or BRCA2, D2, E, F, G, I, J, and L, at least eight FA genes have been cloned *(154,155)*. FA proteins cooperate in a common DNA damage response pathway. FANCA, E, C, F, G, and L are constitutively assembled in a nuclear core complex that functions as a monoubiquitin ligase in which the catalytic subunit is FANCL *(156–159)*. The core complex is required for FANCD2 monoubiquitination on Lysine 561 and chromatin association during S phase and following DNA damage *(160)*. FA cells, which lack ubiquitinated FANCD2, are deficient in the assembly of IR-inducible BRCA2 foci *(161)*. FA core complex is also recruited to the chromatin during S phase *(162)*.

BRCA1 could also influence FANCD2 monoubiquitination. BRCA1/BARD1 heterodimers display ubiquitin ligase activity *(163,164)*. However, FANCD2 is still ubiquitinated in cells lacking BRCA1 but is not translocated to Rad51 foci *(160,165)*. Moreover, FANCD2 monoubiquitination is reduced but not suppressed in cells

expressing a truncated form of BRCA1, suggesting that BRCA1/BARD1 regulates the localization and/or the stability of modified FANCD2.

FA proteins regulate the intra-S-phase checkpoint. FA patients who are deficient for BRCA2 (FANCD1) or FANCD2 have a more severe clinical phenotype than patients from other FA subtypes *(166,167)*. Cells from these patients exhibit a RDS phenotype *(168)*.

ATM phosphorylates FANCD2 on serine 222 following exposure to IR. This phosphorylation is required for the activation of the intra-S phase checkpoint and depends on Nbs1 protein but not on FANCD2 monoubiquitination *(168)*. FANCD2 can also be phosphorylated in an ATM-independent manner, and recent studies have indicated that ATR is required for the normal cellular response to DNA crosslinking agents and UV light. FA cells are hypersensitive to MMC and Cisplatin, and cells deficient for ATR are also defective for MMC induced-FANCD2 monoubiquitination *(12,96)*. The mechanism by which ATR regulates FANCD2 monoubiquitination is not known, but ATR could phosphorylate components of the core complex or FANCD2 directly.

3.2.3. Regulation of the S/M Checkpoint by ATM and ATR

The G2/M checkpoint prevents cells from entering mitosis when DNA is damaged during G2, when cells enter G2 with damaged DNA or if DNA has not been fully replicated during S-phase. The critical target of this checkpoint is the activity of the Cdk1-Cyclin B kinase. Cell-cycle arrest is initiated through ATM-Chk2 pathway and maintenance of the arrest depends on ATR-Chk1 *(84,169–172)*. A-T cells are defective in IR-induced G2 arrest and enter mitosis despite the presence of damage *(173)*. Following IR, ATM phosphorylates and activates Chk2 that is required for G2 arrest *(174–177)*. Treatment of cells with the ATM inhibitor caffeine prevents the phosphorylation and the subsequent activation of Chk2 *(178)*. Once activated, Chk2 phosphorylates and inhibits Cdc25C activity leading to the accumulation of inactive Cdk1-Cyclin B1 and failure to enter mitosis *(179–181)*. Phosphorylated Cdc25C binds 14-3-3, which sequesters Cdc25C in the cytoplasm and facilitates its ubiquitin-dependent degradation *(2,125,182)*. However, cells lacking Cdc25C have a normal G2 checkpoint, possibly because of redundancy and Cdc25A replacing Cdc25C function. Disruption of Chk1-Cdc25A pathway with the chemical Chk1 inhibitor UCN-01 or by antisense oligonucleotides also abrogates the G2 checkpoint induced by IR *(181,183)*. Other ATM and ATR targets have also been involved in the G2/M checkpoint in addition to Chk proteins. Phosphorylation and activation of Chk2 are defective in NBS cells and correlate with the failure to arrest before mitosis *(53)*. Whereas wild-type Nbs1 protein can rescue the G2 arrest, Nbs1 lacking the C-terminus or a form of Nbs1 unable to be phosphorylated by ATM cannot rescue the checkpoint defect. This implies that phosphorylation of Nbs1 by ATM is important for both Chk2 activation and cell-cycle arrest upon DNA damage *(53)*. Mouse embryonic fibroblasts deficient for BRCA1 are genetically instable and defective in G2 checkpoint *(150)*. Cells lacking BRCA1 fail to activate Chk1. Expression of BRCA1 rescues Chk1 activation by regulating the expression of Wee1 and 14-3-3 proteins *(84)*. This suggests that BRCA1 may regulate the G2 checkpoint through Chk1. The G2 checkpoint is also abrogated by caffeine, and substitution of serine 1423, an ATM target, to alanine in BRCA1 also abrogates

the G2 checkpoint, suggesting that phosphorylation of BRCA1 by ATM may regulate the BRCA1-Chk1 pathway *(84,150,184,185)*.

3.3. Chromatin Remodeling

Histones are subjected to many translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation *(186)*. Chromatin is involved in transcription, replication, DNA repair, and recombination and any event impairing the stability of its structure may compromise genome integrity. Recent data link chromatin to the DNA damage response. Changes in chromatin structure can trigger a DNA damage response, and in turn, histones are targets of the DNA damage response.

As mentioned previously, chromatin structure is important for ATM activation, and DNA intercalating agents such as chloroquine or inhibitors of histone deacetylases are able to induce ATM autophosphorylation independently of DSBs *(37)*.

Chromatin assembly has been linked with the DNA damage response. Histone regulator A (HIRA) is a chromatin assembly factor acting independently of DNA synthesis. Overexpression of HIRA induces an S-phase arrest, a process that is dependent on ATM and ATR *(187)*. The chromatin assembly factor CAF1 is required for chromatin assembly coupled to DNA synthesis during replication and repair *(188)*. Disruption of CAF1 in human cells using dominant negative mutants or siRNA triggers cell-cycle arrest in S phase and activates ATR *(187,189)*. Under these conditions, DSBs accumulate, indicating that loss of histone chaperones leads to checkpoint activation and inhibition of DNA replication.

Post-translational modifications of histones play a role in the DNA damage response. Mutations of the N-terminal lysine residues of histone H3 suppress its acetylation and increase the sensibility to DSB-inducing agents *(190)*. Similarly, mutation of the N-terminal lysine residues in Histone H4 induces the activation of a Rad9-dependent checkpoint *(191)*. Furthermore, H2AX inactivation in mouse leads to IR radiosensitivity and genome instability and increases tumor susceptibility *(145,192,193)*. Serine 139 of H2AX is rapidly phosphorylated in response to DSBs. In mammals, although γH2AX is dispensable for the initial recognition of the break, it forms nuclear foci that serve as a hallmark for DSBs and allows the retention of many factors involved in DSBs signaling *(145,194)*. Phosphorylation of H2AX requires ATM in response to IR and ATR in response to UV or DNA replication block *(144,195–197)*. The exact function of H2AX phosphorylation is unclear, but it could induce chromatin changes and facilitate DNA repair through the recruitment of chromatin remodeling factors as it has already been described in yeast *(198)*.

3.4. Telomere Maintenance

Telomeres are the ends of linear chromosomes and comprise protein complexes that prevent the recognition of the chromosome end as damage. When telomeres shorten or lose these protective factors, chromosome fusions and rearrangements occur leading to cell-cycle arrest, senescence, and/or apoptosis *(199)*. Interestingly, many proteins involved in DNA repair and checkpoint pathways are activated in response to telomere dysfunction.

In addition to the telomere-specific binding proteins TRF1 and TRF2, DNA-PK, ATM, and MRN complex are found at telomeres. Together, these proteins promote the formation of a "T-loop" in the DNA, which prevents checkpoint, repair, and recombination activities *(199)*.

The connection between DNA damage and telomere checkpoint has been essentially revealed by using a dominant negative form of TRF2 ($TRF2^{\Delta B/\Delta M}$), which inactivates endogenous TRF2. TRF2 is a small protein that, once bound to telomeric sequences, recruits all the proteins implicated in the maintenance of telomere function *(199)*. $TRF2^{\Delta B/\Delta M}$ expression induces uncapping of the telomere, generation of fused chromosomes, autophosphorylation of ATM, and its association with telomeres *(200)*. This leads to the activation of an ATM-dependent response, inducing cell-cycle arrest and apoptosis *(201)*. Among the proteins that are phosphorylated in response to ATM activation are H2AX, Smc1, Chk1 and Chk2, and p53, and these proteins accumulate in telomeric foci *(202–206)*. A-T cells have an impaired response to telomere uncapping *(200,207)*. However, an ATM-independent pathway has also been described involving the upregulation of p53 and possibly DNA-PK or ATR in the absence of ATM *(200,207,208)*. Interestingly, recent experiments have suggested that TRF2 could play a role at DSBs in addition to telomeres *(209)*.

However, one important question remains to determine how the activation of ATM is prevented on natural chromosome ends. It was recently shown that overexpression of TRF2 abrogates cell-cycle arrest, decreases the phosphorylation of ATM substrates such as Nbs1, and suppresses the upregulation of p53 in response to IR. This suggests that TRF2 could act to protect telomeres from ATM activation. TRF2 directly interacts with unphosphorylated ATM in proximity of serine 1981 and could block ATM autophosphorylation or prevents ATM to interact with other DNA damage proteins *(210)*.

3.5. DNA Repair

The interface between DNA repair and cell-cycle arrest will not be covered in this review and recent reviews can be found. Many of the proteins regulating the cell-cycle response to DNA damage also participate directly or indirectly in regulating DNA repair. DSBs are repaired by two major pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the prominent pathway used in G1, whereas HR is used in S and G2 phases when homologous templates are available. Although ATM and ATR seem to have minor roles in repair, targets of ATM and ATR kinases are essential for repair. This is the case of BRCA1, BRCA2, MRN complex, and FANC proteins, which coordinate DNA repair and cell-cycle arrest during S and G2 phases. Moreover, CDKs have been recently implicated in the regulation of HR *(211)*.

4. CONCLUSIONS

Much progress has been made during the past few years in our understanding of DNA damage response mechanisms. Since the discovery of ATM, many questions remain to be answered. What is the "exact" signal for ATM activation? Does ATM bind directly to the DSB or is it recruited through MRN or another high molecular weight complex? Are there additional molecular mechanisms required for its full activation? However, detection of DNA damage may not be such a simple process and may require more than just one or two proteins to fulfill this role. Supporting this idea is the finding

of "foci" or repair "centers" at damaged DNA sites, where many proteins involved in DNA repair and maintenance aggregate. It is more likely that interactions of these proteins, combined with some unidentified factors might function as DNA damage sensors.

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12 Interactions Between Myc- and Cyclin-Dependent Kinase Inhibitors in Cancer

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Summary

Deregulated cell growth and the inhibition of apoptosis are hallmarks of cancer. The *MYC* family of oncogenes are pivotal players in tumorigenesis and are altered in most tumor types. c-Myc is the founding member of a family of structurally related basic helix-loop-helix-leucine zipper (bHLH-Zip) proteins that function as sequence-specific transcription factors and are aberrantly expressed in most cancers c-Myc is a key regulator of cell proliferation and differentiation, and its expression is both necessary and sufficient to drive quiescent cells into S phase. Following mitogenic stimulation, c-Myc is rapidly induced; remains elevated throughout the cell cycle and, through dimerization with its bHLH-Zip partner Max, regulates the transcription of genes essential for cell growth and division. Conversely, c-*Myc* expression is rapidly suppressed by growth inhibitory signals such as transforming growth factor ß. However, these controls are lost in cancers by translocations, amplifications, and alterations in regulatory signaling pathways, resulting in abnormally high levels of MYC oncoproteins. The precise roles that Myc oncoproteins provide to provoke tumorigenesis are not fully resolved but do include the regulation of target genes that control cell division, differentiation, cell size, and angiogenenesis. These target genes include members of the cyclin dependent kinase inhibitors (encompassing the Ink4 family and the Cip/Kip family of inhibitors), responsible for inhibiting the activity of cyclin/cyclin-dependent kinase complexes that regulate cell cycle traverse. The regulation of these inhibitors by Myc clearly represents an important target in cancer prevention and therapeutics.

Key Words: Myc, CDK, CDK inhibitors, Cyclins, Ink4, Cip/Kip.

1. INTRODUCTION

The genomic integrity of multicellular organisms is ensured by various components of the DNA repair machinery and/or by the decision of cells to undergo apoptosis or senescence. Collectively, these programs prevent cells that have accumulated DNA

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mutations from developing into cancer cells. Once thought to simply provide a proliferative advantage to the malignant cell, the activation of oncogenes is now recognized to also provide new avenues for cancer therapeutics. Foremost among these potential targets are the Myc family of oncogenes (c-Myc, N-Myc, and L-Myc) that function as master regulatory transcription factors overexpressed in approximately 70% of all human cancers *(1–4)*. The array of functions executed by Myc oncoproteins underscore their importance in tumor cell biology, as their enhanced expression drives unrestricted cell proliferation and increases in cell mass and ribosome biogenesis *(5–10)*, inhibits terminal differentiation programs *(11,12)*, and provokes tumor angiogenesis *(13,14)*. Paradoxically, in normal cells, Myc overexpression triggers the apoptotic program *(15,16)*, which acts as a defense mechanism against transformation, and Myc also promotes genomic instability *(17)*. Collectively, these events safeguard cancer cells from progressing toward overt malignancy. Furthermore, Myc functions appear necessary to maintain the tumorigenic state, as even brief inactivation of Myc causes rapid tumor regression because of the induction of apoptosis *(17,18)*. Thus, Myc is widely viewed as an important target in both cancer chemoprevention and cancer therapeutics.

On the flip side of cell growth control are a series of dedicated inhibitors that function to hold the cell cycle in check by inhibiting the activity of cyclin/cyclin-dependent kinase (Cdk) complexes (cyclin D/Cdk4;Cdk6, cyclin E/Cdk2, cyclin A/Cdk2, and cyclin B/Cdk1) that normally phosphorylate key substrates to allow entry and progression through the cell cycle. Cdk inhibitors fall broadly into those that specifically target cyclin-D/Cdk4;Cdk6 complexes, the Ink4 family of inhibitors ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$), and those that broadly inhibit all Cdks, the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$). Notably, at least two of the Inks, $p18^{Ink4c}$ and $p16^{Ink4a}$, as well as $p27^{Kip1}$ play essential roles as guardians against tumorigenesis. For example, gene targeting has established that mice lacking $p16^{Ink4a}$, $p18^{Ink4c}$, or $p27^{Kip1}$ are tumor-prone, especially when exposed to mutagens *(19–23)*. Furthermore, deletion or silencing of the *INK4A* locus and the suppression of *INK4B, INK4C*, and *KIP2* expression are common features of many human malignancies *(24–28)*. In addition, the suppression or loss of heterozygosity (LOH) of *KIP1* is a well-established predictor of poor prognosis in many human cancers *(29–32)*. Finally, there are now obvious connections between Myc and many of the Cdk inhibitors in tumorigenesis. Herein, we describe these interactions and their relevance for tumorigenesis and cancer therapy.

2. MYC'S TRANSCRIPTION FUNCTIONS

Initial studies by Bishop and Varmus and colleagues demonstrated that v-*Myc* was the oncogene of the avian myelocytomatosis (MC29) retrovirus. Subsequent work then revealed that v-Myc's cellular homolog c-Myc, as well as the other Myc family members N-Myc and L-Myc, is activated by chromosomal translocations or amplifications in many human cancers (including breast and prostate, lymphoma, leukemia, colon and cervical carcinoma, melanoma, glioblastoma, and small-cell lung carcinoma). Furthermore, *Myc* genes are often overexpressed in cancers having mutations in other signaling pathways and in those having mutations in tumor suppressors such as p53, which negatively regulate Myc expression *(33)*.

Myc oncoproteins function as transcription factors that repress or activate the expression of targets necessary for cell-cycle entry and traverse and for cell growth. First, their N-terminus harbor domains conserved among Myc family proteins, Myc boxes I and II (MBI and MBII), that regulate Myc's interactions with transcriptional co-activators and co-repressors. Indeed, MBII plays an essential role in transactivation and transrepression of the numerous Myc target genes *(34–36)*. The N-terminus also contains a domain that controls the rates of Myc protein turnover, which have been linked to changes in Myc transcriptional activity *(37–39)* and appear largely mediated by the F-box protein Fbw7 *(40–42)*. The C-terminal domain of Myc harbors a basic helix-loop-helix leucine zipper (B-HLH-Zip) motif, which mediates specific DNA binding and dimerization with another B-HLH-Zip protein coined Max. Myc–Max heterodimers activate transcription by binding to E-box elements (CACGTG), and activation involves the recruitment of multiple co-activators such as TRRAP and BAF53 *(43,44)* and their associated histone acetyltransferases [GCN5, CREB-binding protein (CBP), and p300] *(45,46)* and ATPase/helicases (such as TIP48 and TIP49) *(47,48)*. Furthermore, the central region of Myc harbors another conserved domain termed MBIII, which contributes to Myc's transrepression and transformation functions *(49)*, and yet another domain juxtaposed to Myc's basic DNA-binding motif has been suggested to be a target for monoubiquitylation and to be important for Myc's transactivation functions *(39)*.

In addition to the Myc–Max interaction, Max can also form homodimers or can heterodimerize with several related B-HLH-Zip proteins termed Mad1, Mxi1 (Mads), Mad3, Mad4, Mnt, and Mga *(3)*. However, Mad–Max and Mnt–Max heterodimers function to repress transcription by recruiting histone deacetylases through their ability to interact with the transcriptional co-repressor Sin3 *(50–52)*. Interestingly, Myc–Max dimers can also repress transcription when bound to promoters regulated by the Miz1 transcription factor *(53–55)*. Repression in this scenario occurs either through disruption of the interaction between Miz1 and p300 or by recruiting the DNA methyltransferase DNMT3a *(56)*. Nonetheless, the ultimate balance between transcriptional activation and repression is largely determined by the absolute levels of Myc versus Mad or Mnt proteins, all of which have relatively short half-lives *(57)*.

Myc-induced tumorigenesis requires its transcription functions. A number of genes induced by Myc have been suggested to be important for its biological effects *(3,39,58–61)*, yet important targets also include those that are directly repressed by Myc *(55,62–64)*, and the classification of a true Myc targets is rather controversial, particularly because several putative Myc targets are not regulated by Myc *in vivo (65,66)*. Indeed, as much of 15% of the genome has been suggested to be regulated by Myc (see http://www.myc-cancer-gene.org), and among these are key targets that play direct roles in tumorigenesis, particularly the Ink and Cip/Kip Cdk inhibitors.

3. MYC AND GROWTH CONTROL

Under normal physiological conditions, the expression of *Myc* genes is tightly regulated by signaling pathways emanating from the engagement of growth factors with their receptors and adhesion complexes. Indeed, resting, quiescent cells express very little Myc RNA or protein, whereas high levels of *Myc* are induced, as an

immediate–early response gene, in cells stimulated by mitogens, and in proliferating cells, Myc protein levels are maintained at a constant level throughout the cell cycle. However, *Myc* transcription is quickly repressed by growth-inhibitory signals [e.g., by transforming growth factor-beta (TGF − β)] or following the withdrawal of mitogens, and Myc RNAs and proteins all have very short half-lives, allowing for rapid reductions in pools of Myc. This response is important, as silencing of Myc expression is necessary for withdrawal from the cell cycle and for terminal differentiation, and especially as unchecked Myc expression provokes additional genetic alterations events that lead to cancer.

The crucial roles of Myc in growth control were underscored by the creation of the c-*Myc* and N-*Myc* knockout mice, which die at mid-gestation with obvious defects in their growth and development *(67)*. Most importantly, the analysis of conditional *Myc* knockouts revealed that acute loss of Myc leads to an immediate arrest in G1 in most (but not all) cell types *(68–70)*. Furthermore, Myc overexpression, or even microinjection, is sufficient to drive quiescent cells into cycle and into S phase *(5,71–73)*. Thus, Myc is both necessary and sufficient for cell proliferation.

So how then does Myc regulate cell-cycle entry and traverse? Obvious targets would be expected to include those that are necessary for cell-cycle progression. Indeed, Myc induces the transcription of some of the D-type cyclins *(63)*, cyclin E, and cyclin A *(74)*, and it also induces the transcription of Cdk4 *(75)*. Cyclin D-Cdk4 and cyclin E-Cdk2 complexes phosphorylate many key targets required for S-phase entry, in particular the retinoblastoma protein (pRb) (reviewed in ref. *4*). Furthermore, at least in some cell contexts, D-type cyclins and Cdk4 appear to play important roles in Myc-induced transformation *(76,77)*.

Proteins that inhibit cell-cycle traverse are also important targets of Myc. For example, it is well established that Myc represses the expression of Cdk inhibitors $p21^{Cip1}$, $p27^{Kip1}$, and $p15^{Ink4b}$ and that their repression contributes to Myc's ability to drive cell-cycle progression. Transcriptional induction of *Cip1* and *Ink4b* requires the functions of the transactivator Miz-1, and Myc's interactions with Miz-1 disables in transactivation functions, by for example recruiting transcriptional co-repressors to the *Cip1* and *Ink4b* promoters *(39,55,64,78)*. Suppression of $p27^{Kip1}$ expression by Myc is more complex, as Myc represses *Kip1* transcription, but also more profoundly provokes $p27^{Kip1}$ degradation *(61,63,79,80)*. Thus, Myc coordinately regulates components of the cell-cycle machinery to drive cell-cycle traverse.

4. INK4 AND CIP/KIP CDK INHIBITORS IN CELL-CYCLE CONTROL

The four Ink4 family members $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$, all specifically bind to Cdk4 and Cdk6 and prevent their interactions with the D-type cyclins *(81)*. This effectively cancels the activity of cyclin D-Cdk4/6 complexes in phosphorylating and inactivating pRb, and as a net result, overexpression of any of the Ink4 inhibitors leads to arrest in the G1 phase of the cell cycle in a pRb-dependent manner *(82)*. All four Ink4 family proteins are similar in their structure and harbor several ankyrin repeats that direct their binding and inhibition of Cdk4/6 (reviewed in ref. *83*). Interestingly, although they all essentially perform the same function, each Ink4 has distinct patterns of expression during mouse development and differentiation, with $p18^{Ink4c}$ and $p19^{Ink4d}$ playing key roles in regulating cell-cycle exit during development and differentiation, whereas $p16^{Ink4a}$ and $p15^{Ink4b}$ are rather expressed in response to stress- or

growth-inhibitory signals such as those provoked by "culture shock" or directed by TGF-β. In particular, $p16^{Ink4a}$ is induced as cells undergo senescence *(84)*. Remarkably, the *INK4a* locus also encodes an alternative reading frame (ARF) product termed $p19^{Arf}$ ($p14^{Arf}$ in humans), whose role is to rather interact with and inhibit the functions of Mdm2, an oncogene that harnesses the activity of the tumor suppressor p53, by ubiquitylating p53 and targeting it for destruction by the proteasome *(85)*. Thus, in the face of sustained expression of Arf, such as occurs in response to hyperproliferative signals provoked by oncogenes, p53 protein levels rapidly accumulate, and it induces the transcription of downstream targets that induce cell-cycle arrest or apoptosis *(86)*.

Given their roles, one would predict rather dire consequences of inactivating *Ink4* genes on cell-cycle control in general and on development in particular. Indeed, although their functional redundancy likely dampens the effects of deleting the Inks4s individually, there are notable phenotypes with regard to growth control associated with the knockout of the *Ink4* genes in mice.

Initial targeting studies of the *Ink4a* locus deleted *Ink4a* exon 2, which contains coding sequences of both Ink4a and Arf, and although these mice develop normally, they are highly prone to tumors (100% penetrance), suggesting that Ink4a was a bona fide tumor suppressor *(19)*. However, the creation of the selective knockout of *Arf* revealed that many of the tumor suppressor properties harbored in this locus were specifically contributed by Arf, as these mice are prone to develop a number of malignancies, and *Arf* null cells are immortal in culture and can be transformed by activated Ras alone *(87)*. Subsequently, the creation of knockin mice that harbor disabling and selective mutations in *Ink4a* demonstrated that these mice do display thymic hyperplasia, that *Ink4a*-deficient T cells are hyper-responsive to mitogens, and that these mice also display an increased incidence of late-onset spontaneous malignancies that are exacerbated by mutagens *(21,22)*. In addition, these studies firmly established the role of $p16^{Ink4a}$ in constraining cellular proliferation, as although $p16^{Ink4a}$ null mouse embryonic fibroblasts (MEFs) possessed normal growth characteristics, they remained susceptible to Ras-induced senescence *(88)*. Furthermore, $p16^{Ink4a}$ null MEFs and lymphocytes exhibit increased rates of immortalization *(89)*. Collectively then, these studies established a tumor suppressor role for $p16^{Ink4a}$, and as predicted given its cyclin-D/Cdk4;Cdk6 target, these effects were specifically attributed to targeting of the pRb pathway *(90)*.

Mice lacking $p15^{Ink4b}$ are also viable but exhibit extramedullary hematopoiesis and lymphoid hyperplasia in the spleen and lymph nodes *(91)*. Similar to loss of $p16^{Ink4a}$, the lymphocytes derived from $p15^{Ink4b}$ null mice also demonstrate increased rates in proliferation in response to mitogens *(91)*. Furthermore, although MEFs lacking $p15^{Ink4b}$ show normal contact inhibition and undergo senescence, they display accelerated rates of proliferation in response to serum and show increased plating efficiencies compared with wild-type MEFs. Finally, loss of $p15^{Ink4b}$ also increases susceptibility to transformation by Ras and Myc *(91)*, properties again consistent with functions of a tumor suppressor.

Targeted deletion of $p18^{Ink4c}$ also revealed no effect on normal mouse development, although there is evidence of enhanced epithelial cell growth and increased rates of B- and T-cell proliferation following mitogenic stimulation *(20,91,92)*. As these mice age, there is, however, a rather profound organomegaly and gigantism *(20)*, and Ink4c was found to be essential for the generation of functional plasma cells *(93)*. Furthermore, the

use of the small interfering RNA (siRNA) to knockdown *p18^{Ink4c}* expression indicated that p18^{Ink4c} functions as a negative regulator of cell growth *(94)*. Finally, formal proof that effects of *Ink4c* loss were indeed due to targeting of cylin-D/Cdk4 complexes came from the work of Xiong et al. who generated mutant mice deficient in both p18^{Ink4C} and *Cdk4* and showed that loss of *Cdk4* reversed many of the phenotypes associated with loss of *p18^{Ink4c} (95)*.

Mice lacking *p19^{Ink4d}* are also viable with few developmental defects. However, they do develop testicular hyperplasia that is associated with increased apoptosis of germ cells and male infertility *(96,97)*. Interestingly, *p19^{Ink4d}* mice also exhibit progressive hearing loss, which is due to the disruption of the maintenance of the postmitotic state of sensory hair cells in the auditory epithelium *(98)*.

Unlike the Ink family that targets CDK4/6, the Cip/Kip family that includes p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} binds to both cyclin-D/Cdk4;Cdk6 and cyclin-E/Cdk2 and cyclin-A/Cdk2 complexes. However, they only functionally block the activity of Cdk2 complexes, whereas their role in modifying cyclin-D-Cdk4;Cdk6 complexes is rather a positive one, as they prevent binding of the Ink4 inhibitors, and they also appear to play an important role in the assembly of functional cyclin-D/Cdk4;Cdk6 complexes *(83,99)*, although this requirement is not absolute and can be overcome by other mitogen-activated events *(100)*.

As a downstream target of the tumor suppressor p53, a key role of p21^{Cip1} following DNA damage is to induce cell-cycle arrest, allowing the cell time to repair its damaged genome and to prevent apoptosis *(101–103)*. Although commonly associated with a growth-inhibitory role, p21^{Cip1} can also function in cell proliferation *(104,105)*. This dichotomy may reflect different roles in distinct subcellular locales, where cytosolic p21^{Cip1} functions in cell-cycle progression and nuclear p21^{Cip1} functions to inhibit Cdk complexes to result in cell-cycle arrest *(105,106)*. p21^{Cip1} also plays important roles in cellular senescence, as targeted deletion of *CIP1* in normal human diploid fibroblasts bypasses senescence *(107,108)*. *p21^{Cip1}*-deficient cells and primary MEFs also fail to undergo cell-cycle arrest when exposed to various DNA damaging agents *(109,110)*, and as a consequence, this promotes sensitivity to apoptosis following treatment with chemotherapeutics *(111–113)*. Many of these studies however used systems that also lacked functional p53, and many aspects of p53 function appear intact following loss of *p21^{Cip1}* as, for example, p21^{Cip1}-deficient thymocytes undergo rapid apoptosis following damage *(110)*. Furthermore, the outcome of *p21^{Cip1}* loss clearly depends on cell context, as keratinocytes derived from *p21^{Cip1}*-deficient mice exhibit increased proliferative potential *(114)*.

p27^{Kip1} mediates growth arrest induced by various signals, including those directed by TGF-β and contact inhibition *(115)*. The targeted deletion of *p27^{Kip1}* in mice revealed many important phenotypes relating to growth control, notably an increase in overall size because of extra cell divisions and organ hypertrophy *(116–118)*. Furthermore, *p27^{Kip1}* loss leads to selective effects on augmenting the proliferative rates of hematopoietic cells and leads to pituitary adenomas *(117)*. Furthermore, *p27$^{Kip1+/-}$* mice are tumor prone following exposure to mutagens or γ-irradiation, without showing loss of the wild-type allele; therefore, p27^{Kip1} functions as a haploinsufficient tumor suppressor *(119)*. Finally, *p27^{Kip1}*-deficient females are sterile, which is likely due to defects in cell-cycle exit during maturation of the ovarian corpora lutea *(120)*.

Of all Cip/Kip family members, inactivation of $p57^{Kip2}$ in the mouse results in the most profound phenotype *(121–123)*. These mice die prematurely soon after birth and exhibit many developmental defects similar to the humans suffering from Beckwith–Wiedemann syndrome, including abnormal proliferation in the limbs, lens, and intestine, cleft palate, organomegaly, and even defects in placental development. Deletion of both $p57^{Kip2}$ and $p27^{Kip1}$ has underscored their importance in the cell cycle, as these doubly deficient mice display defects cell-cycle exit and differentiation of lens fiber cells *(122)*. Furthermore, loss of $p27^{Kip1}$ increases the proliferative rate in the labyrinth zone of $p57^{Kip2}$ null placentas *(122)*. Thus, clearly the Kip inhibitors are key regulators of cell-cycle control.

5. LOSS OF INK4/CDK INHIBITORS IN CANCER

The inactivation of the *Ink4a/Arf* locus is the second most common alteration, behind those that disable p53, in cancer (reviewed in ref. *90*), and this can occur through various means: (i) homozygous deletion, generally involving the entire *INK4a/Arf* locus, but only affecting approximately 10% of human tumors *(24,124,125)*; (ii) intragenic mutations, representing both point and missense mutations, a rather infrequent event (5% of human tumors) that occurs in the second exon common for $p16^{Ink4a}$ and Arf, usually affecting the amino acid sequence of both proteins *(24)*; and (iii) epigenetic silencing by methylation, which is by far the most common mechanism. In this scenario, DNA methylation of cytosine residues present in the CpG residues in the Ink4a promoter leads to gene inactivation *(126)*.

There is widespread evidence for inactivation of *INK4A* particularly in human leukemia and lymphoma. First, a hallmark of T-acute lymphocytic leukemia (T-ALL) is biallelic deletion of these genes *(127–129)*. Second, although deletions of *INK4A* are relatively rare in acute myeloid leukemia (AML) *(130)*, they are frequently deleted in chronic myeloid leukemia (CML) *(131)*, non-Hodgkin's lymphoma *(132)*, primary central nervous system lymphomas *(133,134)*, and relapsed ALL *(135–137)*, and this is associated with poor outcome *(136,137)*. In addition, translocations that inactivate these genes in B-ALL have also been reported *(138)*. Methylation of *INK4A* is also relatively frequent in multiple myeloma, although the relevance of this is currently unclear *(139)*. *INK4A* epigenetic silencing is also seen in non-hematological malignancies, where hypermethylation is a common event in solid tumor types such as colorectal, lung, pancreas, and breast *(140)* and also in bladder and cervical tumors and in melanoma and glioma *(141,142)*. Despite its major role as a guardian against transformation in cancer in mice compared with $p16^{Ink4a}$, the role of human $p14^{ARF}$ is somewhat less clear. Indeed, promoter methylation and inactivating missense mutations are rare, although some have been reported in colon cancer *(143,144)*. Furthermore, most cases of deletion of *ARF* usually occur along with concomitant loss of other Ink4 family members *(90)*.

Much of our insight and understanding of the role of the *INK4a/Arf* locus in cancer comes from the use of mouse models. As noted above, targeted deletions of $p16^{Ink4a}$ and especially *Arf* render mice prone to a wide spectrum of spontaneous tumors, including soft-tissue sarcoma, osteosarcoma, melanoma, and histiocytic lymphoma, and lymphoma development is accelerated by carcinogens such as dimethylbenzoic acid (DMBA) *(19,21,22,87)*. Furthermore, malignant melanomas in $p16^{Ink4a}$/*Arf* doubly

deficient mice that are very similar to those seen in the human malignancy can be induced by activated *Ras (145)*. Furthermore, in mice, Arf clearly behaves as a classical tumor suppressor, where one sees loss of the wild-type allele in $Arf^{+/-}$ mice when spontaneous tumors arise in these mice or when they are crossed to other tumor-prone mice, for example, the Eμ-*Myc* transgenic mice, where the onset of lymphomagenesis is dramatically accelerated *(146)*.

Although *INK4A* is one of the most frequently inactivated genes in human cancer, there is now evidence that methylation of *INK4B* may also be important in cancer. The *INK4B* promoter region is hypermethylated in at least 80% of human AML, in about 40% of ALL cases *(147,148)*, and in 60% of chronic myelomonocytic leukemia (CMML) cases *(149)*. Furthermore, *INK4B* is inactivated by deletion in primary cutaneous B-cell lymphomas *(150)* and up to 30% of ALL *(24,151,152)*. Although inactivation of *INK4B* seems to be a frequent event in various hematological malignancies, it is also seen in other cancer types, including ovarian *(153,154)*, pulmonary squamous cell carcinomas *(155)*, pituitary adenomas *(156)*, neurofibromatosis type 1-related malignancies *(157)*, and bladder cancer *(158)*. Interestingly, *Ink4b* knockout mice are also susceptible to tumor formation; they develop a lympho-proliferative disease, extramedullary hematopoiesis, and a low incidence of angiosarcomas *(91)*.

The loss of $p18^{Ink4c}$ in mice results in organomegaly and widespread hyperplasia and increased incidence of tumorigenesis with advanced age or in the presence of carcinogens *(20,23)*. *Ink4c*-deficient mice display many forms of neoplasia, perhaps resulting from enhanced epithelial cell growth, including testicular tumors, pheochromocytomas, angiosarcomas, lymphomas, and renal cell carcinomas *(91)*. Moreover, leukemogenesis induced by an aberrant *abl*-related gene (ARG) kinase involves the suppression of $p18^{Ink4c}$ expression, and blocking ARG kinase activity with the drug ST1571 (an inhibitor of the Bcr-Abl tyrosine kinase) induces cell-cycle arrest through up-regulation of $p18^{Ink4c}$ *(159)*. Furthermore, treatment of tumor cell lines with the histone deactelylase inhibitor Trichostatin A activates $p18^{Ink4c}$ (as well as $p19^{Ink4d}$) *(160)*, and protein kinase C (PKC), known to be increased in many human tumors, may promote human cancer cell growth through suppression of $p18^{Ink4c}$ *(94)*. Recent work utilizing a mouse model of medulloblastoma has also suggested that levels of $p18^{Inkc}$ contribute to the development of this tumor type *(161)*. Taken together, these data suggest that $p18^{Ink4c}$ functions as a tumor suppressor and that its down-regulation is an important regulator in many human malignancies *(23)*.

Unlike other Ink4 family members, $p19^{Ink4d}$ null mice do not develop spontaneous tumors, and carcinogen treatments do not increase the incidence of tumor development, when compared with their normal littermates *(96)*. Indeed, there is a very little evidence to support a role for $p19^{Ink4d}$ in human cancer. However, elevated expression seen in hematopoietic progenitors and leukemic blast cells may contribute to the premature differentiation block in AML *(162)*, and interestingly, $p19^{Ink4d}$ was found to be the only Ink4 family member whose expression is induced by UV light (at least in neuroblastoma cells). Furthermore, ectopic expression of $p19^{Ink4d}$ suppresses UV-induced apoptosis and enhances the DNA repair response *(163)*, suggesting a novel role for this Ink4 family member in the maintenance of DNA integrity, and perhaps cancer prevention.

Given the central function of $p21^{Cip1}$ in cell-cycle regulation, it is perhaps surprising that it plays only modest roles in cancer *(164)*. High levels of the protein are predicted to contribute to chemoresistance in various tumor types *(165,166)*, and hypermethylation

of the gene contributes to its transcriptional silencing in hematopoietic malignancies *(167)*. Polymorphisms that appear to repress *CIP1* expression have also been described in breast cancer *(168,169)*, oral *(170)*, and esophageal cancers *(171)*. In addition, cytosolic localization of $p21^{Cip1}$ has been associated with poor prognosis in breast cancer *(172)*. $p21^{Cip1}$ knockout mice also do develop tumors, including sarcomas and lymphomas, albeit at an advanced age and with rather low penetrance *(173)*. Furthermore, loss of $p21^{Cip1}$ increases the susceptibility to chemically induced skin carcinoma *(174)* and accelerates tumor onset when treated with carcinogens *(175)*.

$p27^{Kip1}$ is expressed at higher levels in quiescent cells and in those undergoing differentiation. Reduced $p27^{Kip1}$ levels is an observed phenotype in many tumor types, perhaps because of abnormal proteolytic degradation *(115)*, and may contribute to tumor development by increasing proliferative rates. Many cancers exhibit low $p27^{Kip1}$ protein levels including colon, breast, prostate, lung, and ovary to name but a few (reviewed in ref. *176*). However, homozygous loss or silencing of the *KIP1* locus is a rare event in human tumors; therefore, the levels of $p27^{Kip1}$ serve as a prognostic marker for disease progression *(177)*. Furthermore, there are only modest effects with regard to malignancy associated with loss of $p27^{Kip1}$ in the mouse; this is perhaps because of the hyperplasia phenotype associated with targeted deletion of $p27^{Kip1}$ where the mice succumb to pituitary adenomas with age *(178)*. $p27^{Kip1}$ deficiency does however cooperate with both oncogenic stimuli *(179–181)* and most recently with a knockin mutation of Cdk4 (Cdk4 R24C) *(182)* in enhancing tumor formation.

p57Kip2 is located on chromosome 11p15, a region frequently deleted in various human cancers, including lung cancers and Wilms' tumors *(183)*. Interestingly, *KIP2* undergoes imprinting such that the paternally inherited allele is transcriptionally repressed and methylated *(184)*, and inactivation of *KIP2* thus includes selective loss of the maternal allele *(185–187)*. As noted above, $p57^{Kip2}$ knockout mice display phenotypes akin to those seen in patients with Beckwith–Wiedemann syndrome, a hereditary disorder that displays predisposition to cancer, and a subset of these patients harbor missense mutations in KIP2. Loss of function and/or expression of *KIP2* is also involved in the proliferation of malignant sporadic adrenal cortical tumors *(188)*, bladder cancer *(189)*, and pancreatic carcinoma *(190)*.

6. REGULATORY CIRCUITS BETWEEN MYC AND CKIS

Deregulated Myc expression prevents cell-cycle withdrawal in response to various antiproliferative signals by activating and repressing transcription of its target genes. Importantly, some of these targets include members of the Cdk inhibitor family, including $p21^{Cip1}$, $p27^{Kip1}$, and $p15^{Ink4b}$. $p15^{Ink4b}$ was initially discovered to be involved in TGF-β growth arrest *(191)*. Prior to this, it was known that TGF-β could down-regulate Myc *(192)* and that enforced expression of Myc could overcome TGF-β-induced cell-cycle arrest *(193)*. Subsequent works both by the Massague and Eilers groups have now shed some light on the mechanism involved, with a central player being the transcriptional activator Miz1 *(53,54)*. Miz1 was previously identified as a partner of Myc *(194,195)*, and it binds to the initiator elements (Inr) that are present near the CAP sites of several genes that are repressed by Myc (e.g., $p21^{Cip1}$, see below) and activates their transcription. Ectopic expression of Miz1 was shown to inhibit the proliferation of rodent fibroblasts and to induce cell-cycle arrest. However,

co-expression of Myc restores the proliferation of Miz1-expressing cells. Miz1 regulates transcription of the *Ink4b* by binding to the initiator element of the $p15^{Ink4b}$ promoter. The Myc–Max heterodimer can form a complex with Miz1 at the initiator and inhibit its transcriptional activation. Therefore, abrogation of Miz1 function and suppression of $p15^{Ink4b}$ are critical functions of Myc *(53,54)*.

To date, there have been no direct associations of Myc with $p19^{Ink4d}$ and $p18^{Ink4c}$, although it has been suggested that the growth arrest of human myeloid cell lines following retinoic acid treatment induces a down-regulation of Myc paralleled by an up-regulation of $p18^{Ink4c}$ by an as yet unknown mechanism *(196)*. Clearly in most cell types, Myc overexpression does not lead to changes in the expression of these two Cdk inhibitors (L. Nilsson and J. Cleveland, unpublished data).

How Myc represses $p21^{Cip1}$ expression has been somewhat controversial (recently reviewed in ref. *197*). Several lines of evidence suggest that $p21^{Cip1}$ can also be repressed by binding Miz1 *(64,198)*. Other groups, however, have suggested that repression is Miz1-independent and instead relies on the inhibition of Miz1 transcriptional activity *(199)*. To add further complexity to this issue, yet another group has suggested that $p21^{Cip1}$ repression involves the recruitment of the DNA methyltransferase corepressor Dnmt3a, with Myc and Dnmt3a forming a ternary complex with Miz1 to corepress $p21^{Cip156}$. This then forces one to consider several alternative mechanisms by which Myc represses $p21^{Cip1}$ transcription.

Myc represses the transcription of $p27^{Kip162}$, but how this occurs has not been resolved. What is clear, however, is that turnover of $p27^{Kip1}$ levels is profoundly affected by Myc and that this appears to occur through Myc's ability to induce the expression of components of the SCF^{Skp2} complex that targets $p27^{Kip1}$ for degradation by the proteasome *(200,201)*. Specifically, Myc induces the expression of Cullin-1

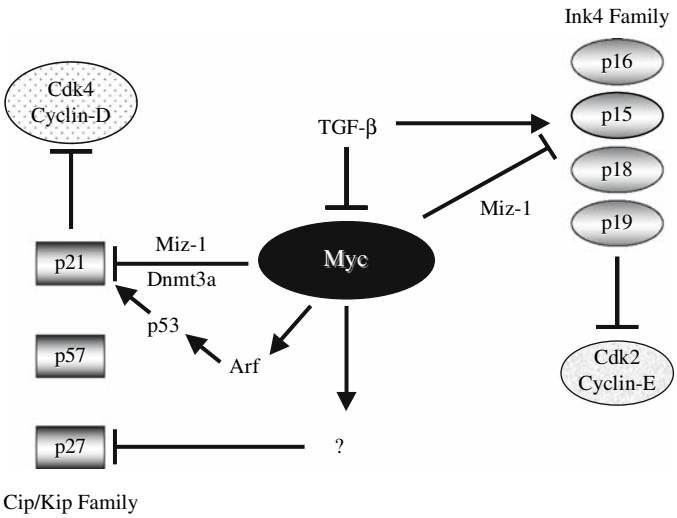

Fig. 1. Regulation of cdk inhibitors by Myc. The Ink4 and Cip/KIP family inhibit CDK4/Cyclin D and CDK2/Cyclin E complexes, respectively. Myc targets $p21^{Cip1}$ repression possibly through binding Miz1 or Dnmt3a. $p21^{Cip1}$ is activated by p53, which is induced by Myc through Arf. The question mark indicates an unknown that Myc targets to repress $p27^{Kip1}$. TGFβ down-regulates Myc, in turn $p15^{Ink4b}$ regulates TGFβ, Myc may also target $p15^{Ink4b}$ repression through Miz1.

and Cks1 *(200)* (Keller UB et al., unpublished data), thus linking effects of Myc overexpression in many malignancies to Cks1 overexpression *(202–207)* and $p27^{Kip1}$ repression *(202,205,208–210)*. Importantly, genetic tests have established that the Myc-to-$p27^{Kip1}$ pathway is a very important regulator of Myc-induced tumorigenesis, as the *Kip1* deficiency markedly accelerates lymphoma development in Eμ-*Myc* transgenic mice *(181)*, whereas lymphoma onset and aggressiveness are impaired in mice lacking *E2f1* or *Cks1*, which are collectively required for Myc-mediated repression of $p27^{Kip1}$ *(80)* (Keller UB et al., unpublished data). By contrast, $p21^{Cip1}$ does not appear to play limiting role in Myc-driven tumorigenesis, as loss of *Cip1* has essentially no effect on lymphoma development in Eμ-*Myc* mice *(181)*.

Myc is clearly linked and directly regulates the expression of at least three Cdk inhibitors (Fig. 1), and its ability to repress the expression of at least $p27^{Kip1}$ is rate limiting for tumor development. Thus, the Myc-to-$p27^{Kip1}$ pathway is certainly an important target in cancer prevention and therapeutics. However, other Cdk inhibitors also need to be considered as therapeutics targets, particularly given the important role of $p16^{Ink4a}$ in inducing the senescence program *(90)*. Perhaps the most effective approach would be one that targets both, which would eliminate escape routes for the cancer cell.

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13 Interplay Between γH2AX and 53BP1 Pathways in DNA Double-Strand Break Repair Response

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Summary

One of the most fascinating themes in the biology of double-strand breaks (DSBs) is that chromatin is emerging as a multifunctional player in the DSB damage response. The phosphorylation of H2AX on Ser 139, named γH2AX, is an early response to the generation of DNA DSBs and extends along megabase-long domains, both sites of the lesion, supporting amplification of signal transduction pathways. In parallel, 53BP1 accumulates on damaged chromatin to interface between methylated histone residues and proteins that belong to the signal-transduction pathways, mediating cell-cycle arrest or apoptosis. Interestingly, the two pathways crosstalk at the chromatin level.

Key Words: H2AX; 53BP1; double-strand breaks; DNA repair; chromatin.

1. GENERAL CHARACTERISTICS OF DOUBLE-STRAND BREAK DAMAGE RESPONSE NETWORKS

Within the living cells, genetic information encoded in DNA sequences is constantly under threat by various genotoxic stresses. Of the various types of DNA lesions that arise, double-strand breaks (DSBs) are the most damaging. In mammalian cells, DSB can result from exogenous agents, such as background radiation and environmental mutagens, or arise from endogenous sources as metabolically produced free radicals. In addition, DSB may emanate as DNA intermediates, normal or aberrant, in several specialized cellular functions, such as DNA replication, V(D)J recombination, meiotic recombination, class switching, and apoptosis.

The consequences of DSBs are of major cost to the cells. If left unrepaired, even one DSB can be lethal. Loss of continuity between the DNA fragment and the centromere

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Table 1
Classification of DNA repair factors according their role in DSB cellular response.

Factor	*Participation in multiprotein complex*	*Function*	*Role in DSB response*
γH2AX	Nucleosome	Chromatin structure	Chromatin mark, transducer
Methyl-H3 Lys 79 and relevant residues	Nucleosome	Chromatin structure	Chromatin mark
53BP1	–	Activator, adaptor–mediator	Sensor, transducer
MDC1	–	Activator, adaptor–mediator	Transducer
Mre11	MRN	Nuclease	Sensor, effector
Rad50	MRN	DNA structural protein	Effector
NBS1	MRN	Activator, adaptor–mediator	Transducer
ATM	Homodimer	Kinase	Sensor (?), proximal kinase
RAD17	Several complexes	Chromatin-binding protein	Sensor
ATRIP	ATR–ATRIP complex	Adaptor–mediator	Transducer
ATR	ATR–ATRIP complex	Kinase	Proximal kinase
DNA-PKcs	DNA-PK	Kinase	Proximal kinase
Ku 80/70	DNA-PK	Sensor	Sensor, transducer
Chk1	–	Kinase	Effector kinase
Chk2	–	Kinase	Effector kinase
BRCA1	BRCA1–BARD1 and several other complexes	E3 ubiquitin ligase, scaffold protein, etc.	Transducer, effector
BRCA2	BASK and several other complexes	Scaffold protein, etc.	Transducer
P53	–	Transcription factor	Effector
Cdc25A	–	Phosphatase (?)	Effector
Cdc25C	–	Phosphatase	Effector
RAD17	Several complexes	Chromatin binding	Sensor
ARTEMIS	–	Nuclease	Effector
XRCC4	XRCC4–LIG4 complex	Adaptor (?)	Adaptor (?)
LIG4	XRCC4–LIG4 complex	Ligase	Effector
DSS1	19S proteasome	Catalytic subunit	Effector
SMC1	Cohesin complex	Structural protein	Effector

during mitosis can result in deletions of a large proportion of the genetic information passed to the progeny cells. If not properly repaired, DSBs can cause alterations in the DNA sequence, chromosomal translocations, genomic instability, and eventually neoplastic transformation. Thus, it is not surprising that the DSB damage response is a highly sophisticated set of reactions that channels cellular activities toward three directions: DNA repair, cell-cycle arrest, and apoptosis.

Two major pathways orchestrate the DSB repair in mammalian cells, one based on homologous recombination (HR), and the other on non-homologous end joining (NHEJ). HR promotes accurate repair by copying information from an intact homologous DNA template, preferentially the sister chromatid when available, and predominates in S/G2 phases *(1)*. NHEJ is independent of homology, or utilizes microhomology to join broken ends, is error-prone, and predominates in G1 *(2–4)*. To provide damaged cells sufficient time to repair, DNA DSB repair systems initiate signal-transduction pathways to activate G1/S, intra-S, and G2/M cell-cycle checkpoints. In the presence of irreparable or excessive damage, checkpoint signaling can also induce apoptotic cell death pathways *(5)*.

To accomplish its multifaceted role, DSB damage response is organized in networks that set in motion cellular subroutines. These networks operate via protein-protein interactions that are communicated by post-translational modifications, mainly phosphorylation and dephosphorylation *(6)*.

Conceptually, the DSB damage response networks need to serve three major necessities: the recognition of the damaged site, the efficient amplification of the damage signal and its transmission to other cell compartments, and the adequate commencing of cellular subroutines that are going to carry out the end results. Reflecting these necessities, the proteins that have evolved to participate in these networks fall into three general classes: sensors, transducers, and effectors. These days, ongoing research reveals that DSB damage response proteins may participate in more than one class, demonstrating that the DSB damage response networks are characterized by elevated sophistication (Table 1).

2. CHROMATIN-BASED EVENTS IN RESPONSE TO DSBs

The lodging of genetic material in the nucleus of eukaryotic cells requires an extreme compaction of DNA that is achieved at different levels of chromatin folding. At the nucleosome level, 147 base pairs of DNA are wrapped 1.7 times around the histone octamer. Further compaction is achieved by the linker histone H1. Nucleosome arrays are further folded into progressively higher-order structures, with the support of non-histone structural proteins. Although the structure of the nucleosome is well characterized, less is known about the molecular nature of more highly folded structures. In this chapter, we focus on two chromatin-based events, γH2AX and 53BP1 focus formation, and their role in the biology of DSBs.

After irradiation, DSB lesions can be visualized as distinct formations called foci, when immunocytochemistry methods are combined with epifluorescent microscopy *(7)*. Currently it is becoming clear that DSB-dependent foci are dynamic chromatin structures juxtaposed to the lesion, where repair, signal transduction, chromatin, and structural proteins are bound onto DNA. Many repair and signaling factors are known to translocate to DSB-dependent foci in a time-dependent manner. These factors are

afterward released from the focus to perform functions in the nucleoplasm or cytoplasm. Experimental evidence have substantiated the classification of these factors to the three categories mentioned above, sensors, transducers, and effectors.

Foci facilitate repair and amplification of the checkpoint signal by orchestrating ordered recruitment, assembly, and activation of further repair and signaling proteins; focus constitution varies considerably. At a given time, different factors can be detected in a focus population within the same cell, as they accommodate different repair complexes depending on the repair system involved, the cell-cycle phase, or the timing of the recruitment and release process. Although the damage response events that occur in a cell are not restricted to the sites of lesion, chromatin-based foci events appear to be crucial in the organization and monitoring of the repair process that takes place at the damaged sites.

2.1. The Biology of γH2AX Foci

2.1.1. γH2AX Foci

The "higher-order chromatin domains model" of γ-phosphorylated chromatin

The first indication that higher-order chromatin structures are involved in the biology of DSBs was in the case of the phosphorylation of the histone variant H2AX *(8)*. Upon the introduction of DSBs into genomic DNA, a characteristic SQ motif that is accommodated in the C-terminus of the histone H2AX becomes phosphorylated in nucleosome arrays that span a distance of up to megabase-long domains *(7)*.

When immunocytochemistry methods are applied to irradiated cells, and cell specimens are observed under the fluorescent microscope, γH2AX forms large, bright, and discrete foci at a random distribution throughout the nucleus but not within the nucleoli area. Focus formation pattern follows fast kinetics; γH2AX foci appear as small and numerous within 1–3 min, become fewer in number but larger and better detected at 15 min, stay steady in size and number between 15 and 60 min, decrease in number at 180 min, and eventually almost disappear at 24–48 h. Various normal and cancer cell lines as well as living organisms respond by the formation of γH2AX to lethal and non-lethal amounts of ionizing radiation (IR) *(7)*.

The demonstration of precise γH2AX localization to the sites of DNA DSBs was achieved by the means of a laser scissors experiment, where DSBs were introduced through a pulsed laser microbeam driven along a predetermined course. Subsequent immunocytochemistry showed that γH2AX forms precisely along this track of DSBs *(7)*.

Remarkably, the phosphorylation of this characteristic SQ motif is conserved over evolution and is part of the DSB damage response. The histone SQ motif is present in different species but resides on different histone variants, all members of the H2A family. In mammals, *Xenopus laevis*, and *Tetrahymena thermophila*, the SQ motif resides on H2AX, in *Drosophila melanogaster* on H2AvD, and in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* on H2A1 and H2A2 *(9)*. In this chapter, for simplicity and clarity purposes, we are going to use indistinguishably the H2AX designation, when we refer to general, conserved features of this cellular response, except where otherwise indicated.

In mammals, the percentage of H2AX with respect to total H2A differs between cell lines, spanning from 2.5 to 30%, whereas in yeast, the ortholog H2A comprises 95%

of the whole complement. Exploiting this divergence, the Bonner laboratory noted a very interesting correlation: although a different amount of H2AX is phosphorylated among different cell lines, the percentage of γH2AX versus H2A per DSB is constant. On the basis of this correlation, the Bonner laboratory proposed a model that predicted that megabase-long domains in chromatin become γ-phosphorylated per DSB *(8)* (Fig. 1A and B).

The expansion of γ-phosphorylated chromatin along megabase-long domains model is based on average values. It predicts a distribution of γ-phosphorylated nucleosomes along an average domain size rather than a "fixed" length on chromatin. In mitotic *Muntiacus muntjak* cells from cultures that were previously exposed to sublethal amounts of IR, γH2AX foci form band-like structures on chromosome arms *(7)*. From a different end, immunoprecipitation experiments in yeast with a γH2AX antibody revealed that γ-modified chromatin extends in the range of several hundred kilobases *(10)*. In budding yeast, it has been showed that a single DSB induces the γ-formation of an approximately 100-kb domain around the lesion *(11)*. It is very likely that these domains reveal higher-order chromatin structures that may be characteristic of the particular species. Yet, little is known about the organization of these chromatin domains.

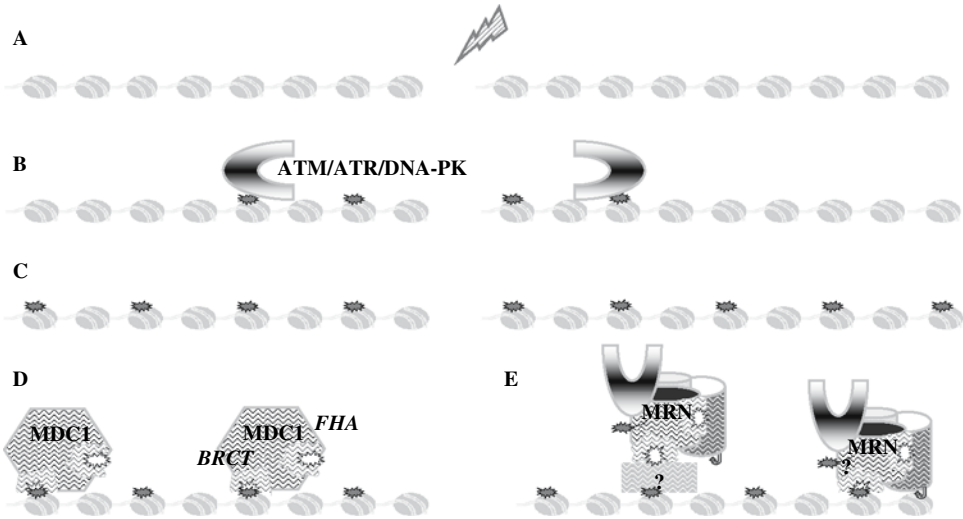

Fig. 1. γ-Phosphorylation alters the affinity properties of megabase-long chromatin domains. When double-stranded DNA damage occurs by irradiation **(A)** or other factors, multiple members of the phosphatidylinositol-3 (PI3) kinases family, namely ataxia telangiectasia mutated (ATM), ATM and Rad3 related (ATR), and DNA-depended protein kinase (DNA-PK), redundantly phosphorylate Ser 139 residue of the histone H2AX (γH2AX) **(B)**. γ-phosphorylation expands along megabase-long chromatin domains, reveals the existence of higher-order chromatin structures that are involved in the biology of double-strand break (DSB) repair, alters the affinity properties of the affected chromatin, and depicts a biological amplification mechanism as DSB sites are surrounded by thousands of γ-modified nucleosomes **(C)**. MDC1 translocates to the lesions sites and binds to γH2AX through its BRCA1 carboxy-terminal (BRCT) domains **(D)**. The MRN complex (Mre11, Rad50, and NBS1) translocates to the lesions sites and binds to damaged DNA through its Mre11 subunit. Forkhead-associated (FHA) domains of NBS1 may facilitate a direct or indirect association of MRN with γH2AX. Subsequently, NBS1 recruits and activates ATM at the damage site **(E)**.

The expansion of γ-phosphorylated chromatin along megabase-long domains depicts a biological amplification mechanism where the DSB site is surrounded by thousands of γ-modified nucleosomes (Fig. 1). A practical implication of this amplification is the notion that even one DSB can be visualized by immunocytochemistry methods. Indeed, during V(D)J recombination, recombination-activating gene (RAG)-mediated cleavage generates DBSs between immunoglobulins and T-cell receptor loci *(12,13,14)*. In developing thymocytes, γH2AX forms nuclear foci that colocalize with the T-cell receptor locus, as determined by immunofluorescence in situ hybridization *(14)*.

This result also demonstrates that immunocytochemistry with γH2AX antibodies is a powerful tool as it can detect the presence of only one DSB per nucleus.

2.1.2. Kinases Responsible for γ-Phosphorylation of H2AX

Phosphorylation of H2AX at Ser 139 is governed by multiple kinases that are members of the phosphatidylinositol-3 (PI3) family, namely ataxia telangiectasia mutated (ATM, ATM and Rad3 related (ATR), and DNA-depended protein kinase (DNA-PK). In *S. cerevisiae*, Tel1 and Mec1, the yeast homologs to ATM and ATR, respectively, are also involved in γ-phosphorylation of H2A, which is the yeast homolog to H2AX *(9,15)*. When yeast strains that bear deletions in *tel1* or/and *mec1* genes are subjected to methyl methane-sulfonate (MMS) treatment that involves generation of putative DSBs, γ-phosphorylation of H2A is impaired *(16)*. A low-level signal is present in the mec1 or tel1 null single mutants, but no signal is detectable in tel1/mec1 double null *(16)*. The above results indicate that both kinases are involved in γH2A phosphorylation and have overlapping roles. In addition, immunoprecipitated Mec1 from yeast cell extracts can phosphorylate a C-terminal yeast H2A peptide *in vitro*, indicating that Ser 129 of the SQ motif of the yeast H2A is a direct target for Mec1 *(10)*.

In human cells, ATM seems to be the major kinase that controls γ-phosphorylation *(17–19)*. ATM knockout cells exhibit impaired γ-H2AX focus formation that can be further eliminated by low-concentration treatment of wortmannin, indicating a redundant role of DNA-PK and/or ATR *(20)*. Low doses of IR activate ATM to γ-phosphorylate H2AX, whereas at higher doses, other kinases contribute or substitute. Fluorescent microscopy of ATM colocalization at the sites of DSBs has been problematic because of the abundance of the former molecules throughout the nucleus. Retention of the ATM molecules at DSBs and colocalization with γH2AX are shown only after in situ extraction of the unbound ATM molecules before immunocytochemistry *(21)*. In accordance with the *in vivo* experiments, immunoprecipitated ATM phosphorylates the SQ motif of H2AX on Ser 139 *in vitro (17)*.

Under hypoxic conditions, H2AX is γ-phosphorylated in an ATR-dependent manner *(22)*. In S phase of the cell cycle, ATR is the kinase to take over γ-phosphorylation, in response to replication arrest and the consequent generation of DSBs, or upon formation of topoisomerase I cleavage complexes after collision of DNA replication forks *(23,24)*. Nevertheless, ATM and ATR exhibit extended redundancy as these kinases are known to have several targets in common, including H2AX *(25)*.

The role of DNA-PK in γ-phosphorylation of H2AX is not yet well understood. Although crude extracts of DNA-PK phosphorylate the SQ motif of H2AX on Ser 139 *in vitro*, several rodent cell lines that are deficient either in the DNA-PK catalytic subunit or in the Ku antigen exhibit no detectable defects in γ-phosphorylation of

H2AX *(8,26)*. However, it has been reported that DNA-PK plays a redundant role in several cases as shown in astrocytoma M059J cell line *(7,27)* and upon formation of topoisomerase I cleavage complexes at replication forks in S phase *(24)*.

By fluorescence microscopy, DNA-PK immunocytochemistry reveals a diffuse pattern throughout the nucleus in both non-irradiated and irradiated cells. However, upon ionizing irradiation, the DNA-PK catalytic subunit becomes autophosphorylated on threonine 2609 and colocalizes with γH2AX in distinct foci *(28)*. Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA DSBs, but the involvement of γH2AX in this mechanism is not clear yet.

H2AX was found to be essentially immobile in chromatin *(29)*. N-terminal fusion constructs of H2AX with green fluorescent protein (GFP) were used to study their diffusional mobility in transient and stable cell transfections. In the absence or presence of DSBs, only a small fraction of GFP-H2AX was redistributed after photobleaching. This fact suggests that a phosphorylation–dephosphorylation cycle takes place during the induction and disappearance of foci rather than that a diffusion-exchange mechanism *(29)*.

2.1.3. The γH2AX DSB-Repair Pathway

Colocalization and interactions of repair and signaling factors with γH2AX foci

Many components of the DNA damage response, including ATM, 53BP1, MDC1, MRN complex, BRCA1, and SMC1, form IR-induced foci (IRIF) that colocalize with γH2AX foci *(9,30–32)*. These factors participate in HR and NHEJ, indicating that γH2AX plays a role in both repair systems in mammals. It has been demonstrated that there is a time-dependent sequential assembly of repair factors and signal mediators on γH2AX foci *(27)*. When cells are treated with wortmannin, a known inhibitor of the PI3 kinase family, γH2AX focus formation is abolished and 53BP1, MNR, and BRCA1 foci are severely impaired. Along the same line, in $H2AX^{-/-}$ cells, initial migration of 53BP1 and MNR to IRIF is not totally abrogated, but further accumulation is diminished *(33)*. This phenotype rises the question as to whether H2AX is a crucial component of double-strand repair and what role it plays in this response.

The Nussenzweig laboratory has proposed the "two-stage recruitment model," according to which γH2AX does not constitute the primary signal that is required for the redistribution of repair complexes to damaged chromatin, but functions as a platform to concentrate repair factors to the vicinity of DNA lesions and to promote interactions between multicomponent complexes *(33)*. The accumulation of repair and signaling factors in proximity to a DSB would facilitate an amplification step of signal transduction and checkpoint pathways, particularly in the case where low numbers of foci per nucleus are present in cells. γH2AX also modulates the accumulation of repair/signaling proteins in chromatin regions distal to a DSB, following their initial, H2AX-independent migration to DSBs. The retention and subsequent increase in the local concentration of factors may be mediated through weak interactions between the SQ motif in the H2AX tail, thousands of which are modified by phosphorylation, and specific domains of repair/signaling proteins.

This model is also consistent with the finding that $H2AX^{-/-}$ cells exhibit reduced ability to arrest the cell cycle at low doses of IR *(33)*. In the case when only a few DSBs are generated in the nucleus, the DNA repair factors that are modified to transduce the signal are limited, and signal amplification at IRIF becomes essential. On the

contrary, the haploinsufficiency of $H2AX^{+/-}$ mice *(13,34)*, which is based on dosage dependence of the *H2AX* gene, could be explained with the hypothesis that chromatin of $H2AX^{+/-}$ cells, which comprises sparsely γH2AX-containing nucleosomes, would not mediate efficient concentration of soluble DNA repair factors on IRIF and further amplification of the signal. Notably, in experimental models where Ser 136 and Ser 139 were substituted with glutamic acid, IRIF fail to form *(34)*. Moreover, these cells exhibit enhanced sensitivity to IR comparable with the wild type, indicating that the actual phosphoserine 139 is essential for biological activity and cannot be substituted.

2.1.3.1. γH2AX Interactions with MDC1. The mediator of DNA damage checkpoint protein 1 (MDC1) is a novel nuclear protein that contains a forkhead-associated (FHA) domain and two BRCA1 carboxy-terminal (BRCT) domains and possesses *in vitro* DNA binding activity *(35)*. MDC1 also presents 20 potential ATM/ATR consensus target phosphorylation motifs (Ser/Thr-Gln), located throughout its N-terminal half, and another 19 consecutive imperfect repeats in its central region *(36,37)*.

In irradiated cultured human cells, MDC1 translocates to the lesion sites rapidly to form foci. MDC1 foci colocalize extensively with γH2AX foci and exhibit similar kinetics *(37–39)*. It has been shown that MDC1 localization to the DSB sites is abolished in $H2AX^{-/-}$ mouse embryonic fibroblasts *(36)*. In support of this observation, MDC1 foci are also abolished in human cells where H2AX expression had been down-regulated by small interfering RNA (siRNA). These experimental results demonstrate that MDC1 focus formation is strictly dependent on γH2AX and place these factors in the same pathway *(40,41)*.

BRCT domains are considered to account for the focus formation of MDC1, as in experiments where these domains were deleted, MDC1 foci were compromised *(42)*. As it has been shown that BRCT domains recognize phosphopeptides *(43)*, it is reasonable to speculate that these domains recognize the γH2AX tail and drive toward the MCD1 focus formation (Fig. 1). In support to this speculation, *in vitro* experiments have shown that a H2AX C-terminus peptide phosphorylated on Ser 139 interacts with MDC1 in human cell extracts, whereas the equivalent non-phosphorylated peptide does not *(44)*.

Down-regulation of MDC1 expression levels by siRNA renders cells hypersensitive to IR *(36)*. At the cellular level, MDC1 down-regulated cells exhibit defects in IR-induced G2/M and intra-S phase checkpoints and show reduced levels of IR-induced apoptosis *(45)*. At the molecular level, the MRN complex and BRCA1 fail to efficiently accumulate in IRIF *(40,46)*. 53BP1 accumulation at sites of DNA damage was found to be MDC1 independent by three different research groups *(36,38,47)* and partially dependent by another *(39)*.

2.1.3.2. γH2AX Interactions with MRN Complex. Colocalization of γH2AX foci with the three-protein MRN complex is evident in IRIF linking H2AX with both DNA-repair and checkpoint cell-cycle responses *(24,27,48)*. Mre11, Rad50, and NBS1 play a crucial role in the biology of DSB repair and operate together in a form of a stable complex *(49)*.

Mre11 has a C-terminal DNA-binding domain and possesses a 3′–5′ exonuclease activity, a single-strand endonuclease activity, and a limited DNA unwinding activity *(50)*. Mre11 is characterized by high affinity for aberrant DNA structures.

Rad50 is an ATPase with a characterized structural role; Rad50 binds DNA through a globular domain, whereas the coiled-coil regions of Rad50 form an extended intramolecular flexible arm. It has been shown by force scanning microscopy that Mre11 and Rad50 heterodimers seriate along broken DNA fragments *(51)*. The intramolecular flexible arm of Rad50 on one DNA fragment "weaves" with the reciprocal Rad50 arm of a distant DNA fragment to hold the two molecules together. It is postulated that Rad50 gives the MRN complex the ability to tether sister chromatids together *in vivo (52)*.

The NBS1 role as a mediator in the DSB damage response is well established *(53)*. NBS1 protein has an N-terminal FHA domain and a BRCT domain *(54)*. In H2AX knockout cells, NBS1 foci (and presumably MRN complex foci) are impaired but not abolished, indicating that γH2AX does not comprise the primary signal for the recruitment of MRN complex at the lesion sites *(33)*. These results indicate that the MRN complex has properties of a sensor protein through the DNA-binding domain of Mre11 *(49)*.

γH2AX-modified chromatin plays a crucial role in the accumulation of NBS1 at the lesion sites *(40)*. In vivo disruption of FHA domains abrogates its interaction with γ-modified chromatin, indicating a direct or indirect association of NBS1 with γH2AX *(54)* (Fig. 1). Moreover, NBS1 interaction with γH2AX is independent of hMre11 or BRCA1 *(55)*. Interestingly, MDC1-depleted cells fail to accumulate the MRN complex in the vicinity of the DSB damaged sites, indicating that this interaction is mediated by MDC1 (see Section 2.1.3.3.).

Several consensus ATM phosphorylation SQ motifs are located within the central region of NBS1 *(56)*. In particular, Ser 278, Ser 343, and Ser 397 are phosphorylated by ATM in response to irradiation. In cells that express mutations at these sites to prevent phosphorylation, no difference was observed in the ability of NBS1 to form foci. Although the role of these phosphorylation events is not clear yet, it has been postulated that they function downstream to mediate the signal to other ATM substrates *(55,57–59)*.

It has been demonstrated that migration of NBS1 molecules occurs independently of their phosphorylation status. A strong indication that NBS1 phosphorylation takes place at the foci, where ATM also migrates in response to DSB, is derived from studies in cells where NBS1 was fused to histone H2B. It was shown that NBS1-H2B molecules were phosphorylated after irradiation only at the lesion sites and not in the residual, undamaged chromatin *(40)*.

NBS1 has an additional function, as activator of ATM. ATM autophosphorylation in response to IR is profoundly impaired in human Mre11ATLD1-expressing cells that contain a C-terminally truncated Mre11 domain *(58,60,61)*.

2.1.3.3. Tripartite Interactions Between γH2AX, MDC1, and MRN Complex.

At present, the interaction between MDC1 and MRN complex is being actively investigated. Down-regulation of MDC1 by siRNA reveals a similar phenotype as in the H2AX knockout and knockdown cells, characterized by impaired, but not abolished NBS1 focus formation *(36,38,62)*. Taking into account that MDC1 focus formation is strictly dependent on γH2AX (see Section 2.1.3.3.), it is reasonable to speculate that the contribution of γH2AX to the accumulation of MRN complex at the lesion sites is mediated by MDC1 (Fig. 2). Consistent with its role as a mediator, FHA domains of

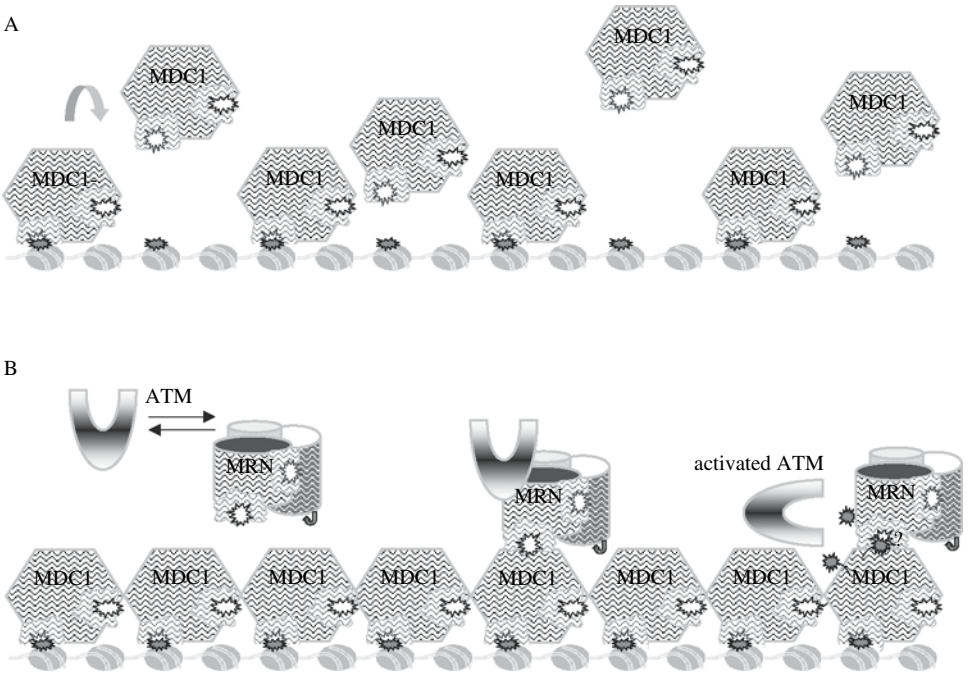

Fig. 2. MDC1 and MRN create a reinforcement loop to enhance ATM activity on γ-modified chromatin. MDC1 relocates to γH2AX foci and binds to phosphorylated Ser 139 through its BRCA1 carboxy-terminal (BRCT) domains **(A)**, where it creates a scaffold to facilitate anchorage of MRN complex **(B)**. In turn, MRN complex enhances ATM activity to facilitate phosphorylation of repair factors, including MRN and MDC1. Multiple phosphorylation of MDC1 may promote multimerization of this protein through its forkhead-associated (FHA) domains and facilitates anchorage of other repair factors that are subsequently recruited to the lesion sites.

MDC1 may be involved in the multimerization of this protein on the DSB-modified chromatin. It is possible that MDC1 multimerization could function to create a scaffold on γ-modified chromatin, where the MRN complex and other checkpoint and DNA repair proteins could anchor and interact with other factors *(37,38,40)* (Fig. 2).

In cells expressing FHA-disrupted NBS1, NBS1 accumulation was abrogated, but microfocus-like structures were formed along the DSB path that was generated by microlaser *(40,47,54)*. These structures are MDC1 independent, demonstrating that there is a small region at the lesion sites where NBS1 associates in a FHA-independent way. Whether these results indicate that NBS1 binds directly to naked DNA or interacts with γ-modified chromatin through alternative means remains to be experimentally approached (Fig. 1).

MDC1 becomes phosphorylated in response to IR in an ATM-dependent manner. This phosphorylation is partially affected in $H2AX^{-/-}$ MEFs, and NBS cells, suggesting that both NBS1 and γH2AX participate in a feedback loop reaction involving ATM activation *(41,55)*. These results are consistent with a model according to which MDC1 relocates to γH2AX foci, where it attracts the MRN complex. In turn, NBS1 activates ATM that is also relocated there, which subsequently phosphorylates MDC1 (Fig. 2). In support to this model, γ-phosphorylation is also affected in MDC1-depleted cells *(35)*. However, the possibility that MCD1 phosphorylation could

occur in parallel at the nucleoplasm by other mechanisms cannot be excluded from these experiments.

In addition to its role as mediator, MDC1 has a function as an activator to ATM as well (see Section 3).

2.2. The Biology of 53BP1 Foci

2.2.1. 53BP1 Foci

53BP1 was originally identified during a yeast two-hybrid screen for p53-interacting proteins *(63)*. The sequence similarity of 53BP1 with the yeast DNA damage checkpoint proteins Rad9 and Crb2/Rhp9 and the localization of 53BP1 to sites of IRIF suggested that 53BP1 would function in the DNA DSB checkpoint pathway. The first experimental evidence showing that 53BP1 is involved in the cellular response to DNA damage was its localization to sites of DSBs in cells exposed to IR *(64)*. Upon DNA damage, 53BP1 relocalizes to discrete nuclear foci that represent sites of DNA lesions and become hyperphosphorylated *(65,66)*. The notion that 53BP1 foci mark sites of DNA DSBs has been documented by colocalization experiments of 53BP1 with γH2AX and other repair factors known to form foci *(62,64–68)*.

In G1, 53BP1 exists in a diffuse nuclear pattern as well as in large nuclear "dots." In S phase, 53BP1 can be found in a discrete, punctuate pattern. The nuclear distribution pattern of 53BP1 in G2 cells appeared in two forms, one similar to S phase but with fewer foci and one that exhibited few, if any, large dots. 53BP1 foci form within minutes of irradiation, and at doses of IR as low as 0.5 Gy. 53BP1 foci colocalize with γH2AX and exhibit kinetics parallel to γH2AX focus formation *(64,69)*. The number of 53BP1 foci increases linearly over time, reaching a maximum at about 15–30 min, and then steadily decreases to baseline levels within the next 16 h. The number of 53BP1 foci, about 20 per cell per Gy of IR, closely parallels the number of DSBs. In addition, the kinetics of resolution of 53BP1 foci is very similar to the kinetics of DNA DSB repair following IR, indicating that there is a relationship between completion of repair and disappearance of 53BP1 foci *(64,70)*.

Several domains have been characterized in 53BP1: a 53BP1 focus formation region (FFR), two tandem tudor folds, two tandem BRCT domains, and an 8-kDa light chain (LC8)-binding domain *(71–76)*. A nuclear localization signal is also present. A 53BP1 FFR has been mapped at residues 1052–1639 *(69,77)*. It was previously shown that γH2AX coimmunoprecipitates with 53BP1 from irradiated cells *(69)*. Interestingly, in a pull-down assay, where six different 53BP1 GST fragments spanning the entire 53BP1 protein were incubated with immobilized C-terminus H2AX peptides, only the fragments 956–1354 showed strong interaction with the γ-phosphorylated one *(72,78)*. In contrast, no binding was detected to the non-phosphorylated peptide bearing identical sequence.

At the C-terminus of the FFR, there are two tandem tudor folds that consist of a 50 amino acid long stretch *(72,76)*. Although not well established, Tudor domains seem to play a role in protein–protein interactions through methylated residues. In support to this notion, tudor folds have structural similarities to chromo domains, that are known to bind to histone tails that contain methylated lysines (see Section 2.2.2.).

The FFR also includes a region required for 53BP1 kinetochore localization in mitotic cells (amino acids 1220–1601) *(76)*. In a different set of experiments

where various GFP-tagged 53BP1 truncations were expressed, the kinetochore-binding domain has been mapped to a 380 residue portion of the protein that excludes the nuclear localization signal and the BRCT motifs *(79)*. It was shown that this region is responsible for loading 53BP1 to kinetochores in prophase and release by mid-anaphase *(79)*. Recently, it was shown that the kinetochore localization region is also essential for 53BP1 focus formation in response to DNA damage, suggesting that both events might be regulated in a similar fashion.

An LC8-binding domain of 53BP1 has been mapped to a short peptide segment immediately next to N-terminal to the kinetochore localization region *(75)*. Unlike other known LC8-binding proteins, 53BP1 contains two distinct LC8-binding motifs that are arranged in tandem. The LC8-binding domain is completely separated from the p53-binding domain in 53BP1. As the LC8 of dynein binds to 53BP1, it has been proposed that 53BP1 can potentially act as an adaptor to assemble p53 to the dynein complex *(75)*.

BRCT domains are a common protein–protein interaction motif that are present in proteins involved in the DNA damage response. 53BP1 has two tandem BRCT domains (amino acids 1714–1850 and 1865–1972, respectively) *(64,66,73)*. A role for 53BP1 in a DNA damage response pathway was first proposed based on the similarity of its BRCT domains to the BRCT domains present in the *S. cerevisiae* Rad9 and *S. pombe* Crb2/Rhp9 proteins *(64,71,72)*. Rad9 in *S. cerevisiae* is required for cell-cycle arrest in response to DNA damage and becomes phosphorylated by Mec1. After phosphorylation, Rad9 interacts with Rad53, which is the homolog of the Chk2 kinase in humans, and this interaction is required for the activation of Rad53.

A comparison of the structure of the BRCT region of 53BP1 with the BRCT tandem repeats reveals that the interdomain interface and linker regions are remarkably well conserved. The crystal structure of the 53BP1 BRCT tandem repeats in complex with the DNA-binding domain of p53 shows that the two tandem BRCT repeats pack extensively through an interface that also involves the inter-repeat linker *(72,74)*. The first BRCT repeat and the linkers together bind p53 on the region that overlaps with the DNA-binding surface of p53 and involves p53 residues that are mutated in cancer and are important for DNA binding.

It has been suggested that the interaction of p53 with 53BP1 reflects the putative adaptor function of 53BP1; 53BP1 may recruit p53 to sites of DSBs, thereby facilitating its ATM-dependent phosphorylation *(72)*. However, this is not the only possible scenario. Recent experimental results suggest that BRCT domains may have phosphopeptide-binding activity, depending on the structure in the vicinity *(43)*. Given the absence of p53 in yeast and the evolutionary conservation of the BRCT domains of 53BP1 in all eukaryotes, the core function of the 53BP1 BRCT domains could relate to some function of 53BP1 that are independent of p53 *(72,78,80,81)*. Because the BRCT domains are not required for localization of 53BP1 to sites of DNA DSBs, it is likely that they play a role in ATM activation, directly or indirectly *(41,71,82)*. The identification of a physiological relevant phospholigand of the 53BP1 BRCT domains will shed light to this question.

Fifteen potential phosphorylation sites (15AQ) have been mapped on 53BP1 N-terminal region, some of which are known to be targeted during the DNA damage response *(66)*. Indeed, 53BP1 becomes hyperphosphorylated on its N-terminus in

an ATM-depended manner in response to IR and mediates DNA damage-signaling pathways in mammalian cells *(41,66,78,81,82)*.

2.2.2. Recruitment of 53BP1 to Sites of DNA DSBs

As mentioned in Sections 2.1.3. and 2.1.3.1., in cells that lack histone H2AX, or where the phosphorylation of H2AX is abrogated, 53BP1 is initially recruited to the damaged chromatin, but cannot be retained *(33)*. A number of laboratories have addressed the question regarding the nature of the factor that recruits 53BP1 to DSBs sites *(47,72)*. The initial recruitment of 53BP1 to the site of a DSB does not seem to require ATM, NBS1, or DNA-PK, since in cells deficient in these proteins, 53BP1 localizes to sites of DSBs with normal kinetics *(33)*. Recently, a role of histone methylation in the DSB damage response has been discovered. The Halazonetis laboratory showed that in human cells, 53BP1 binds to methylated H3 *(72)*. *In vitro* experiments using residues that form the walls of the pocket between the tudor folds showed that the 53BP1 tudor domain binds histone H3 methylated on Lys 79 *(72)*. By deletion analysis, these residues were also shown to be required for recruitment of 53BP1 to DSBs *in vivo*. Competition experiments with H3 peptides showed that the H3 peptide with dimethylated Lys 79 competed with the native histone H3 *(72)*. The corresponding non-methylated peptide did not compete, whereas the peptides with mono-methylated and dimethylated Lys 27, or Arg 26 competed at lower efficiency *(72)*. The enzyme that methylates Lys 79 in human cells is DOT1, an evolutionary conserved methyltransferase. Suppression of DOT1 by siRNA resulted in suppression of H3 methylation on Lys 79 and minimized the 53BP1 recruitment to the lesion sites.

One possible explanation for the reduced affinity of 53BP1 to the damaged chromatin when abolished either the relevant methylated sites or the γ-phosphorylation could be that the tudor domain and the phosphopeptide-binding region act in a cooperative way. As mentioned earlier in Section 2.2.1., the tudor domain and the phosphopeptide-binding region reside in proximity within the FFR of 53BP1, supporting further this hypothesis (Fig. 3).

Surprisingly, the levels of H3 Lys 79 methylation were unaltered upon DSB damage *(72)*. To resolve this discrepancy, the following model was proposed by the Halazonetis laboratory. The introduction of DSBs into chromatin results in disruption of nucleosome stacking, which leads to revelation of H3 Lys 79 and possibly other methylated residues of the histones in the core particle, resulting in exposure of binding sites for 53BP1 and its recruitment to the DSB sites. According to this proposed mechanism, 53BP1 can "sense" DSB lesions through changes in higher-order chromatin structure and participates as a sensor in the DSB damage response *(72)*.

This model, based on the results from human cells, has been supported by parallel work in *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, Rad9, a DNA damage checkpoint protein that has a sequence similarity to human 53BP1, interacted with native H3 *(83)*. Deletion of DOT1 in yeast resulted in radiation sensitivity and a DSB checkpoint defect. In the fission yeast *S. pombe*, it was demonstrated that Set9, a previously uncharacterized SET domain protein, is responsible for H4 Lys 20 methylation *(84)*. Interestingly, this methylation does not have any apparent role in the regulation of gene expression or heterochromatin function. However, loss of Set9 activity or mutation of H4 Lys 20 markedly impairs cell survival after genotoxic challenge and compromises the ability of cells to maintain checkpoint-mediated cell-cycle arrest. Furthermore,

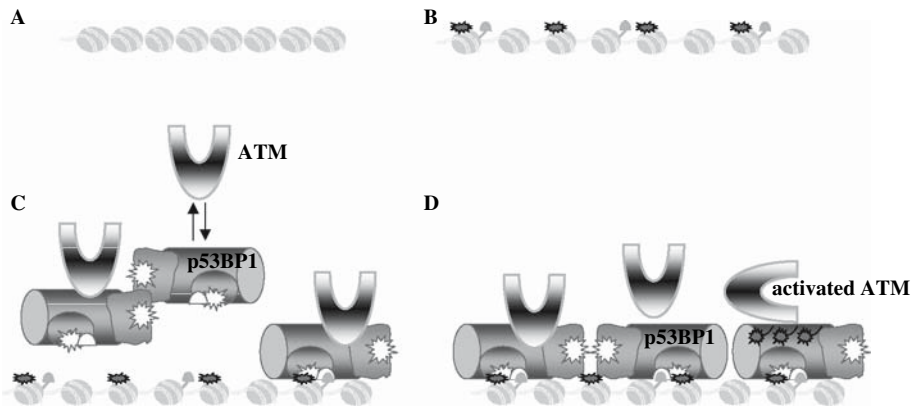

Fig. 3. Recruitment of 53BP1 to damaged sites by chromatin γ-phosphorylation and methylation. Collectively with γ-phosphorylation, the introduction of double-strand breaks (DSBs) into chromatin results in disruption of nucleosome stacking **(A and B)**, which leads to revelation of methylated H3 lysine 79 and possibly other methylated residues of the histones in the core particle **(B)**. 53BP1 relocates to the damaged chromatin where it binds through its focus formation region (FFR). FFR includes a phosphopeptide-binding site and a tudor domain. In the absence of γH2AX or the relevant methylated residues, 53BP1 is recruited to the DSBs sites but not retained, indicating that FFR facilitates cooperative binding **(C)**. 53BP1 creates a scaffold on damaged chromatin to recruit and activate ATM. Activated ATM phosphorylates repair factors including p53BP1 **(D)**.

genetic experiments have demonstrated that Set9 is required for Crb2 localization to sites of DNA damage *(84)*. Although H4 Lys 20 is a different methylation site from H3 Lys 79, it seems that these sites play the same role with respect to the 53BP1/Crb2 recruitment mechanism. This could be explained if the affinity of these factors is not restricted to one methylated site, or multiple histone "marks" may contribute to 53BP1/Crb2 recruitment.

It needs to be mentioned also that BLM helicase has been reported to recruit 53BP1 to DSB lesions independently of its helicase activity *(67)*. Interestingly, a deficiency of the BLM helicase has been reported that markedly increases cancer incidence in humans *(85)*.

3. INTERPLAY BETWEEN γH2AX AND 53BP1

To recapitulate, the introduction of DSBs into DNA results in long stretches of chromatin that are marked by two histone modifications: histone methylation (mainly the H3 Lys 79 in mammals and H3 Lys 20 in *S. pombe*) and the γ-phosphorylation of H2AX. In turn, this DSB-modified chromatin initiates two branches of the DSB damage response: one by the 53BP1 recruitment by methylated H3 Lys 79 (or relevant residues) and the other by the recruitment of MDC1/MRN complex by γH2AX *(31,33)*.

The γH2AX and the 53BP1 branches do, however, clearly interact with each other. The lack of retention of 53BP1 in $H2AX^{-/-}$ cells may indicate a direct mechanism where the FFR of 53BP1 facilitates cooperative binding of Ser 139 phospho groups and H3 Lys 79 methyl groups. Nevertheless, γH2AX seems to play a role in accumulation of 53BP1 by indirect mechanisms as well.

Notably, phosphorylation of 53BP1 is reduced by 40% in $H2AX^{-/-}$ cells, 1 h after low-dose irradiation *(86)*. Although it is not clear whether 53BP1 is phosphorylated only when bound to damaged DNA, these results suggest that γH2AX creates a reinforcement loop to 53BP1 phosphorylation. Notably, in AT cells, 53BP1 relocalizes at DSBs, indicating that ATM-dependent phosphorylation of 53BP1 is not required for 53BP1 focus formation *(69)*. What role this phosphorylation plays is however not clear yet.

Several lines of evidence have shown that 53BP1 is required for a subset of ATM-dependent phosphorylation events *(71)*. An antibody that specifically recognizes the phosho-Ser/Thr-Gln epitope generated by ATM/ATR phosphorylation showed reduced immunofluorescence reactivity in cells treated with 53BP1 siRNA *(41,82)*. However, the magnitude of the defects observed in this system was smaller than that observed in AT cells, indicating the existence of additional 53BP1 network branches that lead to ATM-dependent phosphorylation. In addition, specific antibodies against SMC1 phospho-Ser 966 and phospho-Ser 957 and Chk2 phospho-Ser 33 and phospho-Ser 35 showed suppressed phosphorylation in 53BP1 knockdown cells *(82)*. The above mentioned phosphorylation events have been taken as an indication that 53BP1 functions downstream of ATM activation, as a mediator to several ATM substrates. In support of this conclusion, it has been shown that 53BP1 is required for efficient accumulation of p53 to DSB sites and subsequent p53 activation *(81)*.

According to this line of evidence, 53BP1 functions by creating a scaffold on the revealed methylated H3 Lys 79 chromatin that is further stabilized by interactions with γH2AX to recruit ATM substrates. Recruitment of ATM to the lesion sites would facilitate the phosphorylation of these substrates. This model is consistent with our current understanding of Rad9 function in budding yeast; Rad9 is phosphorylated by Mec1 and binds to Rad53 to recruit it to the lesion sites *(72)*.

The same experimental results could, however, be interpreted in a reverse way. 53BP1 may function upstream of ATM to activate it, in response to DSBs. In fact, a factor that recruits and possibly activates ATM to the DSBs could be 53BP1, as 53BP1 physically interacts with ATM in irradiated, but not in non-irradiated cells, as shown by coprecipitation experiments *(71)*. Consistent with this hypothesis is the observation that 53BP1 forms foci at $ATM^{-/-}$, indicating that it functions upstream of ATM activation *(71)*. According to this model, 53BP1 accumulation to the lesion sites would facilitate the recruitment of ATM, where the ATM substrates would be recruited by γH2AX.

The Halazonetis laboratory has proposed a solution that combines features of two previous opposing models. According to this group, 53BP1 acts both as an activator of ATM and as an adaptor and/or a mediator. 53BP1 functions creating a feedback loop upstream of ATM by activating this kinase, and also downstream of ATM by facilitating the ability of ATM to phosphorylate its substrates, such as Chk1 and Chk2 *(72)*. A dual function of 53BP1 as an activator and adaptor is certainly possible, given that ATM phosphorylates 53BP1 in response to DNA damage at Ser 25 and most likely at other sites as well. Whether these phosphorylation events are critical for phosphorylation of ATM substrates still remains to be demonstrated *(87)*.

To develop experimental support for the "activator" function of 53BP1, the Halazonetis laboratory addressed the question whether 53BP1 contributes to the activation of ATM. It has been demonstrated by the Kastan laboratory that ATM can be activated at a distance from chromatin, in response to the DSB *(88)*. This research

group has shown that ATM is located at the nucleoplasm and exists in a dimer/oligomer form in an inactive form. Upon chromatin aberrations, ATM responds by autophosphorylation on Ser 1981. This phosphorylation disrupts the oligomer form and renders ATM active by exposing its catalytic site *(88)*. Remarkably, ATM is very sensitive in "sensing" chromatin irregularities, even when they are not derived from DSBs, indicating a wide-ranging role of this kinase in response to chromatin abberations. Whether ATM directly senses these DSB-induced chromatin alterations, or requires DNA "sensors" to transmit the damage signal is not clear.

To test the hypothesis that 53BP1 plays a role in ATM activation, the Halazonetis group addressed the question whether 53BP1 contributes to the phosphorylation of ATM at Ser 1981, by suppressing 53BP1 *(72)*. In contrast to what they were expected, their first observation indicated that there is no effect on ATM Ser 1981 phosphorylation in 53BP1 knockdown cells.

Analyzing further into their experimental results, they took into consideration that ATM is activated also by NBSI, as was shown by different research groups *(41,56,57,59)*. The MCD1/MRN complex is recruited to the lesion sites, and this response is mediated by γH2AX, independently to 53BP1 *(59)*. This could provide an explanation to the discrepancy between their woring hypothsis and their experimental results, as the MCD1/MRN contribution could overpower the effect on ATM Ser 1981 phosphorylation.

Exploring the possibility that there are conditions where the two pathways do not overlap, the recruitment of both factors to IRIF was monitored at high and low doses of irradiation *(41)*. The results show that exposure of cells to low dose of IR exhibited recruitment of 53BP1 but not that of MCD1/MRN complex. This piece of evidence emphasizes the notion that 53BP1 plays an essential role at low doses of irradiation *(41)*.

Further pursuing the same question, these investigators investigated the possibility that there is crosstalk between the 53BP1 and the MDC1/MRN pathway. At low-dose irradiation, suppression of MDC1 had no effect on 53BP1 recruitment, but surprisingly, suppression of 53BP1 compels recruitment of MCD1/MRN complex to IRIF *(41)*. Similar results were observed with fibroblasts derived from an individual with XPC (xeroderma pigmentosum group C), in which suppression of 53BP1 resulted in increased localization of MDC1 and NBS1 *(41)*. The increased localization of NBS1 was accompanied by increased phosphorylation of Ser 343, suggesting that in the absence of 53BP1, a greater pool of NBS1 molecules is recruited *(41)*.

Finally, and in accordance with their previous experiments, the Halazonetis group showed that suppression of 53BP1 in NBS1-deficient cells revealed a defect in ATM phosphorylation at low doses of irradiation *(41)*. In addition, downstream targets of ATM, specifically Chk2 Thr 68 and SMC Ser 957, were also found to exhibit impaired phosphorylation. This experimental evidence supports a model according to which 53BP1 has a dual role as an activator and as an adaptor and/or a mediator in the DSB damage response (Fig. 3).

A different line of experiments from the Lukas laboratory indicates another relationship between 53BP1 and MDC1 at later time points post-irradiation. When the departure of 53BP1 from γH2AX foci was monitored by real-time microscopy, it was evident that this departure is accelerated in MDC1-depleted cells *(47)*. In these experiments, GFP-tagged 53BP1 was observed to leave the γH2AX foci after 7–12 h post-irradiation. Surprisingly, in MDC1 knockdown cells, the departure time was reduced to less than 6 h post-irradiation while γH2AX was still present at the sites

of the lesion *(47)*. It has been suggested by these investigators that phosphorylation of MDC1 at foci renders it able to interact directly with 53BP1, or alternatively, with remodeling factors that stabilize chromatin conformation changes that facilitate DSB repair *(47)*.

4. OPEN QUESTIONS AND PERSPECTIVES

DSB-dependent foci are dynamic chromatin structures that facilitate repair and amplification of checkpoint signals by orchestrating a time-dependent ordered recruitment, assembly, and activation of sensors, transducers, and effectors during the DSB damage response. Focus constitution varies considerably at a given time, as different factors can be detected on a focus population within a particular cell, as they accommodate different repair complexes depending on the repair system involved, the cell-cycle phase, or the severity of the damage. Although the damage response events that occur in a cell are not restricted to the sites of lesion, but take place at the nucleoplasm and cytoplasm as well, focus events appear to be crucial in monitoring the repair process that take place at the damaged sites.

During this dynamic series of events, a very reliable point of reference that marks the existence of DSBs from the very early events until the restoration of the damage is γH2AX. It is widely accepted that γH2AX plays a central role in the repair of DSB. Three main pieces of evidence substantiate this conclusion: (i) the early appearance of γH2AX foci after induction of DSB, prior to all other known proteins that form foci; (ii) the delayed disappearance of γH2AX foci that is concomitant with restoration of the lesion (iii) the abrogation of accumulation of several proteins at the lesion sites in H2AX knockout and knockdown systems, whereas ablation of other proteins that form foci does not have a similar impact. As mentioned in Section 2.1.3, 53BP1 and MRN form minute foci in H2AX knockout cells, where further accumulation of these molecules is abrogated.This evidence poses a theoretical question: how sensors such as 53BP1 and MRN are unable to recognize their primary signal in chromatin structures all along the damaged chromatin domains in H2AX knockout cells?

It is reasonable to speculate that γH2AX may recruit other factors that facilitate chromatin changes along the broken fiber that in turn would expose methylated sites for 53BP1 to accumulate or perhaps other structures relevant to MRN recognition. Candidates for this role have recently emerged. Chromatin-remodeling subunits, known to be otherwise involved in transcription, have been shown to be recruited by the γ-phosphorylated chromatin during the DSB repair process. It remains to be experimentally tested whether they indeed play a role is in this context or not.

The overwhelming evidence for the critical function of γH2AX in the DSB response, do not necessarily require acceptance of a "hierarchical" model that opepates in signal transduction pathways that are initiated by chromatin lesions. The fact that 53BP1 recognizes a different type of histone modification, that of methylated H3 Lys 79, and initiates a different branch in the DSB damage response, strongly suggests that the chromatin-initiated network of interactions is "relational" rather than "hierarchical". The existence of an interplay between methylation and 53BP1 from one end and γH2AX and MDC1/MRN from the other supports this notion. It is expected that in the near future, additional factors and interactions will be identified, providing an improved understanding of the role that γH2AX, 53BP1, MDC1 and MRN may play in this highly sophisticated network of DSB damage response.

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14 DNA-Dependent Protein Kinase in Repair, Apoptosis, Telomere Maintenance, and Chemotherapy

Lawrence F. Povirk, PhD

Summary

DNA-dependent protein kinase (DNA-PK) is a high-molecular-weight kinase that binds to and is activated by free double-stranded DNA ends. The primary function of DNA-PK is to promote DNA end synapsis and regulate DNA end processing in the nonhomologous end-joining pathway of double-strand break repair. This regulation is mediated in part by DNA-PK-catalyzed phosphorylation of both itself and the Artemis nuclease, which trims heavily damaged ends that cannot otherwise be joined. In mice, DNA-PK is also required for radiation-stimulated apoptosis, mediated by direct phosphorylation of p53. At telomeres, DNA-PK cooperates with the telomere structural protein TRF2 in a capping function that is essential for preventing chromosome end fusions. The molecular phosphorylation targets for this and other DNA-PK-mediated signaling pathways remain to be identified. In principle, DNA-PK inhibitors should enhance radiotherapy and some forms of cancer chemotherapy, but such inhibitors may have significant toxicities due to the telomeric and other functions of DNA-PK.

Key Words: End joining; radiation; double-strand breaks; DNA repair; Ku; Artemis; repair foci.

1. BIOCHEMICAL PROPERTIES OF DNA-DEPENDENT PROTEIN KINASE

DNA-dependent protein kinase (DNA-PK) was discovered 20 years ago by Anderson and colleagues as a high-molecular-weight serine/threonine kinase activated by DNA ends *(1)*. The DNA end-binding heterodimer Ku, consisting of 70- and 86-kDa subunits, was discovered a few years earlier as an autoantigen in scleroderma polymyositis overlap syndrome *(2)*. Although the 465-kDa polypeptide containing the DNA-PK active site, designated DNA-PKcs, binds to and is activated by DNA ends, its affinity for DNA is low, and under physiological conditions, DNA-PK activity is largely

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dependent on recruitment of DNA-PKcs to DNA ends by Ku *(3–5)*. X-ray crystallography indicates that, when Ku binds to DNA, the DNA helix passes through an orifice in the Ku heterodimer for which the fit is extremely tight, much like a nut on a bolt *(6)*. Ku prefers to bind to the extreme end of DNA, but DNA-PKcs, upon recruitment by Ku, replaces Ku at the tip of the DNA end, forcing Ku to translate inwardly along the DNA by approximately 10 bp *(7)*. The complete DNA-PK holoenzyme sequesters about 28 bp at a DNA end, completely protecting it from DNase digestion *(7,8)*. Although DNA-PKcs also has a central orifice large enough to accommodate double-helical DNA *(9)*, a recent structural study by electron microscopy suggested that, in the ternary DNA/Ku/DNA-PKcs complex, DNA is not inserted into this orifice *(10)*.

DNA-PK is a member of a family of high-molecular-weight protein kinases that are involved in the earliest stages of DNA damage recognition and are more closely homologous to phosphatidylinositol 3-kinase (PIK) than to other protein kinases *(11)*. Other members include the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) kinases as well as their yeast equivalents Tel1 and Mec1, respectively.

The mechanism by which double-stranded DNA ends activate DNA-PK kinase activity is not entirely understood; however, kinetic data suggest that synapsis of two DNA ends is required for activation *(12)*. Based on studies with substrates bearing partially noncomplementary terminal nucleotides, it has been proposed that DNA-PK may denature several base pairs at a DNA end and that the threading of these single-strand termini into cavities in the DNA-PKcs protein may trigger kinase activation *(13,14)*. However, this mechanism cannot be the only means of DNA-PK activation, as under appropriate conditions, DNA-PK can also be activated by double-strand DNA terminated with hairpin loops as well as by single-strand DNA with extensive secondary structure (e.g., single-strand circles from M13 virions, which contain multiple hairpin loops) *(15–17)*. Protein phosphorylation is favored when DNA-PK and the target protein are able to bind to the same DNA molecule, and thus proteins that bind single-strand DNA are favored targets when DNA-PK is activated by M13 DNA *(16)*. Phosphorylation of some target proteins *in vitro* can only be detected under conditions that minimize DNA-PKcs autophosphorylation and inactivation, e.g., very low ATP concentrations.

In vitro, a broad range of DNA-binding proteins are phosphorylated by DNA-PK, preferentially at *S*Q and *T*Q dipeptides *(18–20)*. However, only for a very few proteins has DNA-PK-mediated phosphorylation been demonstrated to be functionally relevant. There is substantial substrate overlap between DNA-PK and the related ATM kinase, and this overlap has complicated efforts to define the specific role of each *(19,20)*. Although DNA-PK is directly activated by DNA ends and is much more abundant than ATM kinase *(21)*, ATM kinase plays a much more prominent role in triggering the overall cellular response to double-strand breaks (DSBs) than does DNA-PK *(22,23)*. In particular, cell-cycle arrest appears to be completely dependent on ATM kinase, with little or no involvement of DNA-PK *(24–28)*. Nevertheless, at least in some cells, DNA-PK plays an important role in DSB-induced apoptosis. In addition, DNA-PK plays a critical, though as yet not clearly defined role in telomere maintenance, distinct from its role in repair.

2. DIRECT ROLE IN DNA DSB REPAIR

DNA-PK is essential for efficient DSB repair by nonhomologous DNA end joining (NHEJ). DNA-PK-deficient mouse and human cells fail to repair a significant fraction of DSBs, even after several days *(29–31)*, and such cells are twofold to fourfold more sensitive to ionizing radiation than normal cells *(31,32)*, with even greater hypersensitivity for cells synchronized in G0/G1 phase *(33)*. There appear to be at least five roles for DNA-PK in this DSB repair pathway (Fig. 1). First, DNA-PK binding initially

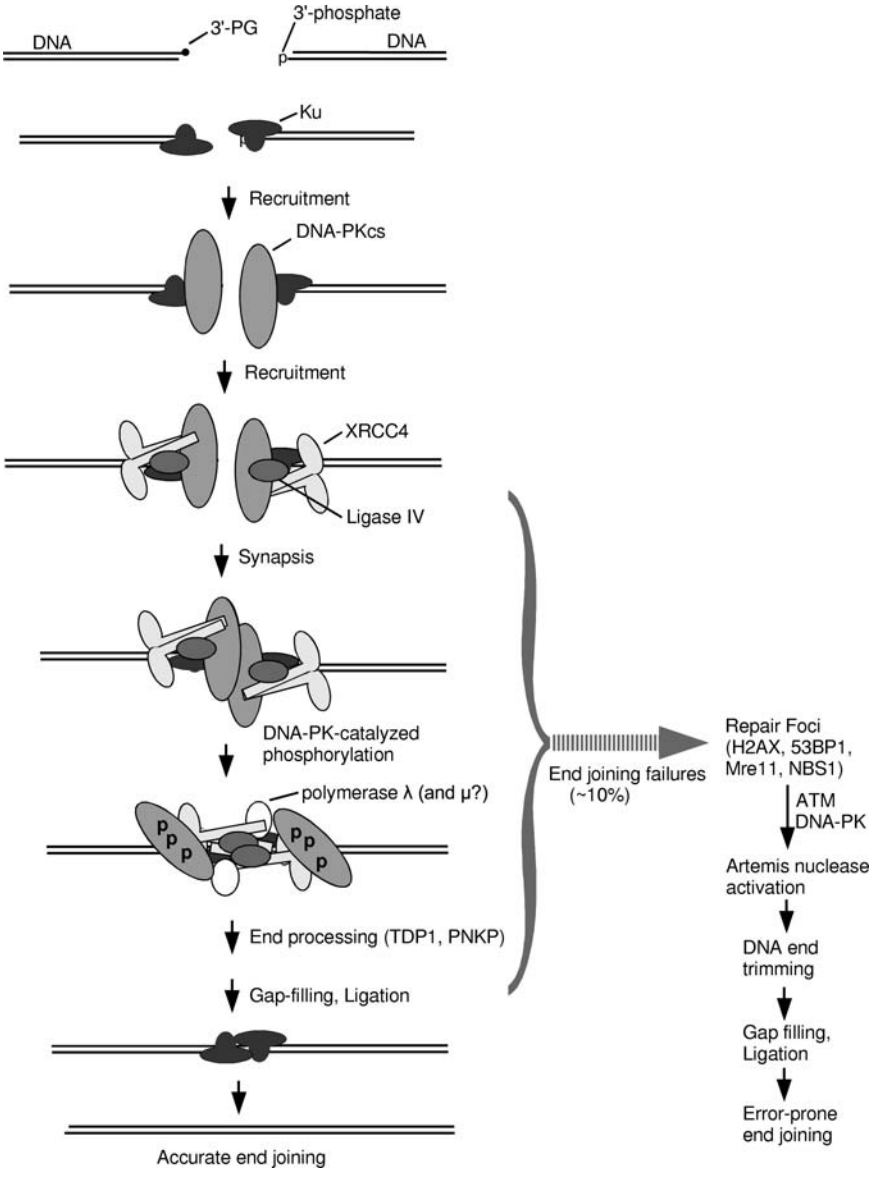

Fig. 1. The nonhomologous end-joining pathway of DNA double-strand break repair. DNA-dependent protein kinase (DNA-PK) protects the DNA ends, recruits XRCC4/DNA ligase IV complex, promotes synapsis, and phosphorylates both itself and the Artemis nuclease.

protects free DNA ends from exonucleolytic degradation *(8,34)*. Second, it promotes synapsis of two DSB ends *(12)*. Third, it aids in recruiting the XRCC4/DNA ligase IV complex *(35,36)*, which is required for fill-in of missing bases in the aligned ends by DNA polymerases λ and μ *(37–39)* as well as for the final ligation. Fourth, it activates the Artemis nuclease by phosphorylating it at multiple sites in the C-terminal domain *(40)*. Activated Artemis trims long 3′ and 5′ overhangs, and this trimming is apparently essential for repair of a subset of DSBs (~10% in the case of ionizing radiation) that are rejoined relatively slowly *(38,41,42)*. Fifth, once two DNA ends are aligned, DNA-PKcs phosphorylates itself (probably in *trans*) at a cluster of *S*Q/*T*Q sites (T2609, S2612, T2620, S2624, T2638, T2647, and S2056) near the middle of the polypeptide *(43–45)*. This autophosphorylation promotes a rearrangement that makes DNA ends more accessible, allowing DNA end processing, gap-filling, and ligation to proceed *(8,38,46–48)*. Thus, DNA-PK may serve to delay end processing until two broken DNA ends have been optimally aligned, thus reducing the possibility of deletions resulting from nucleolytic degradation *(47)*. In addition, DNA-PK and ATM kinase appear to be redundantly capable of phosphorylating histone H2AX in chromatin in the vicinity of each DSB, an event that occurs within minutes of irradiation *(49–51)*. Finally, *in vitro* studies suggest that DNA-PK-mediated phosphorylation of histone H1 may be required for rejoining of DSBs in chromatin *(52)*.

Chinese hamster ovary cells harboring DNA-PKcs alleles with alanine substitutions at the T2609, T2638, or S2056 position are slightly radiosensitive, but a mutant with alanine substitutions at multiple sites in the central *S*Q/*T*Q cluster is more radiosensitive than any of the single-site mutants, suggesting that autophosphorylation of multiple sites is important for repair *(43,44)*. A mutant with six of these sites mutated is more radiosensitive than the parent cell line that lacks DNA-PK entirely *(44)*, suggesting that nonphosphorylatable DNA-PKcs interferes with residual repair that would otherwise occur even in its absence. Foci of T2609-phosphorylated and S2056-phosphorylated DNA-PK can be detected following exposure to ionizing radiation *(43,45)*, suggesting that although mechanistically a single DNA-PK complex per end would appear to be sufficient for end joining, aggregates of multiple DNA-PK complexes accumulate and become phosphorylated in the vicinity of at least some DSBs. S2056-phosphorylated DNA-PKcs can be detected in extracts containing a mixture of kinase-dead and S2056A-mutated alleles, confirming that DNA-PK, like ATM kinase *(53)*, is autophosphorylated in *trans*, at least *in vitro*. Protein phosphatase 5 interacts with and dephosphorylates DNA-PKcs. Either elimination or overexpression of protein phosphatase 5 results in radiosensitivity, suggesting that optimal repair requires a balance between phosphorylation and dephosphorylation of DNA-PKcs *(54)*.

The same basic end-joining pathway involving DNA-PK, Artemis, XRCC4, and DNA ligase IV is utilized in V(D)J recombination, wherein antibody and T-cell receptor genes are created by cleavage and joining of individual V, D, and J segments *(55,56)*. In this process, Artemis nuclease is absolutely required for opening the hairpin loops formed during initial cleavage by RAG1/RAG2 *(41)*. It is presumably because of this requirement that a genetic defect in DNA-PK confers murine severe combined immune deficiency (SCID) *(57)*, whereas lack of Artemis results in human SCID *(58)*.

Diverse cytological and biochemical data suggest that whenever a DSB is formed, there is an initial window of opportunity (~1 h) during which the break can be repaired by simple end joining involving Ku, DNA-PK, XRCC4, and DNA ligase IV *(30,59)*.

This rapid component of end joining probably incorporates conservative end processing by TDP1 and PNKP as well as gap-filling by DNA polymerases λ and μ, although this has yet to be directly demonstrated. Any break that cannot be rejoined by this mechanism, either because of a refractory end structure or because it lacks an end-joining partner, invokes a slower and more complex process characterized by the accumulation of multiple additional factors in "repair foci" *(59–62)*. In G1/G0 cells, these persistent breaks are repaired almost exclusively by an ATM kinase-dependent NHEJ subpathway, incorporating nonconservative end processing by Artemis but still requiring the core NHEJ components as well as many proteins typically found in repair foci, i.e., 53BP1, Mre11, NBS1, and H2AX *(42)*. In late S and G2, many if not most breaks in replicated DNA that escape the rapid phase of NHEJ may instead be channeled into repair by homologous recombination with the sister chromatid *(63)*, but the detailed mechanisms by which these unrepaired DSBs are assigned to slow NHEJ or to homologous recombination remain to be elucidated.

3. DNA-PK AND APOPTOSIS

SCID mice, which harbor a C-terminal truncation of DNA-PKcs, have very low levels of DNA-PKcs protein and at least 100-fold lower levels of DNA-PK activity than normal mice *(57,64,65)*. Attempts to assess the effect of this DNA-PK deficiency on apoptosis yielded diverse results, with reports of a slight reduction in radiation-induced apoptosis in mouse spleen *(66)*, enhanced spontaneous apoptosis and normal radiation-induced apoptosis in intestinal crypt cells *(67)*, and enhanced radiation-induced apoptosis in thymocytes *(68)* and cultured fibroblasts *(69)*. Moreover, concomitant elimination of p53 appeared to enhance the tumorigenic effect of the SCID mutation, and this effect was attributed to abrogation of apoptosis in $p53^{-/-}$ but not in $p53^{+/+}$ SCID cells *(70)*. Thus, these results suggest that there is little, if any, impairment of the apoptotic response as a result of the SCID-associated DNA-PKcs mutation; however, because of the residual truncated DNA-PKcs protein in SCID cells, the results do not rigorously exclude a role for DNA-PK in apoptosis.

In irradiated mice with a complete homozygous knockout of DNA-PKcs, apoptosis in thymocytes was reduced approximately fivefold compared with that in normal mice *(28)*, and in embryonic fibroblasts from the same knockout mice, radiation-induced apoptosis was completely abolished *(71)*. This loss of apoptosis was accompanied by a marked decrease in induction of both p53 and the proapoptotic effector Bax. There appeared to be a distinct bifurcation of p53 function, wherein Bax induction and apoptosis were dependent on DNA-PK but not ATM kinase, whereas $p21^{waf1}$ induction and cell-cycle arrest were dependent on ATM kinase but not DNA-PK, while all these effects were dependent on p53 *(28)*. Studies in murine cell extracts suggest that this DNA-PK-dependent apoptosis reflects ATM kinase-independent phosphorylation of p53, first by DNA-PK at serine 18 (murine S18, equivalent to S15 in human p53), and then by Chk2 at additional, as yet undefined sites *(72)*. Upon phosphorylation, p53 acquires sequence-specific DNA-binding capacity, but it was not determined whether this phosphorylation is sufficient for transcriptional activation. Nevertheless, the hypothesis that DNA-PK and Chk2 cooperate to produce a proapoptotic, phosphorylated form of p53 is also supported by the finding that apoptosis is similarly attenuated in thymocytes and embryonic fibroblasts derived from Chk2 knockout mice *(72,73)*.

There is conflicting evidence regarding whether DNA-PK plays a similar role in apoptosis in human cells. No instances of human SCID associated with DNA-PK mutations have been reported, suggesting that inactivating mutations of DNA-PK are likely embryonic lethal in humans. Thus, studies of human DNA-PK function have centered on comparisons between the human glioma cell lines M059J and M059K. Although these cells were derived from the same tumor, M059K cells have normal DNA-PK function, whereas M059J cells harbor a frameshift mutation in DNA-PKcs and contain no detectable DNA-PK activity or DNA-PKcs protein *(74)*. However, interpretation of data obtained from these cells is complicated by the fact that M059J cells also show abnormally low levels of ATM kinase expression *(75)*.

In extracts of M059J and M059K cells, activation of constitutively expressed p53 for sequence-specific DNA binding shows dependence on Chk2 and DNA-PK, similar to that seen in mouse cells *(72)*. However, despite lack of DNA-PK, M059J cells showed greater apoptosis following ionizing radiation than did M059K cells *(76)*. Similarly, microinjection of an antibody that binds DNA-PK and blocks DNA-PK-mediated DNA end joining increased radiation-induced cell killing and apoptosis *(77)*. Moreover, in HCT116 colon carcinoma cells, homozygous disruption of Chk2 by homologous recombination did not block either fluorouracil-induced p53-dependent apoptosis or activation of p53 for DNA binding. Although this result could be specific for this particular cell type and/or damaging agent, the result nevertheless calls into question whether the proapoptotic activation of p53 by a combination of DNA-PK and Chk2, as described in mouse cells, is important for DNA damage-induced apoptosis in human cells. On the contrary, apoptosis induced by the protein kinase C inhibitor staurosporine was greater in M059K than in M059J cells *(78)*, suggesting that it was at least partially DNA-PK-dependent. In human myeloid leukemia cells treated with the nucleoside analogue gemcitabine, which causes interruption of DNA chain elongation, Ku, DNA-PKcs, and p53 were found in a putative sensor complex bound to gemcitabine-containing DNA, and formation of this complex was accompanied by p53 phosphorylation on S15 *(79)*. These results provide circumstantial evidence of a role for DNA-PK in gemcitabine-induced apoptosis, although a requirement for DNA-PK was not explicitly demonstrated. In human macrophages, DNA-PK is required for activation of Akt (by phosphorylation on T308 and S473) in response to bacterial DNA, suggesting that in some instances DNA-PK can mediate antiapoptotic prosurvival signaling *(80,81)*. DNA-PK-mediated prosurvival signaling through NF-κB in response to DNA damage has also been reported *(82)*. Overall, the evidence suggests that although DNA-PK is involved in proapoptotic signaling in some situations in human cells, its role in apoptosis may be less critical than in mouse cells. It appears that the proapoptotic effect of persistent DSBs (due to lack of repair) in DNA-PK-deficient human cells usually outweighs any deficiency in DNA-PK-mediated apoptotic signaling, at least for agents that directly induce DSBs.

It has been noted that both DNA-PKcs and Ku are substrates for caspase 3 and are efficiently cleaved at an early stage of apoptosis *(83,84)*. Although the functional significance of this phenomenon remains to be demonstrated, such cleavage could in principle contribute to cell disintegration by preventing the rejoining of the DSBs induced by apoptotic endonucleases.

4. DNA-PK, TELOMERES, AND SENESCENCE

At each tip of each eukaryotic chromosome, telomeric DNA is incorporated into a looped structure that prevents DSB repair mechanisms from joining chromosomes end-to-end *(85,86)*. In principle, this structure should preclude binding by Ku in its canonical binding mode, i.e., threading onto a double-stranded DNA end. Nevertheless, both Ku and DNA-PKcs are bound at telomeres (although not necessarily in association with each other) *(87–89)*, and both are required for proper telomere function *(90–92)*. Telomeric Ku is not bound directly to telomeric DNA but rather to TRF1, a primary structural component of the telomere *(92)*. DNA-PKcs may be bound to the telomere through KIP, which was originally identified as a DNA-PKcs-binding protein and subsequently shown to bind to the reverse transcriptase subunit of telomerase *(93)*.

Although $Terc^{-/-}$ knockout mice, which lack the catalytic subunit of telomerase, can survive for several generations, telomeres in stem cells of these mice become progressively shorter with each generation and the mice begin to develop senescence-like phenotypes *(94,95)*, as do $Terc^{-/-}$ embryonic stem cells after long-term culture *(96)*. In double-knockout $Terc^{-/-}$ DNA-$PKcs^{-/-}$ mice, telomere loss and onset of age-associated pathology (such as atrophy in a variety of tissues) are accelerated, and the mice are completely infertile by the second generation *(97,98)*. These results suggest that DNA-PKcs contributes to maintenance of stable telomere length, although it has not yet been determined whether DNA-PKcs kinase activity is required for this function. It was proposed that in $Terc^{+/+}$ cells, the accelerated telomere loss associated with DNA-PKcs deficiency was reversed by telomerase. In human HeLa cells, RNAi-mediated knockdown of Ku86 ($\sim$50%) also resulted in telomere loss, as well as telomere fusions and an increase in spontaneous apoptosis *(99)*.

Murine embryonic fibroblasts and other cells derived from SCID mice as well as from mice with knockouts in Ku80, Ku70, or DNA-PKcs all show elevated levels of telomere fusions (detected cytogenetically), even when they have wild-type telomerase and normal telomere length *(90,91,100)*. Thus, the end-protection function of the telomere requires both Ku and DNA-PKcs, and the requirement for DNA-PKcs is not solely due to its role in maintaining telomere length. High levels of telomere fusions are also induced by the DNA-PK inhibitor IC86621, suggesting that the kinase activity of DNA-PK is required for normal telomere function *(101)*. Intriguingly, the levels of telomere fusions induced by IC86621 were much higher than levels in cells that lack DNA-PK entirely. IC86621-induced fusions, like those seen in DNA-PKcs- or TRF2-deficient cells *(102)*, involved only telomeres with ends replicated by leading-strand synthesis and were exclusively of the chromatid type. These results suggest that DNA-PKcs activity is required for TRF2-mediated telomere capping following replication. Thus, even though the normal looped telomere structure is not expected to activate DNA-PKcs, the double-stranded end transiently exposed at the telomere by replication could account for the DNA-PK activation that is apparently required for this capping function. Paradoxically, whereas lack of DNA-PK promotes fusions in cells with telomeres of normal length, DNA-PK is required for the telomere fusions and subsequent apoptosis induced by critically short telomeres *(97)*. Thus, the telomere fusions in DNA-PKcs-deficient cells must be formed by some aberrant end-joining pathway that does not require DNA-PKcs.

5. DNA-PK AND CHEMOTHERAPY/RADIOTHERAPY

DNA-PK-deficient cells, including cells containing catalytically inactive DNA-PK *(103)*, are radiosensitive; therefore, specific inhibitors of DNA-PK are expected to confer radiosensitivity. The most frequently used DNA-PK inhibitor is wortmannin, a fungal toxin that attaches covalently at the ATP-binding site of PIK and all PIK-related protein kinases, irreversibly inactivating kinase activity. Pretreatment with wortmannin inhibits DSB repair and renders cells about threefold more sensitive to ionizing radiation *(104–106)*. Although PIK is about 100 times more sensitive to wortmannin than are DNA-PK and ATM kinase, the fact that DNA-PK-deficient cells are not radiosensitized argues that DNA-PK is the target for radiosensitization. Like genetic NHEJ deficiency, wortmannin treatment eliminates split-dose recovery *(104,105)*, that is, the wortmannin-treated cells sustain the same degree of cell killing when a given dose of radiation is divided into two fractions separated by several hours as when the same amount of radiation is administered in a single dose. Sensitization to radiation and DSB-inducing chemotherapeutic agents (e.g., bleomycin and the topoisomerase II inhibitor etoposide) has also been achieved by siRNA-mediated silencing of DNA-PKcs *(107)*, expression of a dominant-negative Ku fragment *(108)*, and suppression of Ku expression with antisense oligonucleotides *(109,110)*. In cases where cross-sensitivity to other agents has been tested, there appears to be little or no sensitization to cross-linking agents such as cisplatin and mitomycin C *(109)*, consistent with a specific effect on DSB repair.

Unexpectedly, mouse and hamster cells deficient in Ku or DNA-PK were found to be more resistant than normal cells to cisplatin. This difference was only seen when the cells were treated at high density and was eliminated by chemical or genetic disruption of gap junctions *(111)*. Thus, these results suggest a cell-interdependent mode of killing involving a death signal that requires DNA-PK kinase activity and intercellular communication through gap junctions. Although this phenomenon has not been demonstrated in human cells, it could in principle explain some clinical studies in which higher DNA-PK levels were correlated with better response to cytotoxic cancer therapy. For example, in one study, abundance of DNA-PK in individual esophageal tumors was associated with improved response to combined chemotherapy/radiotherapy *(107)*. Similarly, in leukemic cells derived from patients with chronic lymphocytic leukemia, sensitivity to doxorubicin and etoposide was correlated with high DNA-PKcs expression *(112)*.

In view of its multiple roles in DSB repair, telomere maintenance, and apoptosis (Fig. 2), DNA-PK could be a reasonable candidate for pharmacological or genetic manipulation in cancer radiotherapy and chemotherapy. However, attempts at pharmacologically maximizing DNA-PK-mediated apoptosis will, at a minimum, require a much more detailed understanding of the specific apoptotic pathways involved as well as some means of constitutively activating DNA-PK or increasing its expression. Moreover, much of the evidence for the proapoptotic functions of DNA-PK (including gap junction-mediated cytotoxicity) has come from rodent cells, and most of these findings have yet to be confirmed in human cells.

A more promising strategy is the development of new small-molecule DNA-PK inhibitors that are much more specific than wortmannin and have little effect on related kinases such as ATM or PI3K. In principle, these agents can be used to block DSB repair in tumors and thus potentiate cell killing by ionizing radiation and DSB-inducing

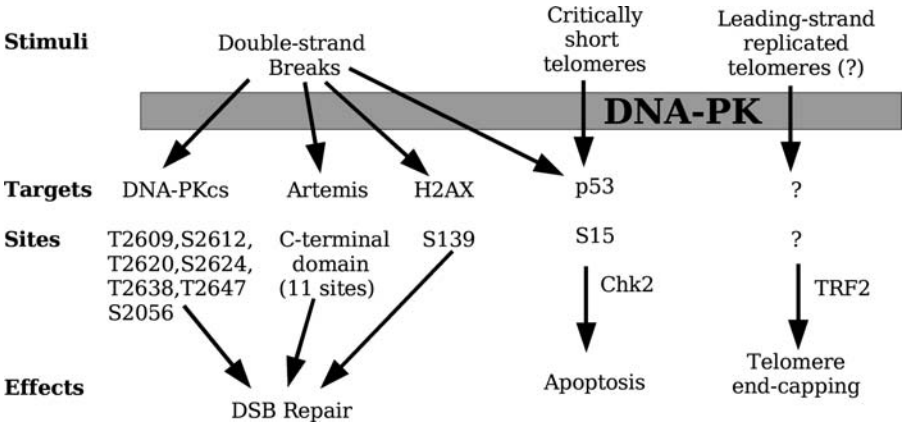

Fig. 2. Signaling pathways mediated by DNA-dependent protein kinase (DNA-PK).

chemotherapeutic agents. Preclinical radiosensitization/chemosensitization studies with two such inhibitors, IC87361 and NU7441, have yielded quite encouraging initial results. IC87361 markedly increased the radiosensitivity of human melanoma and lung cancer cells in culture and increased radiation-induced growth delay in xenografts in mice *(113)*. NU7441 similarly increased sensitivity of human colon cancer cells to both radiation and the topoisomerase II inhibitor etoposide and enhanced etoposide-induced growth delay in xenografts *(114)*. As predicted, sensitization by NU7441 was accompanied by a marked increase in the persistence of radiation- and etoposide-induced DSBs, as judged by γH2AX foci. Neither of these DNA-PK inhibitors produced any obvious acute toxicity, suggesting that the potentially toxic effects of DNA-PK inhibition may be tolerable for the short periods of exposure during chemotherapy/radiotherapy, at least in mice. However, the significant apparent differences between rodent and human cells in Ku and DNA-PK function *(8,18,48,115)* raise the possibility that some human toxicities might not be manifest in animal models. DNA-PK inhibitors would presumably block repair in normal cells as well, so it is likely that there will be at least some increase in toxicity of radiotherapy and chemotherapy toward normal tissue as well, and indeed the combination of NU7441 and etoposide resulted in some loss of body weight *(114)*. Moreover, if, as expected, these inhibitors promote telomere dysfunction, the possibility of carcinogenesis as a long-term side effect must be considered. Nevertheless, results obtained thus far suggest that these agents warrant further development and testing.

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IV Resistance and Sensitization

15 Resistance/Signaling Pathways

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Summary

Prior to the early 1990s, the mechanisms by which growth factors and cytokines modulate cell behavior were largely unknown. In the mid-1980s, with the discovery of the first mitogen-activated protein kinase (MAPK) pathway, and with subsequent discoveries of other MAPK family pathways in the early 1990s, our understanding of the hormonal control of cell biology was provided with a greater degree of molecular underpinning. In light of these findings, the ability of cytotoxic drugs and ionizing radiation to control the activity of MAPK family (and other) signaling pathways was first investigated in the mid-1990s. It was discovered that radiation and many noxious drugs, in a cell-type-dependent manner, can activate multiple intracellular signal transduction pathways: the activation of some pathways has been reported to be DNA-damage-dependent, that of others by generation of lipids such as ceramide, whereas others have been noted to be dependent on mitochondria-derived reactive oxygen/nitrogen species and the activation of growth factor receptor tyrosine kinases. The precise roles of growth factor receptors and signal transduction pathways in cellular responses following exposure to noxious stresses are presently under intense investigation. Generally, in a cell-type and dose-dependent manner, inhibition of the extracellular-regulated kinase 1/2 (ERK1/2), and to a greater extent phosphatidyl inositol 3 kinase (PI3K)/AKT, pathways can enhance cell killing. The modulation of cell survival by the ERK1/2 and AKT pathways has been correlated to the expression of mutant active RAS isoforms and to growth factor receptors of the ERBB and insulin/insulin-like growth factor (IGF) families. The activation of the c-Jun NH_2-terminal kinase 1/2 (JNK1/2), ERK1/2, and PI3K/AKT pathways in tumor cells has also been linked to the expression of paracrine ligands—ligands that can promote cell growth and survival after exposure to a noxious stress and ligands that are generally only expressed at high levels in transformed cells. This chapter will discuss the signaling pathways activated by cytotoxic drugs and ionizing radiation and the roles each pathway can play in cellular responses of tumor cells.

Key Words: Radiation; signaling; kinase; phosphatase; reactive oxygen/nitrogen species; receptor.

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1. SELF-LIMITING EFFECTS OF CANCER THERAPIES: AGENTS THAT CAUSE INCOMPLETE KILLING AND THE COMPENSATORY ACTIVATION OF GROWTH FACTOR RECEPTORS AND SURVIVAL SIGNALING PATHWAYS

As judged by the findings of many groups using a wide variety of noxious xenobiotic agents, cellular stresses cause pleiotropic activation of multiple receptor proteins in the plasma membranes of cells. The activation of death receptors by xenobiotic agents, such as the FAS and tumor necrosis factor α(TNFα) receptors, has been linked to the generation of ceramide, resulting in the clustering of activated death receptors in the plasma membrane, and the activation of apoptotic pathways. However, lethal cellular stresses also promote activation of growth factor receptors that when activated by their natural ligands are normally linked to cell growth and survival. Hence, conflicting signals are generated.

For example, xenobiotic agents can activate ERBB family receptors, and signaling by ERBB family of receptors is, in general, believed to be pro-proliferative and cytoprotective *(1,2)*. Because both ERBB receptor expression and autocrine growth factor levels are often increased in carcinoma cells compared with normal tissue, many laboratories have studied signaling by the ERBB family in tumor cell growth and survival control after exposure to therapeutic agents. Thus, when signaling from ERBB family receptors is blocked, by use of inhibitory antibodies (e.g., C225, 4D5 Herceptin, and monoclonal antibody 806), small molecular weight inhibitors of receptor tyrosine kinases [e.g., PD183805 (also called CI1033), PKI166, AG1478, PD153035, ZD1839, PD169414, OSI774, AG825, and AG879], dominant-negative truncated receptors (e.g., dominant-negative EGFR-CD533), or antisense approaches (antisense EGFR), that tumor cell growth can be reduced and the sensitivity of these cells to being killed by noxious stresses increased *(3–21)*.

In part, the above findings can be explained by the fact that growth factor receptors, including ERBB1, ERBB2, ERBB3, VEGF-R (Flt-1), and the insulin-like growth factor-1 (IGF-1), are all known to be activated, in a cell-type-dependent manner, after radiation/drug exposure *(22–24)*. Recent publications have tended to argue that reactive oxygen and nitrogen species (ROS/RNS) play an important role in the activation of ERBB family receptors and the signaling by protective downstream pathways following exposure to ionizing radiation *(25,26)*. In the case of receptor tyrosine kinases, the primary target of ROS/RNS is most likely to be protein tyrosine phosphatase (PTPase) enzymes that contain an ROS/RNS-sensitive cysteine residue within their active site, which is a residue that is essential for phosphatase activity *(27)*. As PTPases are approximately 100-fold more active as enzymes than protein kinases, a small reduction in cellular PTPase activity will result in an increase in the steady-state tyrosine phosphorylation of the protein kinases, for example, receptor tyrosine kinases, whose phosphorylation the PTPases act to suppress. Many cytotoxic drugs also have been shown to directly or indirectly generate ROS/RNS as part of the mechanism by which they kill both non-transformed and tumor cells, for example, cisplatinum, histone deacetylase inhibitors, rituximab, doxorubicin, and NSAIDs *(28–32)*. Hence, both cytotoxic drugs and radiation have the potential to both kill tumor cells and simultaneously activate protective pathways, for example, ERBB receptor signaling into downstream survival signal transduction pathways, by which their toxicity is blunted.

In addition to a direct effect on the receptor tyrosine kinases themselves, the actions of ERBB receptor autocrine ligands have also been shown to play important roles in the activation of the downstream protective pathways following exposure to noxious stresses. For example, transforming growth factor alpha (TGFα) has been shown to mediate secondary activation of ERBB1 and the downstream extracellular-regulated kinase 1/2 (ERK1/2), AKT, and c-Jun NH_2-terminal kinase 1/2 (JNK1/2) pathways after irradiation of several carcinoma cell lines *(33,34)*. Radiation caused an ERK1/2-dependent cleavage of pro-TGFα in the plasma membrane within 2 h that led to its release into the growth media *(35)*. Similar findings have been noted by other groups *(36,37)*. In addition, signaling by RAS proteins, ERK1/2, and p53 can, over many hours/days in various cell systems, act to increase the expression of autocrine factors such as HB-EGF and epiregulin *(38)*.

2. A PARADIGM FOR DOWNSTREAM PROTECTIVE PATHWAYS: THE "CLASSICAL" MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY, ERK1/2

"MAP-2 kinase" was first reported by the laboratory of Dr Thomas Sturgill in 1986 *(39)*. This protein kinase was originally described as a 42-kDa insulin-stimulated protein kinase activity whose tyrosine phosphorylation increased after insulin exposure and which phosphorylated the cytoskeletal protein MAP-2. Contemporaneous studies from the laboratory of Dr Melanie Cobb identified an additional 44-kDa isoform of this enzyme, termed ERK1 *(40)*. As many growth factors and mitogens could activate these enzymes, the acronym for this enzyme was subsequently changed to denote mitogen-activated protein kinase (MAPK). Additional studies demonstrated that the p42 (ERK2) and p44 (ERK1) MAPKs regulated another protein kinase activity ($p90^{rsk}$) and that they were themselves regulated by protein kinase activities designated MKK1/2 [MAPK kinase (MAP2K)] also termed MEK1/2. MKK1 and MKK2 were also regulated by reversible phosphorylation. The protein kinase responsible for catalyzing MKK1/2 activation was initially described as the proto-oncogene RAF-1 *(41)*. This was soon followed by another MEK1/2 activating kinase, termed MEKK1, which was a mammalian homolog with similarity to the yeast *Ste 11* and *Byr 2* genes *(42)*. However, further studies have shown that the primary function of MEKK1 is to regulate the JNK1/2, rather than the ERK1/2, pathway *(43)*.

Plasma membrane receptors transduce signals through the membrane to its inner leaflet, leading to the recruitment and activation of guanine nucleotide exchange factors, which increases the amount of GTP bound to membrane-associated GTP-binding proteins, in particular RAS family proteins *(44–46)* (Fig. 1). There are four widely recognized isoforms of RAS: Harvey (H), Kirsten (K4A and K4B), and Neuroblastoma (N) *(47)*. Receptor-stimulated guanine nucleotide exchange of "RAS" to the GTP-bound form permits RAF proteins and P110 phosphatidyl inositol 3 kinase (PI3K) to associate with "RAS", resulting in kinase translocation to the plasma membrane environment where activation of these kinases takes place. RAS contains a GTPase activity that converts bound GTP to GDP resulting in inactivation of the RAS molecule. Transforming/oncogenic mutation of RAS results in a loss of the protein's inherent GTPase activity, generating a constitutively active RAS molecule that can lead to elevated activity within downstream signaling modules, notably the ERK1/2 and PI3K pathways.

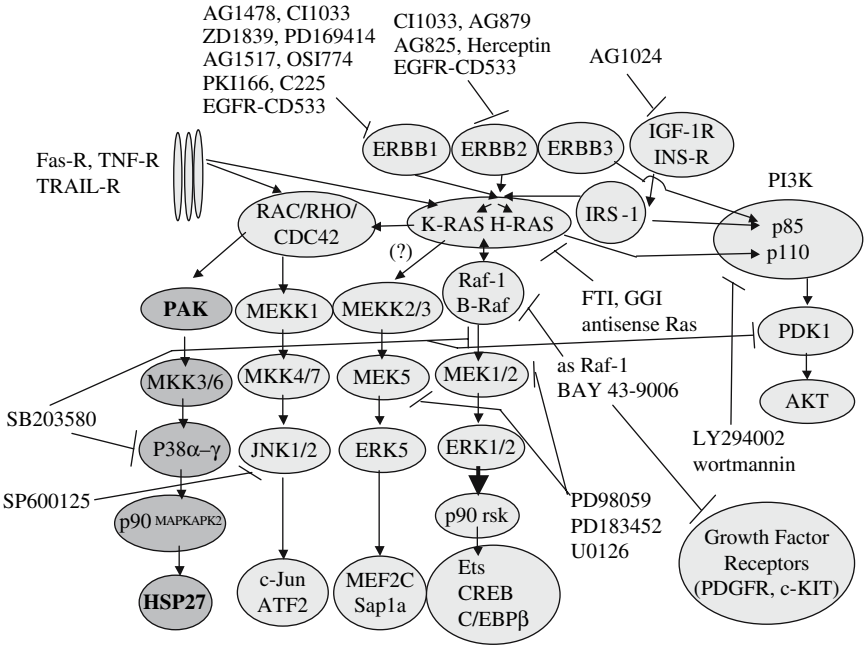

Fig. 1. Some of the characterized signal transduction pathways in mammalian cells. Growth factor receptors, for example, the ERBB family, and insulin receptor can transmit radiation-induced signals down through GTP-binding proteins into multiple intracellular signal transduction pathways. Predominant among these pathways are the mitogen-activated protein kinase (MAPK) superfamily of cascades (ERK1/2, ERK5, JNK, and p38) and the phosphatidyl inositol 3 kinase (PI3K) pathway. Growth factor receptors, RAS proteins, and downstream pathways are often partially or constitutively activated in tumor cells, and inhibitors have been developed to block the function of these molecules thereby slowing cell growth and promoting cell death responses. Multiple inhibitors for the ERBB family receptors have been developed. Inhibitors of RAS farnesylation (and geranylgeranylation) are in clinical trials as are inhibitors of the ERK1/2/5 pathway. It should be noted that MEK1/2 inhibitors also are capable of inhibiting the "Big" MAPK pathway through blocking activation of MEK5.

Mutated active H-RAS was one of the first oncogenes discovered, in bladder cancer cells *(48)*, and approximately one-third of human cancers have RAS mutations, primarily the K-RAS isoforms. Early studies examining RAS transformation noted that RAS proteins were prenylated and proteolytically processed causing their translocation into the plasma membranes of cells *(49)*. RAS proteins that could not be prenylated were not located in membranes and could not cause transformation. Subsequently, pharmaceutical companies developed inhibitors of RAS prenylation [farnesyltransferase inhibitor (FTI) and geranylgeranyltransferase inhibitor (GGTI)] that block the prenylation of RAS proteins as well as other small GTP-binding proteins including RAC/RHO/CDC42 *(50)*. Antisense techniques have also been used to manipulate RAS expression *(51)*. Inhibition of small GTP-binding protein prenylation has been shown to both growth arrest and radiosensitize tumor cells in a mutated active RAS-independent fashion, arguing that RAS may not be the primary target of this therapeutic approach *(52)*. In the clinic, as single agents, FTIs have not proven highly successful, and the long-term future of such agents remains unclear. It is possible that FTIs in

combination with cytotoxic agents, whose activity they may magnify, may represent the likeliest avenue for the use of FTIs in the clinic.

RAF-1 is a member of a family of serine–threonine protein kinases comprising also B-RAF and A-RAF. All "RAF" family members can phosphorylate MKK1/2 and activate the ERK1/2 pathway *(53)*. Thus, the "RAF" kinases act at the level of an MAPK kinase kinase (MAP3K). The NH_2 domain of RAF-1 can reversibly interact with Ras family members in the plasma membrane, and the ability of RAF-1 to associate with RAS is dependent on the RAS molecule being in the GTP-bound state *(54)*. Additional protein serine/threonine and tyrosine phosphorylations are also known to play a role increasing RAF-1 activity when in the plasma membrane environment *(55,56)*. The initial biochemical analyses of purified RAF-1 demonstrated constitutive Y340–Y341 phosphorylation when the protein was co-expressed with SRC. PI3K-dependent phosphorylation of S338 may facilitate RAF-1 tyrosine phosphorylation by *src* family members, leading to full activation of RAF-1 *(57,58)*. Of note, activating B-RAF mutations have more recently been shown to play an important role in tumorigenesis in melanoma, thyroid, colorectal, and cholangio-carcinoma, and B-RAF is now considered to be a genuine proto-oncogene, with transforming potential after its mutation *(59–62)*.

Phosphorylation of RAF-1 at S259 by either the AKT or the cAMP protein kinase [protein kinase A (PKA)] can inhibit RAF-1 activity and its activation by upstream stimuli *(63–66)*. Phosphorylation of RAF-1 at S43 by cAMP-dependent protein kinase (PKA) inhibits the interaction of RAF-1 with RAS molecules, thereby blocking RAF-1 translocation to the plasma membrane and its RAS-dependent activation *(67)*. In contrast to RAF-1, the B-RAF isoform does not contain an equivalent to S43 but contains multiple sites of AKT (and potentially PKA)-mediated phosphorylation in addition to the B-RAF equivalent of S259 *(68)*. B-RAF can be activated by both RAS and cAMP through the RAP1 GTPase *(69,70)*. Thus, the regulation of the MEK1/2–ERK1/2 pathway is very complex, and in some cell types, may be both inhibited by cAMP/PKA, through RAF-1, and being stimulated by cAMP/RAP1, through B-RAF *(71)*.

As there are several "RAF" isoforms, it is likely that inhibition of one "RAF" family member, for example, by a specific antisense approach, will not have a profound prolonged growth inhibitory or drug/radiation sensitizing effect on tumor cells because of a compensatory utilization of another "RAF" family member. In light of this, although antisense oligonucleotides to RAF-1 have been shown *in vitro* and *in vivo* to enhance the radiosensitivity of tumors cells *(72)*, such studies have not been highly successful in the clinic because of poor response rates. The development of a relatively non-specific small molecule Raf-1/B-Raf inhibitor is thus more likely to have therapeutic usefulness *(73,74)*.

3. DOWNSTREAM PROTECTIVE PATHWAYS: PI3K/AKT SIGNALING AND THE INTERACTIONS OF PI3K/AKT SIGNALING WITH RAF/ERK1/2 PATHWAY SIGNALING

PI3K enzymes consist of two subunits, a catalytic p110 subunit and a regulatory p85 subunit, and several different classes of PI3K enzymes exist *(75,76)*. The p85 subunit of PI3K enzymes contains a phospho-tyrosine (SH2) binding domain *(77)*. The major

catalytic function of the PI3K enzymes is in the p110 subunit that acts to phosphorylate inositol phospholipids [phosphatidyl inositol 4,5 *bis*-phosphate (PIP2)] at the third position within the inositol sugar ring. Mitogens such as TGFα and heregulin stimulate tyrosine phosphorylation of ERBB family receptors, providing acceptor sites for the SH2 domain of p85 *(78,79)*. Binding of p85 to active ERBB receptors (predominantly ERBB3) results in p110 PI3K activation. Other studies have suggested that mutant oncogenic H-RAS, or serpentine receptors that are stimulated by mitogens, that the p110 subunit of PI3K can directly bind to RAS-GTP, leading to catalytic activation of the kinase *(80–82)* (Fig. 1).

The molecule inositol 3,4,5 trisphosphate is an acceptor site in the plasma membrane for molecules that contain a plecstrin-binding domain (PH domain), in particular, the protein kinases PDK1 and AKT (also called PKB) *(83)*. PDK1 is proposed to phosphorylate and activate AKT. Signaling by PDK1 to AKT and by PDK1 and AKT downstream to other protein kinases such as PKC isoforms, GSK3, mTOR, $p70^{S6K}$, and $p90^{rsk}$, has been shown to play a key role in mitogenic and metabolic responses of cells as well as protection of cells from noxious stresses *(84–88)*.

The anti-apoptotic role of the PI3K/AKT pathway has been well documented by many investigators in response to numerous noxious stimuli, and in some cell types, the anti-apoptotic effects of ERBB signaling have been attributed to activation of the PI3K/AKT pathway *(89,90)*. ERBB signaling to PI3K/AKT has been proposed to enhance the expression of the mitochondrial anti-apoptosis proteins $BCL\text{-}_{XL}$ and MCL-1 and caspase inhibitor proteins such as c-FLIP isoforms *(91–93)*. Enhanced expression of $BCL\text{-}_{XL}$ and MCL-1 will protect cells from apoptosis through the intrinsic/mitochondrial pathway, whereas expression of c-FLIP isoforms will block killing from the extrinsic pathway through death receptors *(94)*. In addition, AKT has been shown to phosphorylate BAD and human pro-caspase 9, thereby rendering these proteins inactive in apoptotic processes *(95,96)*. Inhibitors of ERBB signaling have been shown to decrease the activity of the PI3K/AKT pathway in various cell types and to increase the sensitivity of cells to a wide range of toxic stresses including cytotoxic drugs and radiation *(97)*. Activation of AKT was shown to protect cells from death in the presence of ERBB receptor inhibition *(98)*. These findings strongly argue that PI3K/AKT signaling is a key cyto-protective response in many cell types downstream of ERBB family receptors.

Inhibition of PI3K signaling in cells has been achieved by using several drugs, notably wortmannin and LY294002. Wortmannin inhibits PI3K isoforms with IC50 values in the low nanometer range and LY294002 with IC50 values in the low micrometer range *(99)*. More recently, inhibitors of AKT function (through both the PH domain of AKT and the ATP binding site) as well as inhibitors of PDK-1 (also, primarily through the PH domain of PDK-1) have been developed *(100–103)*. The therapeutic usefulness of PI3K inhibitors, and potentially those of AKT and PDK-1, may be limited because of systemic toxicities and effects on cellular glucose metabolism. For example, wortmannin was noted to cause hemorrhages, and the development of effective PI3K inhibitors without dose-limiting side effects is being undertaken by many pharmaceutical companies.

4. DOWNSTREAM CYTOTOXIC PATHWAYS: THE C-JUN KINASE AND STRESS-ACTIVATED PATHWAY, JNK1/2/3

JNK1 and JNK2 were initially described biochemically to be stress-induced protein kinase activities that phosphorylated the NH_2 terminus of the transcription factor c-JUN; hence, the pathway is often called the stress-activated protein kinase (SAPK) pathway *(104–106)*. Multiple stresses increase JNK1/2 (and the subsequently discovered JNK3) activity including UV and γ irradiation, cytotoxic drugs, bile acids, and compounds that primarily generate ROS (e.g., H_2O_2). Phosphorylation of the NH_2-terminal sites Ser63 and Ser73 in c-JUN increases its ability to transactivate AP-1 enhancer elements in the promoters of many genes *(107,108)*. It has been suggested that JNK1/2 can phosphorylate the NH_2 terminus of c-MYC, potentially playing a role in both proliferative and apoptotic signaling *(109)*. In a similar manner to the previously described ERK1/2 MAPK pathway, JNK1/2 activities were regulated by dual threonine and tyrosine phosphorylation that were found to be catalyzed by a protein kinase analogous to MKK1/2, termed stress-activated extracellular-regulated kinase 1 (SEK1), also called MKK4 *(110,111)*. An additional isoform of MKK4, termed MKK7, was subsequently discovered. As in the case of MKK1 and MKK2, MKK4 and MKK7 were regulated by dual serine phosphorylation. Recent studies have also indicated that AKT can phosphorylate and inhibit the activity of MKK4, demonstrating cross-talk between the PI3K and JNK pathways *(112)*.

In contrast to the ERK1/2 pathway, however, which appears to primarily utilize the three protein kinases of the "RAF" family to activate MKK1/2, at least 10 protein kinases are known to phosphorylate and activate MKK4/7, including the Ste 11/Byr2 homologs MKKK1–4, as well as proteins such as TAK-1 and TPL-2 *(113–115)*. Cleavage of MEKK1 by caspase molecules into a constitutively active molecule may play an amplifying role in the execution of apoptotic processes *(116)*.

Upstream of the MAP3K enzymes are another layer of JNK1/2 pathway protein kinases, for example, Ste20 homologs and low molecular weight GTP-binding proteins of the RHO family, in particular CDC42 and RAC1 *(117–119)* (Fig. 1). It is not clear how growth factor receptors, for example, ERBB1, activate the RHO family low molecular weight GTP-binding proteins; one mechanism may be through the RAS proto-oncogene, whereas others have suggested through PI3K and/or PKC isoforms *(120)*. In addition, others have shown that agonists acting through the TNFα and FAS receptors, through sphingomyelinase enzymes generating the messenger ceramide, can activate the JNK1/2 pathway by mechanisms that may act through RHO family GTPases *(121)*.

Thus, there appear to be multiple distinct mechanisms by which toxic drugs and radiation could activate the JNK1/2 pathway. Initial reports demonstrated that ceramide generation and that the clustering of death receptors on the plasma membrane of cells played an important role in JNK1/2 activation *(122–125)*. This was linked to a pro-apoptotic role for JNK1/2 signaling. Other studies have argued that JNK1/2 activation can be dependent on DNA damage and activation of the ataxia telangiectasia mutated (ATM) and c-ABL proteins *(126–128)*. Studies by our group of laboratories have shown that low-dose radiation activates JNK1/2 in two waves in carcinoma cells. The first wave of JNK1/2 activation was dependent on activation of the TNFα receptor, whereas the second wave of JNK1/2 activity was dependent on ERBB1 and autocrine TGFα. It is

also possible that JNK1/2 activation could be a secondary event to the loss of protective pathway activities, and this could occur through two mechanisms: (i) drug/radiation causes a loss of protective pathway activity resulting in the loss of a negative regulatory signal, which suppresses pro-apoptotic JNK1/2 signaling, for example, loss of JNK phosphatase expression and loss of MKK4 inhibitory phosphorylation; and (ii) JNK1/2 activation is dependent on the subsequent activation of effector pro-caspases: cleavage of the upstream activator MEKK1 can lead to constitutive activation of this enzyme and the downstream JNK1/2 pathway, which in some cell types plays a key role in full commitment to apoptotic cell death *(129–131)*.

5. A SIMPLISTIC SCENARIO FOR CELL SURVIVAL SIGNALING AFTER EXPOSURE TO A NOXIOUS STRESS: INHIBITION OF RAF/MEK/ERK1/2 FUNCTION AND/OR PI3K/AKT FUNCTION TO CIRCUMVENT PROTECTIVE SIGNALING PROCESSES INDUCED BY CYTOTOXIC INSULTS THUS PROMOTES DRUG/RADIATION LETHALITY

Data from several groups have argued that a key protective pathway, responsible for blunting the toxicity of therapeutic drugs, downstream of plasma membrane receptors is the PI3K pathway. Inhibition of p110 PI3K function by using the inhibitors LY294002 and wortmannin radio- and chemo-sensitizes tumor cells of multiple diverse expressing mutant RAS molecules or wild-type RAS molecules that are constitutively active *(132–135)*. It is possible that these inhibitors may also exert a portion of their sensitizing properties by inhibiting proteins with PI3K-like kinase domains such as ATM, ATR, and DNA-PK proteins whose function is to modulate cell survival after DNA damage *(136–138)*. Dominant-negative AKT and small molecule inhibitors of AKT have also been shown to sensitize tumor cells exposed to various noxious agents, suggesting that AKT is a key downstream target for cytoprotective PI3K signaling *(100–103)*.

In many cell types, ERK1/2 signaling does not appear to play a role in controlling cell sensitivity to cytotoxic drugs or ionizing radiation, and in some cells, elevated ERK1/2 signaling has been linked to the promotion of cell killing. However, others have argued that the duration of suppression of ERK1/2 activity may play an important role in whether signaling by this pathway has protective or toxic effects *(139)*. The dual positive and negative nature of ERK1/2 signaling in the control of cell survival has also been observed for other DNA-damaging agents such as adriamycin and UV radiation *(140–142)*.

The mechanisms by which PI3K, AKT, and ERK1/2 signaling protect cells from toxic stresses are multi-layered. The activity and activation of apoptotic cysteine proteases has been shown by many groups to be modulated directly and indirectly by PI3K and AKT signaling *(143)*. For example, activation of PI3K/AKT can promote phosphorylation and inactivation of caspase 9 and BAD, increase expression of inhibitor of apoptosis proteins (IAPs: XIAP), and enhance expression of the caspase 8 inhibitors c-FLIP-$_{L/S}$ *(144–147)*. Similarly, ERK1/2 signaling has been shown to promote phosphorylation and inactivation of caspase 9, BAD, BIM, and the FAS receptor, increase expression of Bcl-$_{XL}$, and elevate expression of the caspase 8 inhibitors

c-FLIP-$_{L/S}$ *(148–153)*. AKT and ERK1/2 have also been shown in a cell-type-dependent fashion to suppress the generation of ROS and the toxic lipid ceramide *(154,155)*.

Hence, as toxic (therapeutic) stresses increase activation of pro-apoptotic pathways, they simultaneously activate parallel survival pathways such as AKT and ERK1/2 that act to blunt apoptotic pathway function. This has been exploited in the laboratory with excellent success in many tumor cell types and in the clinic with more modest success, for example, the combination of ionizing radiation with PI3K or MEK1/2 inhibitors or cytotoxic drugs with PI3K or MEK1/2 inhibitors. A portion of the reason why inhibiting one survival pathway or one survival growth factor receptor has not proven to be "the magic bullet" in the clinic is because tumor cells are metastable with respect to their survival signaling. For example, inhibition of the estrogen receptor in established breast cancer cells *in vitro* can generate a surviving line which is dependent on ERK1/2 signaling, rather than estrogen receptor signaling, for cell growth and survival *(156,157)*. Inhibition of ERK1/2 signaling in turn promotes outgrowth of tumor cells that are dependent on PI3K signaling for growth and survival and not the estrogen receptor or ERK1/2 pathway. Thus, inhibition of two (or more) parallel survival pathways may be required to promote prolonged tumor cell killing in vivo.

An example of how simultaneous inhibition of several survival pathways kills tumor cells can be found in the combination of inhibitors of the ERK1/2 pathway with the Chk1/PKC/PDK-1 inhibitor UCN-01 *(158–160)*. Treatment of tumor cells with UCN-01 causes activation of the ERK1/2 pathway. Inhibition of the induced ERK1/2 activity by using various MEK1/2 inhibitors results in a rapid apoptotic response, dependent on mitochondrial dysfunction and the release of cytochrome c into the cytosol. Other groups have combined tyrosine kinase inhibitors with inhibitors of the protective mTOR pathway to achieve similar results *(161)*. An additional example of this concept is the combination of the cyclin-dependent kinase inhibitor flavopiridol with the PI3K inhibitor LY294002. Treatment of leukemia cells with flavopiridol causes compensatory activation of the PI3K survival pathway: simultaneous inhibition of kinases using flavopiridol and LY294002 results in a synergistic increase in cell killing *(162)*.

Activation of the JNK1/2 pathway has been in general linked by many groups to cell-killing processes; however, it is of note that activation of the JNK1/2 pathway per se does not induce cell death, for example, by growth factors. Intense activation of JNK1/2 for a short period of time ($\sim$ 1 h) or prolonged low levels of JNK1/2 activity can cause proliferation and/or differentiation through modulation of transcription factors, for example, AP-1 complex, whereas prolonged intense activation of the JNK1/2 pathway has been linked to cell killing *(122–131,163)*. Activation of JNK1/2 can promote mitochondrial injury, that is, BAX association with mitochondria, as well as facilitate death receptor signaling by multiple mechanisms and has been strongly linked to the toxicity of ionizing radiation *(164,165)*. The toxicity of JNK1/2 pathway signaling is also regulated by the relative activities of protective signaling pathways such as PI3K-AKT and ERK1/2 (*166*, reviewed in ref. *1*). Thus, activation of JNK1/2 may not cause cell death when AKT and ERK1/2 remain active: conversely, activation of JNK1/2 in the presence of inhibited or inactivated ERK1/2 and AKT promotes cell killing.

Collectively, these findings argue that the future of signaling modulators in cancer therapy may be linked to a careful appreciation of how compensatory signaling pathways

interact, so that multiple inhibitors can be combined in a relatively precise manner to achieve a maximum amount of tumor cell killing while preventing the activation of survival pathways that will lead to loss of tumor control and therapeutic failure.

6. CONCLUSIONS

The manipulation of signal transduction pathways to reduce cell survival after exposure of cells to noxious insults has been extensively explored over the last 10 years. Some of these concepts are slowly being translated into the clinic, for example, combining ERBB1 or ERBB2 inhibitors with radiotherapy or drugs such as cisplatinum or paclitaxel. Many of the initial studies have proven less successful in patients than was expected based on *in vitro* and orthotopic animal tumor model systems, in part because tumor cell signaling is metastable, and inhibition of one signaling survival pathway can lead to the activation of a compensatory survival signaling module. The development of therapies that combine kinase inhibitors in a specific manner to cause cell death, combined with traditional modalities such as radiotherapy, may represent the future of therapeutic intervention based on the inhibition of multiple survival signaling pathways.

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16 Ceramide and Multidrug Resistance

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Summary

Chemotherapy resistance represents a formidable barrier to the successful treatment of advancing cancer, and patients and oncologists would welcome its possible correction. Unfortunately, many frontline chemotherapeutic agents drive biochemical events that promote tumor cell resistance. Averting these events is key to halting and perhaps reversing this process. Only recently have we begun to appreciate the role of lipids in both chemotherapy response mechanisms and resistance process. Because much emphasis is being placed on "targeted therapeutics," we believe that targeting the enzymes of lipid metabolism represents a promising direction in the treatment of cancer. This chapter explains how the harnessing of ceramide metabolism could be key in tempering multidrug resistance.

Key Words: Ceramide; chemotherapy; multidrug resistance; glucosylceramide synthase.

1. INTRODUCTION

Drug and biocide resistance arising in biological systems is widespread in nature. Organisms develop defense mechanisms against deleterious effects of myriad toxins. Posing a serious threat to human health and the environment are drug-resistant bacteria and viruses, pesticide-resistant insects, and herbicide-resistant plants. Many human cancers are also drug resistant; although some such as melanoma can be intrinsically resistant *(1)*, cancers more often acquire resistance through selection pressure in the face of adversity, for example, chemotherapy.

Poor response to chemotherapy is a major clinical problem, and drug resistance is usually the cause *(2,3)*. Approximately 40% of cancer patients with resectable disease and 80% of cancer patients with unresectable disease have a poor response to chemotherapy and radiotherapy. In breast cancer alone, nearly 50% of patients demonstrate primary and/or secondary resistance to doxorubicin *(4)*. In prostate and ovarian malignancies, the prevalence of drug resistance prevents significant cure with current chemotherapeutic regimens; in melanoma, which is characterized by high risk for early metastasis, the commonly employed frontline anticancer drugs do not improve prognosis *(5)*. In small cell lung cancer, acquired resistance to multiple drugs has

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reduced the cure rate of chemotherapy to less than 10% *(6)*. A clinically effective means of tempering or reversing resistance would significantly improve response to chemotherapy and thereby decrease the mortality of human cancer. This chapter will explore how interfering with ceramide metabolism might modulate tumor cell resistance to chemotherapy and thereby serve as the basis for clinically effective approaches to drug-resistant cancer.

2. CERAMIDE IN GROWTH ARREST AND CELLULAR SENESCENCE

To the small fraternity of lipid biochemists worldwide, news that ceramide was involved in the killing action of chemotherapy drugs brought a resurgence in cancer studies. In the not-too-distant past, life was composed of proteins, lipids, carbohydrates, and nucleic acids; today, these elements are being redefined by knockouts, clones, and chain reactions, leaving little room for mere fat. Lipids, like Mahler, can be difficult to appreciate—unless they prove effective in the treatment of cancer.

Ceramides or "*N*-acylsphingosines" are neutral lipids that can be either generated by a de novo pathway through condensation of serine and palmitoyl CoA and a series of enzymatic steps or formed by degradation of sphingomyelin through the sphingomyelinase pathway (Fig. 1). The amide-linked acyl moieties of ceramide can consist of various chain lengths of typically 16–26 carbons. Ceramides influence numerous cellular

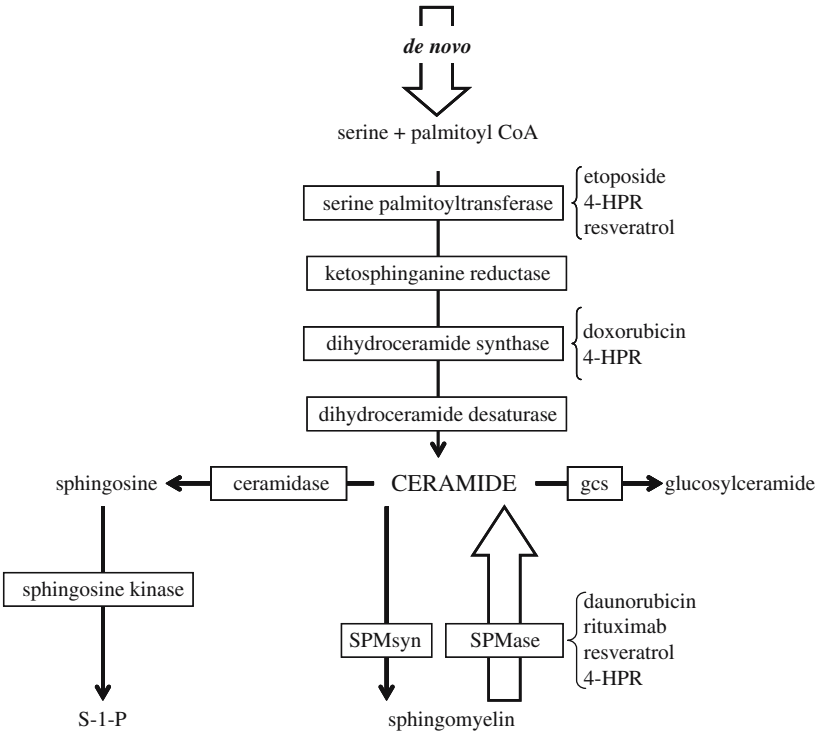

Fig. 1. Ceramide metabolism. Shown at top and bottom (bold arrows) are de novo and sphingomyelinase (SPMase) pathways of ceramide formation. The points of action of a few selected anticancer agents are shown in braces. All enzymes are shown in boxes. S-1-P, sphingosine-1-phosphate; GCS, glucosylceramide synthase.

responses, among them growth arrest, cell death pathways, chemotherapy responses, and cellular senescence. Cellular senescence, which is characterized by an irreversible growth phase, can be prompted by ceramide. Mouton and Venable *(7)* demonstrated, using human diploid fibroblasts, that ceramide induces biochemical changes in character with senescence when administered to low-passage cells. In particular, they showed that ceramide (short-chain C_6 analog) exposure induced expression of beta-galactosidase, a biochemical marker of senescence. Similarly, in young proliferating fibroblasts, beta-galactosidase can be induced by exposure to ceramide, tumor necrosis factor alpha (TNFα), or okadaic acid *(8)*. Administration of exogenous ceramide to fibroblasts and endothelial cells provided data to suggest that differences in the regulation of apoptosis are associated with senescence in different histotypes *(9)*. In a study by Obeid and colleagues *(10)*, C_6-ceramide-induced G0/G1 arrest of the cell cycle in Wi38 human diploid fibroblasts was accompanied by inhibition of cyclin-dependent kinase 2 by way of activation of p21, a class of cyclin-dependent kinase inhibitory proteins. Using breast cancer as a model, Gewirtz and colleagues *(11)* studied the role of ceramide in blocking escape from radiation-induced cell death. MCF-7 cells exposed to fractionated radiation undergo a senescence-like growth arrest but then recover proliferative capacity. This recovery, it was shown, could be blocked, and cell killing by radiation could be potentiated by the addition of ceramide-generating vitamin D_3 analogs *(11)*. In a recent study by Venable et al. *(12)*, the authors showed that increases in senescence-associated ceramide appear to be associated with the action of neutral sphingomyelinase. Finally, in a very interesting study of age-related acceleration of apoptosis, Perez et al. *(13)* showed in a mouse model that elevated oocyte death of aged females is coincident with enhanced ceramide sensitivity.

3. CERAMIDE SIGNALING PATHWAYS

Excellent reviews are available on second-messenger signaling events associated with ceramide's action *(14,15)*; therefore, we will briefly caption some of the paramount ceramide targets. Protein phosphatases 1 and 2A are activated by ceramide *(16)*; these phosphatases regulate both growth and apoptosis. Downstream targets include Bad *(17)*, Bax *(18)*, Akt *(19)*, and Bcl2 *(20)*. Kinase suppressor of ras (KSR) represents another target of ceramide through the intermediary action of protein phosphatase 2A *(21,22)*. Protein kinase C (PKC) translocation is also mediated by ceramide, and several members of the PKC family are affected, including PKCα, δ, ε, ξ, and β2 *(23–28)*.

4. CERAMIDE GENERATION BY CHEMOTHERAPEUTIC AGENTS

Many anticancer chemotherapeutics activate enzymes that catalyze ceramide production (reviewed in ref. *29*). Ceramide can be produced de novo by steps that begin with the enzyme serine palmitoyltransferase and end with the enzyme dihydroceramide desaturase; alternatively, ceramide can be produced by hydrolysis of sphingomyelin *(30–33)*, which cleaves the phosphocholine group from the ceramide backbone. As shown in Fig. 1, some drugs may have more than one effect on ceramide metabolism: 4-HPR (fenretinide) activates serine palmitoyltransferase, dihydroceramide synthase, and sphingomyelinase *(34,35)*. Anthracyclines such as doxorubicin and daunorubicin can also stimulate both de novo ceramide production *(36,37)* and sphingomyelin hydrolysis *(38)*, although these pathways may be cell-type specific. Resveratrol, a

hydroxylated stilbene found in skins of red grapes, stimulates serine palmitoyltransferase and neutral sphingomyelinase, promoting ceramide production and apoptosis in metastatic breast cancer cells *(39)*, whereas etoposide, an epipodophyllotoxin derived from the May apple and used in the treatment of lymphoma, leukemia, and small cell lung carcinoma, promotes ceramide formation de novo during apoptosis *(40)*. Laurent and colleagues *(41)* have linked the antiproliferative activity of rituximab, a chimeric human immunoglobulin G1 anti-CD20 monoclonal antibody used to treat B-cell lymphoma, to activation of acid sphingomyelinase. Findings that anticancer agents promoted formation of ceramide, a proapoptotic messenger *(42–44)*, heralded a wave of enthusiasm and in-depth studies on the role of ceramide and glycolipid metabolism in cancer treatment *(29,45–48)*.

5. DRUG RESISTANCE IN CANCER

Cancer cells develop multiple mechanisms to evade drug toxicity *(2,49,50)*. The biology underlying drug resistance in cancer cells encompasses multiple factors including overexpression of P-glycoprotein (P-gp) and multidrug resistance-associated protein *(2,51)*, changes in topoisomerase II *(52)*, and modifications in glutathione *S*-transferase activity *(53)*. Chemoresistance also may be related to the expression of important apoptosis-associated proteins such as the bcl-2 family of proteins *(54)* and the tumor suppressor protein p53 *(55)*, synthesis of vaults *(56)*, and overexpression of caveolae *(57)*. Many recent studies *(58–62)* suggest that the dysfunctional metabolism of ceramide contributes to multidrug resistance. For example, the glycosylated form of ceramide (glucosylceramide) is elevated in multidrug-resistant cancer cells *(58,59,63,64)*; this might be expected to dull ceramide's cytotoxic impact by sequestration of the apoptosis inducer (ceramide) as a glycolipid. In other work, Kok and colleagues *(65)* demonstrated altered glycolipid metabolism in multidrug-resistant ovarian carcinoma; Sonnino et al. *(66)* reported similar findings in 4-HPR-resistant ovarian cancer cells. The work of Okazaki et al. *(67)* revealed a strong connection between low ceramide levels and elevated levels of glucosylceramide synthase (GCS) and sphingomyelin synthase in doxorubicin-resistant leukemia cells. These reports of changes in the cerebroside metabolism of multidrug-resistant cells were followed by studies showing that GCS influences cellular responses to chemotherapy. Thus, GCS is a likely target for manipulating chemoresponse.

The remaining sections of this chapter focus on ceramide metabolism through GCS and its relationship to chemotherapy resistance, with particular emphasis on a new concept: the connection between natural product chemotherapy, ceramide metabolism, and expression of P-gp, product of the *MDR1* gene. One of the most consistent alterations in drug resistance is overexpression of P-gp *(2)*. This membrane-resident protein, which has a mass of 170 kDa, functions as an energy-dependent pump that reduces the intracellular concentration of anticancer drugs, chiefly natural product agents including anthracyclines, *Vinca* alkaloids, and paclitaxel.

6. GCS AND CELLULAR RESPONSE TO CHEMOTHERAPY

Resistance of cancer cells to chemotherapy is associated not only with elevated levels of cerebrosides and gangliosides *(58,59,63–66)* but also with enhanced expression of GCS *(68,69)*. Interestingly, overexpression of GCS and MDR1 coincides in multidrug-resistant breast cancer, leukemia, melanoma, colon cancer, and epidermoid carcinoma

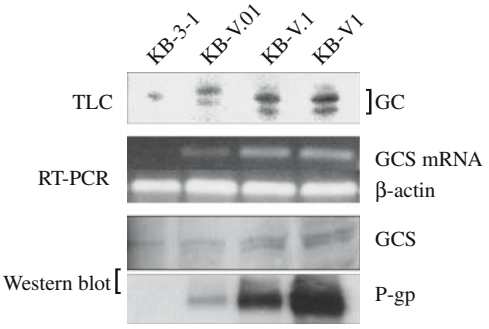

Fig. 2. Ceramide glycosylation and MDR1 expression increase in parallel under chemotherapy selection pressure. KB-3-1 and vinblastine-resistant sublines were analyzed for glucosylceramide (GC) content by thin-layer chromatography (TLC) and autoradiography, and GC synthase (GCS) and MDR1 expression by reverse transcriptase–polymerase chain reaction (RT–PCR) and Western blot. GC migrates as a doublet because of heterogeneity in chain length of the ceramide moiety. Reproduced with permission *(69)*.

(head/neck) cells *(69)*. This relationship was shown quite elegantly in the head/neck carcinoma cell line KB-3-1 and in vinblastine-resistant sublines KB-V.01, KB-V.1, and KB-V1, listed in order of increasing multidrug resistance (Fig. 2). As illustrated, glucosylceramide, GCS mRNA, GCS protein, and P-gp increased with increases in vinblastine selection pressure. This clearly shows that selection pressure for natural product drug resistance not only activates the *MDR1* gene *(2,70,71)* but also enhances ceramide metabolism through GCS. Kok et al. *(72)* have reported similar findings in HT29 colon cancer cells selected for colchicine resistance. In their study, increases in glucosylceramide coincided with upregulation of MRP1; however, GCS expression and activity were not upregulated in the colchicine-resistant model.

Altering GCS activity, either chemically or genetically, has been helpful in illustrating the impact of ceramide metabolism on cellular response to anticancer drugs. In breast cancer cells, for example, sensitivity to anthracyclines, *Vinca* alkaloids, and taxanes can be decreased or increased by manipulating GCS expression or activity *(61,68,73)*. Transfection of drug-sensitive MCF-7 breast cancer cells with GCS cDNA using a Tet-on expression system *(74)* conferred resistance to doxorubicin *(61)*. Resistance closely paralleled the level of expression of GCS, which could be manipulated by doxycycline addition *(61)*. Enforced overexpression of GCS also potentiates resistance to TNFα *(62)*, a tumor-killing cytokine that regulates apoptosis through ceramide generation from sphingomyelin *(75)*. Conversely, GCS antisense transfection of multidrug-resistant MCF-7-AdrR breast cancer cells knocks down high GCS levels and greatly increases cell sensitivity to doxorubicin, vinblastine, and paclitaxel *(68)*. Antisense GCS (asGCS) transfection also can decrease the growth rate of neuroepithelioma cells and reduce the tumorigenicity of melanoma cells *(76,77)*; recently antisense oligodeoxyribonucleotides to GCS have been used to enhance doxorubicin sensitivity in multidrug-resistant breast and ovarian cancer cells *(78)*.

These studies on the genetic manipulation of ceramide metabolism highlight the importance of GCS in regulation of cellular response to chemotherapy. This relationship has been reinforced by investigations that used chemical inhibitors of GCS, such as 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), which is a structural analog of the natural GCS substrate *(79)*. PPMP and related agents enhance sensitivity

to ceramide-generating anticancer agents (doxorubicin, paclitaxel, and vincristine) in multidrug-resistant breast cancer, neuroblastoma, and leukemia cells *(80–82)*. In neuroblastoma cells, D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) as a single agent caused only a transient elevation in ceramide; however, when PDMP was combined with paclitaxel, ceramide levels remained high *(81)*. In vincristine-resistant leukemia cells, the combination of D,L-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP) and vincristine raised ceramide levels above those observed with vincristine alone and enhanced apoptosis *(82)*. Nicholson et al. *(83)* have demonstrated that PPMP and PPPP preferentially kill drug-resistant variants of KB-3-1 cells; this suggests that manipulation of glycolipid levels could be key in targeting drug resistance.

We have shown that the cytotoxicity of 4-HPR, a potent ceramide generator *(34,35)*, can be enhanced in 10 different tumor cell lines by the addition of PPMP and other modulators of ceramide metabolism *(84)*. Interestingly, a combination of three inhibitors of sphingolipid metabolism elevates ceramide levels and reverses resistance to radiation in squamous cell carcinoma *(85)*, suggesting that the manipulation of ceramide metabolism may offer new opportunities for overcoming radioresistant cancer. Kolesnick and Fuks *(86)* review radiation- and ceramide-induced cell death.

Results with a few GCS inhibitors such as the imido sugar derivative *N*-nonyldeoxygalactonojirimycin, used at millimolar concentrations *(87)*, and a few cancer cell types such as GM95 mouse melanoma *(88)* do not support the targeting of ceramide metabolism to enhance radiotherapy and chemotherapy. However, most reports demonstrate that the sequestering of ceramide by GCS leads to chemotherapy resistance; conversely, inhibiting glycosylation of ceramide potentiates downstream cell death programs. The following section examines mechanisms by which ceramide might promote drug resistance.

7. INFLUENCE OF CERAMIDE ON MULTIDRUG RESISTANCE

The ability of chemotherapeutic agents to activate ceramide formation through de novo and sphingomyelinase pathways has been well documented, as has the ability of ceramide to promote apoptosis. However, the influence of ceramide on multidrug resistance is far from understood. Recent reports show that ceramide influences gene expression. For example, ceramide upregulates sphingomyelinase *(89)*, p21 *(90)*, and cyclooxygenase-2 expression *(91)* and downregulates glutathione *S*-transferase expression *(92)*. Ceramide also enhances GCS expression in cultured cells *(93,94)*, prompting Shayman and colleagues *(93)* to speculate that exogenous sphingomyelinase and other agents that produce large increases in ceramide also induce elevations in GCS, because of increased availability of the enzyme's lipoidal substrate. Because some anticancer agents are potent ceramide generators, we examined whether these drugs affected GCS expression.

Doxorubicin activates ceramide production in a number of systems *(29)*. We have recently shown that treatment of breast cancer cells with either C_6-ceramide or doxorubicin increases GCS expression (Liu et al., manuscript submitted for publication). This indicates that levels of GCS message can be enhanced by ceramide, the GCS substrate, and by chemotherapeutic agents that generate ceramide. We also evaluated breast cancer cells transiently transfected with GCS promoter pGCS-Luc1 *(95)*. GCS

promoter activity increased approximately fourfold in cells exposed to C_6-ceramide and twofold in cells exposed to doxorubicin, but it remained unchanged in cells exposed to C_6-dihydroceramide, the saturated precursor of ceramide *(96)*. These data suggest that ceramide's influence on the expression of GCS might drive cellular resistance to ceramide-generating anticancer agents.

8. GCS AND THE *MDR1* PHENOTYPE

Studies using asGCS and other GCS blockers indicate that ceramide glycosylation influences chemosensitivity by affecting both ceramide residence time and P-gp function *(68,73,76,77,97,98)*. We reported that the use of asGCS to stably transfect multidrug-resistant MCF-7-AdrR cells, which are rich in GCS and P-gp, decreased GCS expression, enhanced chemotherapy uptake, and greatly diminished the expression of *MDR1*, based on mRNA and P-gp protein *(98)*. These data suggest an association between GCS and glycolipids and *MDR1* expression. Ladisch et al. *(77)* found that asGCS transfection of melanoma cells reduced cellular glycolipids levels, GM3 ganglioside in particular, and inhibited melanoma formation in mice. In our model, asGCS transfection caused a fourfold reduction in ganglioside levels of MCF-7-AdrR cells *(98)*. To determine whether the loss of *MDR1* was a clonal artifact, we treated MCF-7-AdrR cells for prolonged periods with either PPMP or tamoxifen, specific and nonspecific inhibitors of GCS, respectively *(79,80,99)*: both agents produced striking decreases in *MDR1* expression (Fig. 3). With tamoxifen, the reduction was time-dependent; by 72 h, mRNA levels were minimal. The high levels of P-gp in MCF-7-AdrR cells are much greater than those that are clinically relevant *(100)*. For this reason perhaps, and because of slow protein turnover, treatment of MCF-7-AdrR cells with PPMP did not significantly reduce P-gp levels. However, we showed a 50% reduction in P-gp levels of multidrug-resistant KB-V0.01 cells treated for 7 days with PPMP (unpublished data, Cabot laboratory).

Use of the PDMP to reduce glucosylceramide levels in human leukemia cells resulted in elevated ceramide levels and in increased rhodamine-123 uptake *(97)*. Similarly, in doxorubicin-resistant ovarian cancer cells, PPMP markedly enhanced rhodamine-123 uptake and also inhibited *MDR1* expression at the mRNA level *(101)*. Verapamil, an agent long known to enhance cell sensitivity to chemotherapy *(102)*, decreased *MDR1* gene transcription in multidrug-resistant leukemia cells *(103)*. We have reported that several of the classical P-gp blocking agents as well block glycolipid metabolism *(80,99)*. In a study of vincristine-resistant epidermoid carcinoma cells, Zhang and

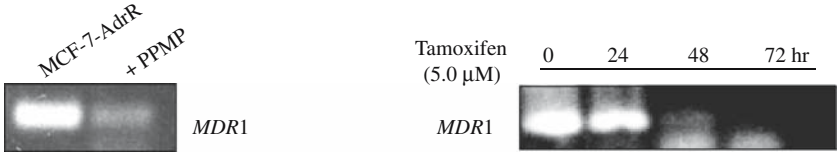

Fig. 3. Influence of glucosylceramide synthase (GCS) blocking agents, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and tamoxifen, on MDR1 expression in multidrug-resistant human breast cancer cells. Cultured MCF-7-AdrR cells were exposed to either D-*threo* PPMP (5.0 μM) for 48 h or tamoxifen (5.0 μM) for times shown. MDR1 mRNA was evaluated by reverse transcriptase–polymerase chain reaction (RT–PCR).

coworkers *(104)* found that expression of GCS was associated with multidrug resistance and that inhibition of GCS with PPMP and verapamil reduced *MDR1* gene expression. Therefore, whether by antisense downregulation or chemical inhibition, these works show that targeting GCS influences *MDR1* expression and draw a curious parallel between drug-resistance modifiers, glycosphingolipids, and the multidrug-resistant phenotype.

9. CONCLUSIONS

Although numerous studies have demonstrated that GCS influences cellular response to anticancer agents, the mechanisms remain uncertain. Most studies on ceramide, chemotherapy, and drug resistance emphasize circumvention of apoptosis by GCS-mediated sequestering of ceramide (Fig. 4, top). Chemotherapy enhances ceramide formation; increased levels of ceramide lead to apoptosis and/or enhanced expression of GCS. GCS is a scavenger of ceramide; high levels of GCS therefore can counteract apoptosis by sequestration of ceramide in the form of glucosylceramide. Chemoresistance can be circumvented in some systems by inclusion of GCS blocking agents.

GCS appears to have dual roles in multidrug resistance. It dulls the cytotoxic response to ceramide-generating chemotherapeutics, and it may also enhance expression of the *MDR1* phenotype. As shown in Fig. 4, we propose that GCS upregulates MDR1 through either cerebroside-driven (glucosylceramide) or ganglioside-driven pathways; upregulation of MDR1 then leads to increased production of P-gp. Overall, the data suggest that the acquisition of multidrug resistance is associated, in part, with changes in cellular glycolipids. A functional role for gangliosides in multidrug resistance has recently been noted *(97)*, and other works have proposed that drug sensitivity and metastatic potential are linked to ganglioside metabolism *(83–109)*. We already know that chemotherapeutic agents can directly activate the *MDR1* gene *(70,71)*; Fig. 4 introduces another pathway that emphasizes GCS as a potential upstream target for downstream drug resistance.

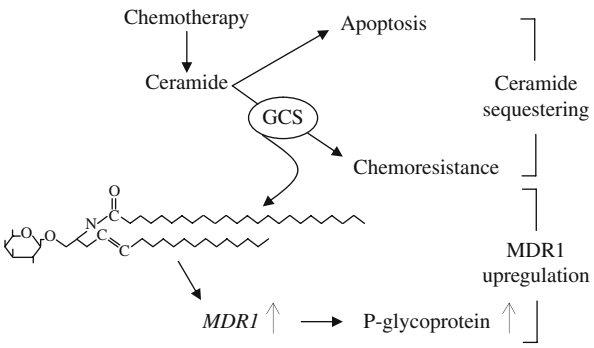

Fig. 4. Suggested mechanisms for the impact of glucosylceramide synthase (GCS) on cell response to chemotherapy. Chemotherapy enhances formation of ceramide (Fig. 1), which signals events leading to apoptosis and/or upregulates expression of "its enzyme," GCS. The latter step can produce chemoresistance through ceramide sequestering in the form of glucosylceramide (molecular structure shown). Glucosylceramide and/or other cerebrosides and gangliosides upregulate MDR1 expression leading to production of P-glycoprotein.

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17 Chemo- and Radiosensitization Through Inhibition of PI3K/Akt Signaling

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Summary

Tumorigenesis and tumor progression are the consequences of disturbed balance between cell proliferation, differentiation, and programmed cell death. Additional processes including loss of cellular polarity, increased motility, and invasiveness lead to metastases resulting in spread of the tumor beyond the original site and account for the majority of morbidity and mortality from cancer. The phosphatidylinositol 3-kinase (PI3K) pathway, composed of multiple intracellular signaling proteins, controls various pivotal cellular functions related to cancer biology including cell proliferation, cell survival, migration, and vascularization. Major components of this pathway, including PI3K isoforms, *p*hosphatase and *ten*sin homolog (PTEN), phosphoinositide-dependent kinase 1 (PDK1), Akt, and mTOR, are frequently dysregulated by mutation, amplification, or rearrangement in multiple cancer lineages. These genetic aberrations render cancer cells sensitive to the inhibition of PI3K pathway at various levels. Targeting the PI3K/Akt pathway demonstrates marked efficacy in preclinical models. The PI3K signaling pathway has emerged as a high-quality therapeutic target in cancer treatment.

Key Words: Phosphatidylinositol 3-kinase; Akt; signal transduction; molecular-targeted therapy; chemosensitization; radiosensitization.

1. INTRODUCTION

Phosphatidylinositol 3-kinases (PI3Ks) are a group of lipid and protein kinases, catalyzing phosphorylation of membrane phosphatidylinositols (PtdIns) at the D3 position of the inositol ring. The resulting products of PI3K, D3-phosphoinositols

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PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 play a critical role in the regulation of cellular signaling networks *(1)*. There are multiple PI3K isoforms present in the mammalian genome. On the basis of their *in vitro* substrate selectivity, these isoforms can be divided into four classes *(2,3)*. Class I PI3Ks efficiently phosphorylate PtdIns(4)P and PtdIns(4,5)P_2 *in vitro*; however, the latter may present as a preferable substrate *in vivo*. Class I PI3Ks are heterodimers composed of a p110 kDa catalytic subunit and an adaptor/regulatory subunit. The Class I PI3Ks are further categorized into two subclasses, classes IA and IB, based on structural and functional differences. Class IA consists of three p110 catalytic isoforms, p110 α, β, and δ, constitutively associated with a regulatory subunit, which can be p85α, β, p55α, p50α, or p55γ. The regulatory subunits of Class IA contain two Src-homology 2 (SH2) domains and one SH3 domain. The SH2 domains bind to specific phosphorylated tyrosine residues in receptor proteins and in other signaling molecules. SH3 domains mediate protein–protein interaction through proline-rich sequences. Class IA PI3K isoforms transduce signals from tyrosine kinase-coupled receptors, including those with intrinsic tyrosine kinase activities. Class IB PI3K, of which the sole member is p110γ, associates with a p101 regulatory subunit. p110γ can be activated *in vitro* by the βγ subunits of heterotrimeric G proteins. Therefore, Class IB PI3K primarily transduces signals from G-protein-coupled receptors, although these receptors can also activate Class IA PI3K through recruitment and activation of intracellular tyrosine kinases, particularly Src. Class IB PI3K also transduces signals of Rac, Rho, and cdc42 small GTPases.

Class II PI3Ks exclusively phosphorylate PtdIns and PtdIns(4)P *in vitro*. Class II isoforms, PI3K-C2 α, β, and γ, are larger molecules (~200 kDa) whose defining feature consists of a C-terminus C2 domain with no adaptor/regulatory subunits. The C2 domain binds to phospholipids *in vitro* in a Ca^{2+}-independent manner. Class II PI3Ks are predominantly associated with the cellular membrane fraction and can be activated by tyrosine kinase-coupled receptors, such as insulin receptor (INR), epithelial growth factor receptor (EGFR), or platelet-derived growth factor receptor (PDGFR). However, the activation mechanisms, the *in vivo* substrates, and the cellular functions of Class II PI3Ks remain to be fully elucidated.

Class III PI3K, a constitutively active enzyme that phosphorylates only PtdIns as a substrate *in vitro*, is likely responsible for the generation of the majority of PtdIns(3)P in resting cells. A single Class III PI3K catalytic subunit has been identified in all eukaryotic species, which is a homolog of Vps34P in yeast and of Age 1 in *Caenorhabditis elegans*. Class III PI3K regulates the trafficking of proteins through lysosomes or vacuoles. Class III PI3K has recently been implicated in the regulation of autophagy, an evolutionarily conserved process whereby cells utilize intracellular energy sources to survive during environmental stress. Prolonged autophagy, however, results in programmed cell death. In contrast to apoptosis where a role in chemotherapy and radiation therapy-induced cell death is well documented, the role of autophagy in therapy-induced death is under investigation. Our recent data indicate that both entry into and survival during autophagy are highly regulated processes with multiple mediators in addition to cellular responses to stress.

Class IV PI3Ks are composed of lipid and protein kinases whose catalytic domains closely resemble those of other PI3Ks. Among these enzymes are PI4K, phosphoinositide phosphate (PIP) kinase, DNA-dependent protein kinase (DNA-PK), ataxia teleangiectasia mutated (ATM), ataxia teleangiectasia related (ATR), and mTOR. Class

IV PI3Ks play an important role in the regulation of the phosphoinositide cycle, DNA repair, and protein synthesis. Furthermore, in contrast to Classes I–III PI3Ks, which are primarily lipid kinases that can phosphorylate proteins in close physical complexes, several members of the Class IV PI3Ks such as DNA-PK, ATM, ATR, and mTOR are exclusively protein kinases.

2. PI3K/AKT SIGNALING IN CELL BIOLOGY

PI3K signaling mechanisms and their functional processes have been most extensively studied for Class IA PI3Ks. Under resting conditions, p85 regulatory subunits bind and inhibit p110 catalytic activity while stabilizing p110. This creates a pool of p110 poised to be activated by removal of the inhibitory constraints of p85. When a GFR is triggered by its ligand, such as insulin, EGF, or PDGF, the receptor kinase is activated, causing a rapid increase in tyrosine phosphorylation on the receptor or other intracellular proteins. PI3Ks are recruited to the activation site through an interaction of the SH2 domain in PI3K regulatory subunits with tyrosine-phosphorylated residues. This interaction relieves the inhibitory effect of the p85 subunit on p110 catalytic activity resulting in the accumulation of PI3K enzymatic products PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 at the cellular membrane *(4,5)*. The action of PI3K is directly opposed by the tumor suppressor *p*hosphatase and *ten*sin homolog (PTEN, also known as MMAC1 and TEP). PTEN encodes a dual specificity phosphatase capable of dephosphorylating PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 at the D3 position. Therefore, PTEN functions as the key negative regulator of PI3K signaling *(6–10)*. Two other phosphatases, SHIP1 and SHIP2, dephosphorylate the 5′ site of the inositol ring generating PtdIns(3,4)P_2, which regulates a separate subset of downstream signaling molecules.

Accumulated D3 phosphatidylinositols at the cellular plasma membrane bind to a subset of pleckstrin homology (PH) domains and recruit PH domain-containing signaling molecules to the activation nidus. A smaller subset of FYVE, Phox (PX), C1, C2, and other lipid-binding domains also bind D3 phosphatidylinositols. Among PH domain-containing proteins, the serine/threonine kinases Akt (also called protein kinase B, PKB) and phosphoinositide-dependent kinase 1 (PDK1) are of particular interest in transducing PI3K signaling as they have been demonstrated to be the primary downstream mediators of D3 phosphatidylinositols by genetic screens in the model organisms *C. elegans* and *Drosophila melanogaster*. PDK1 possesses an N-terminal catalytic domain and a C-terminal PH domain. Binding to D3 phosphoinositides enables PDK1 to colocalize with its cellular substrates at the membrane, such as Akt, and other AGC family protein kinases including PKA, p70S6K, p90RSK, and PKC. In this regard, PDK1 is an important mediator of PI3K signaling *(2,11)*.

Akt isoforms (Akt1, Akt2, and Akt3 in the human proteome) are composed of an N-terminal PH domain, a central kinase catalytic domain, and a C-terminal hydrophobic regulatory domain. Binding of the PH domain to membrane phosphoinositides not only brings Akt into the proximity of PDK1 but also triggers a conformational change in Akt, allowing PDK1 access to the catalytic loop of Akt. PDK1 phosphorylates Akt on its Thr308 activation site, and further phosphorylation on Ser473, in the C-terminal regulatory domain, is required for full activation of Akt *(2,12,13)*. The identity of the kinase (PDK-2) that phosphorylates Ser473 has been elusive, although integrin-linked

kinase (ILK), Akt itself, PKCβ II, and PKCα have been proposed as possible kinases. Recently, the mTOR–rictor complex, but not the mTOR–raptor complex, has been demonstrated as a kinase phosphorylating Akt at Ser473 *(14)*.

The biological consequences of Akt activation in cancer are significant and contribute directly to the survival, proliferation (an increase in cell number), and growth (an increase in size) of these cells. In fact, a role for Akt has been well established in nearly all the collective changes that occur genetically/epigenetically in cells during tumorigenesis, called the six "Hallmarks of Cancer" outlined by Hanahan and Weinberg *(15,16)*. The gain of unlimited replicative ability of tumor cells has been suggested to occur through enhancement of telomerase activity and putative phosphorylation of hTERT by Akt *(17)*. PI3K and Akt also regulate small G proteins contributing to changes in cellular polarity, motility, and invasiveness. Akt has been implicated in binding to intracellular SMADs relieving the growth inhibitory effects of TGFβ.

Akt delivers a broad survival signal through phosphorylation of multiple downstream targets including transcription factors and signaling proteins (Fig. 1). Phosphorylation of Forkhead transcription factors (AFX, FKHR, and FKHRL1) by Akt results in their cytoplasmic retention through interaction with 14-3-3 family proteins, which blocks the transcription of proapoptotic genes including *FasL* and *Bim (18,19)*. Additionally, Akt phosphorylates cyclic AMP-response element-binding protein (CREB), which increases

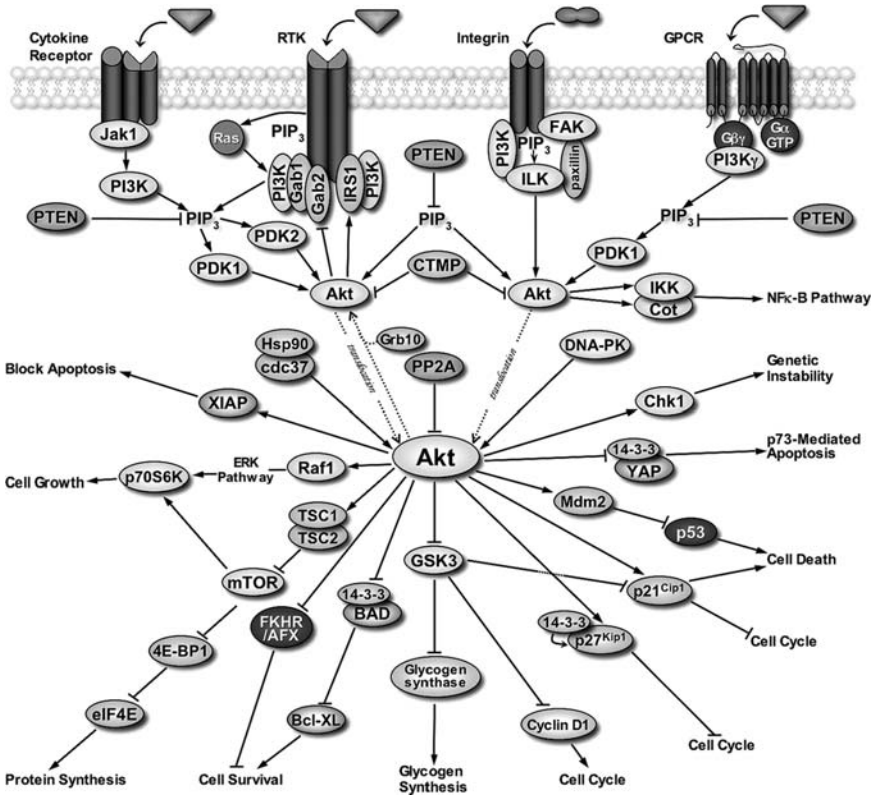

Fig. 1. The PI3K/Akt signaling pathway. *Adapted by L. Nolden from http://www.cellsignal.com Product Pathways*.

the transcription of antiapoptotic genes such as *Bcl-2* and *Akt* itself *(20–22)*. Phosphorylation of I-κB kinase by Akt causes degradation of I-κB, promotes nuclear translocation and activation of NF-κB, and induces transcription of prosurvival genes including *Bcl-XL (23–25)*, caspase inhibitors, and c-Myb *(26)*. Akt phosphorylates the ubiquitin ligase MDM2 and promotes its nuclear translocation thereby destabilizing p53. The loss of p53 alters DNA damage and stress-induced cell death allowing replication and survival of cells with DNA aberrations, thus potentially contributing to tumorigenesis *(27,28)*. Akt directly phosphorylates the BCL2 family member, pro-apoptotic protein BAD (at Ser136), and promotes its association with 14-3-3 proteins, releasing BAD from BCL2 or BCL-XL complexes thus increasing cell survival *(29)*. Similarly, Akt phosphorylates apoptosis signal-regulating kinase-1 (ASK-1) and reduces its ability to regulate stress- and cytokine-induced cell death *(30,31)*. Taken together, the activation of Akt downregulates the expression or activity of multiple pro-apoptotic proteins, whereas it upregulates those of antiapoptotic proteins.

Akt promotes cell-cycle progression through its deregulation of cell-cycle checkpoint controls, particularly at the G1/S transition. Akt phosphorylates cyclin-dependent kinase inhibitors $p21^{Cip1/WAF1}$ (on Thr45) and $p27^{Kip1}$ (on Thr157) inducing the cytoplasmic retention of these molecules and preventing their interaction with nuclear effectors, conferring a proliferative advantage *(32)*. In addition, Akt phosphorylates GSK3 − α (on Ser21) and GSK3 − β (on Ser9), thereby decreasing their respective activities. Inactivation of GSK3 by Akt enhances glycogen synthesis, a key step in tumor development. Inhibition of GSK3 also stabilizes cyclin D1 and Myc, two proteins that drive S-phase entry. Furthermore, phosphorylation and cytoplasmic retention of Forkhead receptor transcription factor releases the repression of cyclin D1 expression, contributing to the overall upregulation of cyclin D1. Tumor cell growth (size), which is required for cellular division, occurs through mTOR leading to increased protein synthesis. Recent studies have revealed that Akt activity also regulates cell-cycle transition through G2/M. Increased Akt activity is present during the G2/M phase in epithelial cells. Akt promotes G2/M cell-cycle progression by inducing phosphorylation-dependent 14-3-3θ binding and cytoplasmic localization of WEE1Hu, thus preventing inhibitory phosphorylation of cdc2 *(33)*. In addition, phosphorylation of Chk1 peptide by Akt indicates the existence of crosstalk between the PI3K/Akt pathway and key regulators of the DNA damage checkpoint machinery *(34)*.

mTOR (also known as FRAP1 or RAFT1), a central regulator of cell growth, belongs to Class IV of the PI3K family and also function as a major downstream effector of Akt *(35)*. Its Ser2448 residue can be directly phosphorylated and activated by Akt *(36–38)*. Recent studies reveal that the tuberous sclerosis complex (TSC)-1/2, PTEN, and LKB1 tumor suppressor proteins also modulate mTOR activity *(39,40)*. Intriguingly, mutational inactivation of TSC, PTEN, and LKB1 results in a series of hamartoma syndromes, tuberous sclerosis, Cowden's syndrome, and Peutz-Jaegers' syndrome, characterized by an increased propensity to develop tumors in particular cell lineages. These experiments of nature provide genetic evidence that these proteins regulate a similar set of biochemical and functional processes. TSC2 contains multiple potential Akt phosphorylation sites. Akt phosphorylates and inhibits TSC, which subsequently derepresses the small GTP-binding protein Rheb leading to the activation of mTOR. In the contrasting situation of energy depletion, changes in the intracellular ATP/AMP ratio induce the activation of AMP-activated protein kinase (AMPK),

a process largely dependent on the activity of the upstream kinase LKB1. AMPK phosphorylation activates TSC1/2, thereby decreasing mTOR function downstream. mTOR regulates protein synthesis by activating the p70 ribosomal S6 kinase (p70S6K) and inhibiting the elongation-initiation factor 4E binding protein-1 (4E-BP1). Activated p70S6K phosphorylates S6 and enhances translation initiation of mRNA that has 5′-terminal oligopyrimidine tracts. 4E-BP1 acts as a translational repressor of mRNA that bears a 5′ CAP structure and inhibits, through direct binding, the function of elongation factor eIF4E. Phosphorylation of 4E-BP1 by mTOR decreases the association of 4E-BP1 with eIF4E, thus initiating mRNA translation. Protein synthesis is a process, in large part, controlling cell growth and aberrations in protein synthesis contribute to the high growth rate of cancer cells *(41,42)*.

3. DYSREGULATION OF PI3K/AKT SIGNALING IN CANCER

Deregulation of the PI3K/Akt pathway can confer a number of the functional capabilities required for cells to undergo tumorigenesis. As discussed in Section 2, activation of Akt promotes cellular survival, stimulates nutrient uptake and glycolysis, and leads to substantive growth and proliferation of the cell. Its phosphorylation of downstream effectors reduces transcription of proapoptotic genes while promoting the transcription of prosurvival factors, rendering the cell insensitive to normal apoptotic and senescent program. Furthermore, the PI3K/Akt pathway regulates cellular motility, production of proteases, as well as proangiogenic factors leading to the development of tumor vasculature.

The importance of Akt as a major hub and interchange between numerous signaling cascades positions it and its component pathway members as a prime group of molecular targets in cancers where this pathway is overactive. Interestingly, Akt itself is amplified in only a small group of cancers, and only rare mutations in the *Akt* gene have been reported to date. Hyperactivity through this network appears to occur, rather, through sustained activation of growth factor RTKs, mutational activation of upstream regulators such as Ras, loss of the primary negative regulator PTEN, or through mutation, overexpression, rearrangement, and subsequent constitutive activation of both the catalytic and the regulatory subunits of PI3K.

3.1. Alterations Upstream of PI3K/Akt in Cancer

Overexpression and constitutive upregulation of cell surface receptors in various cancers leads to ligand-dependent stimulation of receptor tyrosine kinases, G-protein-coupled receptors, and cytokine and integrin receptors, subsequently increasing downstream signaling through PI3K/Akt *(12,43)*. This sustained activation through upstream effectors results in hyperactivated signaling through PI3K and has been shown to contribute to the pathophysiology of a number of malignancies.

A well-examined case in point is that of the erbB2 (Her2/*neu*) tyrosine kinase receptor *(44–48)*. ErbB2 homodimerizes and heterodimerizes with other members of the human EGFR (HER/EGFR) family to elicit a broad spectrum of downstream signaling effects dictating cell growth, antiapoptosis, and cell invasion *(44)*. ErbB2 is overexpressed as a result of gene amplification in various cancers, including breast *(45)*. Overexpressed erbB2 has been shown to be constitutively associated with erbB3 in primary breast tumors, breast cancer cell lines, and transgenic mouse models, and

these heterodimers strongly activate the PI3K/Akt pathway *(46,47)*. This powerful stimulation occurs through the ability of erbB3, through seven tyrosine residues, to bind the SH2 domains of the PI3K regulatory subunit p85 thus enabling the survival and proliferation of erbB2-overexpressing tumor cells *(48)*.

Activating mutations in Ras, an upstream regulator of PI3K, occur in nearly a quarter of all human malignancies and lead to receptor-independent, constitutive upregulation of the PI3K/Akt pathway *(15,49,50)*. Ras directly binds and activates the catalytic subunit of PI3K, p110, and although Ras signaling through other cascades, such as mitogen-activated protein kinase (MAPK), has been implicated in tumor cell resistance to radiotherapy and chemotherapy, PI3K has been identified as the key downstream mediator of Ras-induced resistance *(51)*.

3.2. Alterations of PI3K, Akt, and PTEN in Cancer

Evidence of the significant contribution of aberrant PI3K/Akt signaling to tumorigenesis can be inferred from the sheer number of diverse malignancies with mutations, amplifications, deletions, and/or loss of signaling of PI3K pathway components (reviewed in refs *1* and *52*). The PI3K/Akt signaling cascade has been implicated in as many as 30% of all human malignancies *(53–57)*. Activation of PI3K/Akt in ovarian cancer, for example, has been shown to be as high as 60–70% in samples directly isolated from patients *(58)*.

3.2.1. PTEN

Loss of the PTEN tumor suppressor can occur through mutation *(59,60)*, deletion *(61)*, epigenetic silencing (promoter methylation) *(62)*, transcriptional silencing *(63)*, or protein instability *(62)*. Mutation of PTEN is one of the most prevalent events in most human cancers, rivaling the mutational frequency of p53 or Rb *(64)*. Loss of PTEN leads to hyperactivation of the PI3K/Akt pathway, drives inappropriate proliferation, and confers resistance to apoptosis in tumor cells *(65,66)*. Germline mutations in PTEN give rise to autosomal dominant hamartomatous tumor syndromes such as Cowden's disease and Bannayan–Riley–Ruvalcaba syndrome *(67,68)*. Constitutive activation of Akt resulting from loss-of-function mutations in PTEN is common in sporadic glioblastoma *(69,70)*, prostate cancer *(71–73)*, melanoma *(74)*, and endometrial carcinoma *(75)*. Additionally, PTEN mutations contribute to a small portion of lung cancer *(76)*, breast cancer *(9,10,61)*, and lymphomas *(77)*.

3.2.2. PI3K Subunits

Overactivation of PI3K in human cancer has been shown to occur through mutations in the p110 and p85 subunits, rearrangement of p85, as well as overexpression through genetic amplification *(56,78–80)*. Somatic mutations in *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K, are prevalent in a high percentage of tumors, such as breast (40%) *(54,55,81)*, colorectal cancers (CRCs) (32%) *(56)*, glioblastoma (27%) *(70)*, and others *(53,82,83)*, and likely contribute directly to tumor progression *(55)*. Study in colorectal tumors showed that mutations of *PIK3CA* were primarily found in high-grade, advanced tumors, indicating that these mutations may be important in progression to aggressive and/or invasive

phenotypes. When three of the most frequent mutations were individually introduced in chicken embryo fibroblasts, the resulting mutant p110α, but not wild-type p110α, was sufficient to cause oncogenic transformation, constitutively activate Akt and downstream pathway signaling, and increase phosphorylation of p70S6K and 4E-BP1 *(84)*. Additionally, activating mutations in *PIK3R1*, encoding the p85 subunit, have been observed in ovarian and colon carcinomas, and rearrangements have been identified in lymphomas *(78)*.

The *PIK3CA* gene is located on chromosome 3q26, a region that is frequently amplified in human tumors including breast (27%) *(85)*, ovarian (40%) *(86)*, and cervical (50%) *(87)* carcinomas. Amplification of this region occurs frequently in low-grade and low-stage ovarian tumors and is likely indicative of key early events in the development of this disease *(79,80)*. In lung cancer, specific study of the PIK3CA locus during progression revealed its amplification in high-grade, but not low-grade, preinvasive lesions *(88)*. In both cases, gain of PI3K function through amplification of this region is thought to imply a commitment to tumor progression.

3.2.3. Akt

Rare mutations in the *Akt* gene have only recently been described, but amplification of Akt in human malignancies is a known event leading to hyperactivation of downstream signaling. The family is composed of three isoforms: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ *(66)*; each Akt is derived from distinct genes and amplified in various tumors, with each isoform appearing to have selective effects in different types of cancer. Akt1 is rarely amplified, reported only in glioblastoma and gastric carcinomas *(70,89)*, but overactivation of Akt1 has been observed in a high percentage of prostate, breast, and ovarian carcinomas, and this increase in Akt1 kinase activity is associated with poor prognosis *(90)*. Amplification of the 19q13 chromosomal region, which includes Akt2, occurs in approximately 25% of ovarian cancers *(58,91–93)*, particularly high-grade aggressive tumors *(94)*, and is also amplified in breast and pancreatic tumors (10%) *(91,94,95)*. Somatic mutation of Akt2 has been reported in breast and pancreatic carcinomas *(85)*. Interestingly, the Akt2 kinase is activated in nearly 40% of ovarian tumors *(58)*. Overexpression of Akt2 in NIH 3T3 cells is oncogenic *(96)*, and breast cancer cells expressing Akt2 exhibit increased metastatic potential *in vivo (97)*. Likewise, Akt3 is suggested to contribute to the aggressive clinical phenotype of estrogen-negative breast and androgen-insensitive prostate cancers as both Akt3 mRNA levels are elevated and enzymatic capacity is increased in these cell types *(98)*. Genetic amplification of *Akt3* has not been reported. The three Akt isoforms are amplified with comparatively less frequency than other pathway components, and constitutive upstream signaling is more frequently causative of the hyperactive state of Akt observed in malignancies.

3.3. Downstream Alterations

Alterations of downstream targets of Akt in cancer are less thoroughly described to date. Loss of heterozygosity (LOH) and subsequent mutation of the tumor suppressor TSC1 contributes to sporadic tumor formation in the bladder *(99)*. Amplification of *RPS6KB1 (p70S6K)* has been reported in breast cancer (~ 30%), but it is unclear whether these increases occur independently or as a consequence of the frequent

17q22-24 chromosomal amplification (particularly in BRCA1/2-associated tumors), where the neighboring *ERBB2* could be the driver of the amplicon *(100)*. Whatever the mechanism, amplification is associated with overexpression of p70S6K in these tumors. As patient tumors are better characterized with increasing resolution at the DNA, RNA, and protein level, our understanding of the number of genetic aberrations and the complexity of changes that can lead to dysregulation of this pathway in various cancers will expand. Additionally, aberrations in unidentified components of the pathway and upstream/downstream pathway crosstalk have yet to be elucidated.

3.4. Activation of PI3K/Akt Pathway and Correlation to Therapy Resistance

Refractoriness to anticancer therapy is often due to failure of the apoptotic program, gain of proliferative capacity, growth and decreased sensitivity to antigrowth signals in tumor cells, all events that can be mediated through the activation of Akt and its downstream substrates. Akt activity, as reflected by phosphorylation, is linked to poor disease prognosis in many cancer lineages *(101–105)*, and several studies have examined the contribution of the PI3K/Akt pathway toward targeted/radio/chemotherapeutic tumor resistance *(106–108)*.

In breast cancer, loss of PTEN in patients with erbB2-expressing tumors predicts resistance to trastuzumab (Herceptin), an erbB2-targeting antibody *(109)*. Combinatorial therapeutics such as trastuzumab plus paclitaxel overcome this resistance through a more effective inhibition of Akt phosphorylation and pathway activation *(110)*. In one-third of patients with estrogen receptor α (ERα)-positive tumors, de novo or acquired resistance develops and resistance to the standard adjuvant therapy tamoxifen is predicted by the activation of Akt *(111)*. This has led to an ongoing phase III trial of inhibition of mTOR (the only target with validated drugs currently available) and inhibition of estrogen signaling. Models of ERα-positive tumors overexpressing erbB2 *in vivo* revealed that tamoxifen treatment led to growth stimulation and de novo resistance, in part, through activation of the Akt pathway. In MCF7 breast adenocarcinoma cell lines, overexpression of erbB2 in the presence of erbB3 led to a PI3K-dependent increase in Akt phosphorylation and conferred increased chemotherapeutic resistance against multiple agents including doxorubicin, paclitaxel, 5-FU, etoposide, and camptothecin *(112)*. Studies of radioresistance in breast cancer cells have also uncovered a causal role for PI3K/Akt, and combination with a PI3K inhibitor, *in vitro*, led to sensitization of cells to radiation treatment *(113)*.

Non-small cell lung cancer (NSCLC) cell lines with high levels of PI3K/Akt activity have shown increased cellular resistance to cisplatin, paclitaxel, VP16, and ionizing radiation *(106)*. Combination of a specific PI3K inhibitor potentiates both chemo- and radiotherapy in resistant NSCLC *(106)*. mTOR, an effector downstream of Akt (Fig. 1), is targeted by rapamycin (Sirolimus) and its derivatives, which are currently in clinical trials. Indeed, these are the only selective PI3K pathway inhibitors in current clinical trials. Resistance to rapamycin was studied *in vitro* in NSCLC and attributed to PI3K/Akt stimulation. Despite inhibition of mTOR signaling by rapamycin, a negative-feedback mechanism leads to the increase in phosphorylation of both Akt and eIF4E, and this resistance is attenuated through combination with a PI3K inhibitor *(114)*. Resistance to gefitinib (Iressa) in advanced NSCLC and imantinib mesylate (Gleevec) in SCLC have also been attributed to activation of Akt or loss of PTEN expression (in the case of gefitinib) *(115,116)*. Inhibition of the PI3K/Akt pathway through the

use of inhibitors sensitizes both NSCLC and SCLC to traditional chemotherapeutic agents *(106,117)*.

Increased signaling and activation of Akt, either before or after treatment, may indicate resistance to therapy. Paradoxically, however, high Akt activity is associated with increased patient responses to gefitinib in lung cancer likely as a surrogate for the presence of activating mutations in the EGFR targeted by gefitinib. Broadly combining PI3K/Akt inhibitors with standard therapeutics to synergize treatment effects and/or overcome issues of treatment resistance has also been suggested in ovarian *(118,119)*, prostate *(120,121)*, bladder *(49,122)*, colon carcinomas *(121)*, as well as glioma *(123)*, among others *(124)*. Possible mechanisms for synergistic effects include enhancement of apoptosis and attenuation of PI3K-like kinases DNA-PK, ATM, and ATR (activated by DNA damage/radiation) by agents that target the PI3K kinase family. However, it is important to note that many of these studies are based on the actions of LY294002 and wortmannin, which inhibit multiple members of the PI3K kinase family with high efficiency. Inhibition at the level of Akt compared with downstream inhibition (e.g., mTOR) confers a theoretical advantage because of the bulk of the downstream network branches that would be targeted (Fig. 1); thus, the PI3K/Akt signaling network may be more extensively and effectively inhibited when the pathway is targeted at the level of PI3K or Akt.

4. CANCER THERAPEUTICS TARGETING PI3K/AKT SIGNALING

Overactivation of the PI3K/Akt pathway is frequently a major driver of tumor progression. In addition, the PI3K/Akt pathway contributes to the development of resistance to chemotherapy and radiation treatment *(121,125,126)*. Activation of the PI3K/Akt pathway in neoplastic tissues, resulting from a genetic aberration of the cancer cells or resulting from DNA damage or other mechanisms of activation induced by radiation and/or chemotherapy treatments, mediates survival and results in resistance to radiation and chemotherapy treatments. As tumors with overactive PI3K/Akt signaling are often rendered resistant to apoptosis, inhibition of this pathway may be necessary to induce tumor regression in response to chemotherapy and/or radiotherapy.

Small-molecule drugs have been developed and are in development to target the PI3K/Akt pathway at every level, including upstream of PI3K at the level of GFRs and downstream at the level of Akt targets.

4.1. Inhibition of PI3K

Several cancer models have indicated that inhibition of PI3K increases response to chemotherapy and/or radiation.

Wortmannin, a small-molecule PI3K inhibitor not used clinically because of its insolubility in aqueous conditions and low stability, has demonstrated efficacy in sensitizing multiple tumor cell lines to both ionizing radiation and bleomycin treatment *(127)*. Inhibition of PI3K with LY294002, another PI3K inhibitor not used clinically because of short half-life, enhances paclitaxel-induced apoptosis in ovarian cancer models *(119)*.

Cancer cells of the pancreas often express constitutively active Ki-Ras and overexpress multiple receptor tyrosine kinases, two genetic aberrations that may upregulate activity of the PI3K/Akt pathway *(128)*. Two pancreatic adenocarcinoma cell lines shown to be resistant to treatment with the chemotherapeutic gemcitabine (a DNA

chain terminator) showed substantial enhancement of apoptosis when treated with PI3K inhibitors wortmannin and LY294002 *(128)*.

Recently, isoform-specific PI3K inhibitors have been identified *(129)*. The development of isoform-specific PI3K inhibitors will facilitate analysis of distinct isoform-specific cell-signaling pathways in tumorigenesis. Furthermore, the discovery and characterization of novel PI3K inhibitors may provide compounds suitable for clinical use. These isoform-specific inhibitors offer the opportunity to compare the efficacy and toxicity of inhibition of specific PI3K isoforms to that of pan-PI3K inhibitors. As clinical utility reflects the balance between efficacy and toxicity, there may be advantages to isoform-specific inhibitors. Indeed, as indicated in Section 3.2, *PIK3CA*, which produces p110α, is selectively targeted in cancer, suggesting it is an optimal therapeutic target. Furthermore, insulin signal appears to process primarily through p110β, suggesting that a selective p110a inhibitor may have decreased toxicity.

4.2. Inhibition of Akt

Activated PI3K facilitates subsequent activation of Akt, which in turn phosphorylates many substrates to effect cell proliferation, cell-cycle progression, and cell survival.

Currently there are four Akt inhibitors in clinical trials. The first of these is perifosine, which like other members of the alkylphospholipid (APL) class of compounds, cause dose-dependent inhibition of Akt phosphorylation, although the exact mechanism of action is not known. APLs have, however, been demonstrated to bind to PH domains and to selectively interfere with the PH domain of Akt potentially providing selectivity. Miltefosine, another APL, is used topically to treat cutaneous breast cancer metastases *(130)*. This efficacy may be due to the high frequency of *PIK3CA* mutations in breast cancer. European clinical studies evaluating miltefosine as an oral anticancer agent were terminated at phase II because of cumulative gastrointestinal side effects *(130)*. Perifosine was better tolerated than miltefosine in preclinical studies and is currently in phase I/II trials *(130)*.

A number of pan-Akt inhibitors have been developed. These demonstrate a high degree of efficacy *in vitro* inducing apoptosis in multiple cell lineages, particularly those with abnormalities in the PI3K pathway. Compound XL418 and compound GSK690693 have recently entered Phase I studies. Additionally, a series of potential isoform-specific inhibitors has recently been identified.

Intriguingly, a number of drugs that are in use in other diseases or that have been assessed because of potential other mechanisms have been demonstrated to inhibit Akt signaling. These include triciribine *(131)* and more recently amiloride *(132)*. Whether these molecules inhibit Akt in patients and are useful probes of the function of the PI3K pathway will require further investigation. A Phase I trial is in progress to evaluate triciribine in patients with metastatic cancer whose tumors are p-Akt positive.

4.3. Inhibition of Hsp90

Hsp90 ensures proper folding and stability of many proteins including HER2, Erk, Akt, mutant B-Raf, and mutant p53. Radicicol and geldamycins, such as 17-allylamino 17-demethoxygeldanamycin (17AAG), inhibit Hsp90 by binding to the ATPase domain of Hsp90 *(133)*, thus destabilizing proteins that depend on Hsp90 for proper form and function *(130)*. These proteins are then depleted through the ubiquitin-proteosome

pathway. In this manner, 17AAG modulates the PI3K/Akt pathway *(130)*. Phases I and II studies of 17AAG are in progress. In these studies, 17AAG induces loss of both total Akt and phospho-Akt from tumors, suggesting that targeting the PI3K pathway may contribute to their function.

4.4. Inhibition of PDK1 Activation of Akt

PDK1 phosphorylation of Akt on its Threonine 308 residue is required for Akt activation. Inhibition of PDK1 is thus an attractive strategy for inhibiting the survival pathways mediated through Akt. Studies have shown that inhibition of PDK1 with UCN-01 results in inhibition of Akt and cell growth *(130)*. UCN-01 also enhances the antitumor effects of several chemotherapeutic cancer agents including antimetabolites, camptothecins, and cisplatin *(130)*. Phase I and Phase II clinical trials with UCN-01 have been carried out in patients with refractory solid tumors *(134)*, clinical chronic leukemia *(135)*, and chronic lymphoma and Phase II clinical. Whether the effects of UCN-01 in patients are a consequence of inhibition of PDK1 and Akt is unknown, however, because of the pleiomorphic action of UCN-01.

4.5. Targeting Ras, Rac, and Rho

4.5.1. Inhibition of PI3K/Akt with Prenylation Inhibitors

G proteins, such as Ras, Rac, and Rho, are important mediators of signal transduction at the cell membrane and are anchored to the membrane by a post-translationally added prenyl group. Ras, a G-protein activated in many cancers, for example, is generally prenylated with a farnesyl group, and other important G proteins such as Rac and Rho are prenylated with a geranylgeranyl group *(136)*. As prenylation of these important signaling molecules is essential to their function, inhibitors of the farnesyltransferase and geranylgeranyltransferase I enzymes that mediate prenylation have been developed as anticancer agents *(137)*.

Inhibition of PI3K/Akt signaling has been demonstrated as a critical target for farnesyltransferase inhibitor-induced apoptosis *(138)* and geranylgeranlytransferase I inhibitor (GGTI)-induced apoptosis *(139)*. FTI-277, a farnesyltransferase inhibitor, has been shown to inhibit growth-factor-induced PI3K/Akt2 activation *in vitro* and in vivo *(138)*. Furthermore, ectopic expression of wild-type Akt2 sensitizes cells to FTI-277 *(138)*. Two GGTIs, GGTI-298 and GGTI-2166, have been shown to induce apoptosis in both cisplatin-sensitive and cisplatin-resistant human ovarian epithelial cancer cells by inhibition of the PI3K/Akt pathway as well as the survivin pathway *(139)*, which is essential for proper cell division and also inhibits apoptosis *(140)*. As upregulation of Akt and survivin are frequently associated with chemoresistance, FTIs and GGTIs could prove to be valuable agents to overcome resistance to chemotherapy. Several prenylation inhibitors are at varying stages of clinical development *(141–143)*.

4.5.2. Additional Ras Inhibitors

Short antisense synthetic oligonucleotides are another treatment in development for targeting activated Ras *(143)*. ISIS 2503, an antisense oligonucleotide targeting H-Ras, is in phase II clinical trials *(143,144)*. Whether this will inhibit downstream activation of PI3K and Akt needs to be assessed in patient samples.

4.6. Inhibition of mTOR Activity

Akt activation results in subsequent activation of mTOR, a key mediator of protein translation, cell size, and cell-cycle progression. Phosphorylation of Akt, mTOR, and 4E-BP1 increases progressively from normal breast epithelium to hyperplasia and from abnormal hyperplasia to tumor invasion. Akt-induced drug resistance can be mediated by mTOR, indicated by reversal of tumor chemoresistance upon treatment with mTOR inhibitors *(130)*.

The current mTOR inhibitors consist of rapamycin and related analogs. These proteins interfere with the activity of the mTOR–raptor complex but not the mTOR–rictor complex blocking-specific functions of mTOR. Indeed, rapalogs block mTOR-mediated p70S6K phosphorylation without interfering with phosphorylation of the PDK2 site of Akt or of the effects of mTOR on 4E-BP1 and eIF4E *(14)*. This may contribute to the relative non-toxicity of rapamycin and its analogs. However, it may also limit the efficacy of rapalogs in the treatment of cancer patients.

Treatment of cancer cells (*in vitro*) with an inhibitor of mTOR, rapamycin, yields a higher rate of cell death when used in conjunction with traditional cytotoxic agents, such as cisplatin, camptothecin, 5-fluorouracil, and cyclophosphamide *(145)*. Rapamycin analogs with better pharmacological properties such as CCI779 and RAD001 have recently been developed. Currently, CCI779 and RAD001 are in phase II and Phase III clinical trials.

Feedback downregulation of Akt activity through mTOR/p70S6K-dependent loss of IRS-1 expression is well-established (146) and thus, potential upregulation of Akt signaling with mTOR inhibitors must be carefully evaluated. A treatment regimen combining an mTOR inhibitor with an inhibitor of Akt may provide for greater therapeutic efficacy.

4.7. Targeting EGFR Family Receptor-Mediated PI3K/Akt and MAPK Signaling

Much of cell signaling in epithelial cells is initiated through activation of epidermal growth factor receptors. The EGFR family includes EGFR (HER1/erbB1), HER2/neu (erbB2), HER3/erbB3, and HER4/erbB4. Binding of a ligand to the receptor facilitates its homo- and heterodimerization with other EGFR family members and subsequent activation of the receptor's tyrosine kinase located on the cytosolic side of the cell membrane. HER3 lacks intrinsic tyrosine kinase activity resulting in its functioning primarily as a linker molecule. Intriguingly, HER2, which lacks a functional ligand binding site, is constitutively in the active conformation resulting in its being the preferred binding partner for other members of the EGFR family. The tyrosine kinase activity results in autophosphorylation and subsequent activation of downstream signaling pathways that regulate cell proliferation and survival, such as the PI3K/Akt pathway and the Ras/Raf/MAPK pathway, which shows significant crosstalk with the PI3K/Akt pathway.

The EGFR pathway has been shown to contribute to radiation and chemotherapeutic resistance in many tumor types *(147–150)*. DNA damage can activate EGFR and downstream pathways *(151)*, and it has been observed that both radiation and chemotherapy can activate EGFR signaling, likely the reason for increased crossresistance between sequential administration of radiation and chemotherapy observed

in glioblastoma cells *(152)*. It appears that Ras-mediated activation of PI3K/Akt and MAPK (p44/p42), through EGFR, may play a critical role in mediating this effect *(152)*.

Antitumor drugs targeting the EGFR family of tyrosine kinase receptors have been developed, and more are in development. These agents include monoclonal antibodies that antagonize the receptors as well as other chemical inhibitors of the tyrosine kinase receptors.

Cetuximab, an anti-EGFR monoclonal antibody in clinical use, is approved for treatment of CRC and NSCLC and is in clinical trials for treatment of pancreatic, head and neck, breast, renal, and prostate cancers *(153)*.

ZD1839 (also known as gefitinib and Iressa), a reversible EGFR inhibitor in clinical use, is approved for treatment of NSCLC and is in clinical development for use in head and neck, CRC, breast, gastrointestinal, prostate, and esophageal cancers and has shown clinical activity in NSCLC and prostate cancer *(153)*. Its failure to increase patient survival in clinical trials, however, has resulted in most patients being transferred to a related inhibitor, erlotinib, which has demonstrated activity. Subset analysis indicates importantly that gefitinib does have high activity in patients with activating mutations in the EGFR, amplification of the EGFR, or phosphorylation of Akt *(123,154)*. These may represent a surrogate for constitutive activation of the EGFR and "addiction" of tumor cells to signaling through the EGFR pathway.

Unlike the other EGFR family members, which have a series of ligands that trigger homo- and heterodimer formation upon binding, the HER2 receptor has no ligands. Its activation occurs through heterodimer formation with other EGFR family members or through spontaneous homodimer formation under conditions of marked overexpression *(155)*. High levels of HER2 result in the progression of breast cancer tumorigenesis and metastasis and may also protect cancer cells from traditional cytotoxic therapies *(125)*. Trastuzumab (anti-HER2 monoclonal antibody), a drug in clinical use that inhibits breast cancer cell proliferation (in tumors overexpressing HER2) but does not induce apoptosis alone, was shown to enhance radiation-induced apoptosis of human breast cancer cells (*in vitro*) in a HER2-level-dependent manner *(125)*.

Phosphorylation of at least two important HER2 downstream molecules, Akt and MAPK, is increased in breast cancer cells with high levels of HER2, and cells are resistant to radiotherapy as shown by reduced induction of apoptosis *(125)*. *In vitro* exposure of these cells to trastuzumab downregulated the levels of HER2, reduced phosphorylation levels of Akt and MAPK, and sensitized these cells to radiotherapy *(125)*. On the basis of the results of chemical inhibition of each of the PI3K and MAPK pathways, the radiosensitization induced by PI3K inhibition was much stronger relative to MAPK inhibition, suggesting that the PI3K pathway may be the major pathway for trastuzumab-mediated radiosensitization of breast cancer cells *(125)*. Recent studies suggest that patients with decreased PTEN levels are less responsive to trastuzumab *(110)*. *In vitro* studies suggest that trastuzumab results in inactivation of HER2 and dissociation of Src from the HER2 activation complex *(110)*. Src-dependent phosphorylation sites on PTEN *(156)* are dephosphorylated, increasing PTEN activity. In patients with low PTEN levels, it is hypothesized that insufficient PTEN is present to mediate the effects of trastuzumab and that high phospho-Akt levels persist, leading to drug resistance *(110)*.

As described previously in this subsection, gefitinib is selective for EGFR; however, as the members of the EGFR family can form heterodimers, drugs targeting one

receptor may be effective in cells overexpressing other members of the EGFR family of receptors *(157)*. In a panel of human breast cancer and other epithelial tumor cell lines, HER2-overexpressing tumors were particularly sensitive to gefitinib. Growth inhibition of these tumor cell lines was associated with the dephosphorylation of EGFR, HER2, and HER3, accompanied by loss of association of HER3 with PI3K, and downregulation of Akt activity *(157)*. Erlotinib (Tarceva), another EGFR tyrosine kinase inhibitor in clinical use, has been shown to enhance radiosensitivity *(158)*. A series of reversible and non-reversible pan-EGFR inhibitors are currently under development and evaluation.

4.8. Patient Selection

The PI3K/Akt pathway is targeted genomically in multiple tumor lineages. Indeed, as described in Section 3.2.1, the PI3K pathway is mutationally activated in tumors with a greater frequency than any other pathway. Studies of trastuzumab in breast cancer, erlotinib and gefitinib in lung cancer, retinoic acid in PMML, imatinib mesylate in CML, gastrointestinal stromal tumors, dermatofibrosarcoma protuberans, and hypereosinophil syndrome have demonstrated that mutational activation, genomic amplification, or genomic rearrangement identifies a population of patients most likely to respond to therapeutic manipulation of the target. Indeed, modeling of the registration trial for trastuzumab suggests that the efficacy of trastuzumab would not have been detected had all breast cancer patients rather than just those with overexpression of HER2 been treated. This suggests that targeting therapy against the PI3K/Akt pathway in patients with genetic abnormalities in the pathway will result in greater efficacy, a greater likelihood of demonstrating activity, a decreased number of patients and decreased time of enrollment in trials, and thus decreased trial costs and more rapid implementation of effective drugs. Furthermore, in terms of the interests of pharmaceutical companies, this will result in protection of patent life. Thus, selection of patients with mutations in *PIK3CA, PTEN*, or other pathway components is likely to result in greater efficacy. At a minimum, patient samples should be collected before and after treatment to allow identification of patients likely to respond in subsequent clinical studies.

5. CONCLUSION

The emerging data strongly support a central role for aberrant PI3K/Akt pathway activation in many human cancers. The extended reach of this network, through its evergrowing list of substrates together with its direct effects toward cell survival, growth, motility, invasion, neovascularization, and proliferation, makes the PI3K/Akt pathway an attractive target for cancer treatment. Robust PI3K/Akt signaling in various tumor lineages has been linked to chemo- and radiotherapeutic resistance. PI3K/Akt inhibitors used in parallel to standard therapy have proven synergistic and effective in counteracting resistance in the preclinical setting. These demonstrate the greatest activity in models with genetic aberrations in the PI3K pathway, suggesting that selection of patients based on these aberrations is likely to identify those most likely to benefit from therapy. Targeting this central player in pathogenesis is a desirable approach that has become well accepted as a cancer therapeutic strategy. As such, multiple compounds targeting different components of the PI3K/Akt pathway have

entered or will begin clinical trials, either as single agents or in combination regimen with conventional cytotoxic agents. However, the utility of a drug is based on its therapeutic index, the ratio between efficacy and toxicity. As the PI3K/Akt pathway plays a critical role in normal cellular physiology, some components of the PI3K pathway may prove to be non-druggable. Thus, it will be important to develop inhibitors against multiple components of the pathway to identify targets with an adequate therapeutic index. These questions will only be answered with the development of pharmacologically relevant, highly selective drugs targeting components of the PI3K pathway.

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18 The Advancement of Epidermal Growth Factor Receptor Inhibitors in Cancer Therapy

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Summary

Epidermal growth factor receptor (EGFR) signaling is involved in various cellular processes ranging from normal growth and differentiation to oncogenesis. Targeted disruption of this pathway affords therapeutic potential in oncology, particularly in combination with conventional anticancer treatment modalities. Preclinical data across a spectrum of tumor model systems show promise for this approach, and a broad series of clinical trials that examine EGFR inhibition are currently underway or recently complete. Insights gained from early preclinical and clinical experience regarding mechanisms of EGFR action and response to inhibitors are helping to shape the rational incorporation of these new agents into modern cancer therapy.

Key Words: Epidermal growth factor receptor; radiation; apoptosis; small molecule inhibitors; monoclonal antibodies.

1. INTRODUCTION

Conventional cancer treatment involves the judicious application of surgery, radiation, and chemotherapy. Although incremental advances continue to emerge using these classic treatment modalities, increased knowledge regarding specific cellular, molecular, and genetic mechanisms of tumor initiation, growth, and progression has stimulated opportunities for new cancer treatment approaches. Improved understanding of molecular pathways involved in tumor cell growth has yielded a class of cancer therapeutic agents capable of more specific disruption of critical cellular proteins. This approach is commonly referred to as molecularly targeted biologic therapy, because unique biologic features of the underlying malignancy are exploited for therapeutic effect. As validation of this strategy, several molecularly targeted anticancer therapies have recently completed clinical trial testing and are currently entering clinical practice.

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This chapter will focus on a particularly promising molecular targeted therapy—epidermal growth factor receptor (EGFR)-signaling inhibition. The EGFR was identified as a potential anticancer target more than two decades ago following recognition that EGFR signaling is an important regulator of tumor cell growth *(1)*. Several strategies to inhibit EGFR signaling have been subsequently tested in preclinical systems, including anti-EGFR monoclonal antibodies (mAbs) to the extracellular receptor domain, small molecule tyrosine kinase inhibitors (TKIs) targeting the intracellular kinase domain, mRNA disruption, and gene targeting *(2)*. Advances in high throughput pharmacologic screening of mAb production have facilitated the development of an array of molecules with therapeutic potential. Two broad classes of agents that modulate EGFR signaling have emerged in clinical oncology practice: mAbs and TKIs.

To date, clinical testing of anti-EGFR agents has resulted in the approval of one mAb, cetuximab (Erbitux, ImClone Systems, New York, New York), and two TKIs, gefitinib (Iressa, Astra Zeneca, London, England) and erlotinib (Tarceva, OSI/Genentech, South San Francisco, California). Although not all clinical trials have shown positive results to date, EGFR-signaling inhibition as a therapeutic approach has been validated for several clinical applications including chemotherapy-refractory colon cancer, lung cancer, and advanced head and neck cancer *(3–5)*. Efforts to study the efficacy of these agents across a variety of other cancer disease sites are ongoing.

This chapter will focus on the preclinical development of EGFR-signaling inhibitors and examine current clinical indications for their use. Current hypotheses regarding the anticancer activity of EGFR-signaling inhibition will be presented, with particular focus on the interaction between EGFR signaling and the balance between cell survival and cell death.

2. EGFR STRUCTURE AND FUNCTION

2.1. Structure

Growth factors are soluble effector molecules of cellular proliferation, differentiation, adhesion, survival, and migration. Growth factor receptors therefore play a key role in transmitting signals from the extracellular compartment to the intracellular compartment. The EGFR was one of the earliest growth factor receptors to be characterized and sequenced *(6,7)*. This 170-kDa glycoprotein belongs to a family of four closely related transmembrane receptors named the ErbB or HER family. Receptor structure across the family is conserved, with a characteristic cysteine-rich extracellular ligand-binding domain, a hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase domain *(8,9)* (Fig. 1). Family members share considerable homology in the kinase domains but are divergent in the extracellular and C-terminal domains. As the prototypical ErbB family member, the EGFR is also known as ErbB1 or HER1. The remaining family members include ErbB2 (HER2/neu), Erb3 (HER3), and ErbB4 (HER4) *(10,11)*. ErbB family members are expressed across a wide array of tissues in a temporally and spatially distinct manner. More than 10 ligands are known to bind to the EGFR, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF, amphiregulin, betacellulin, epiregulin, and neuregulin *(9,12,13)*.

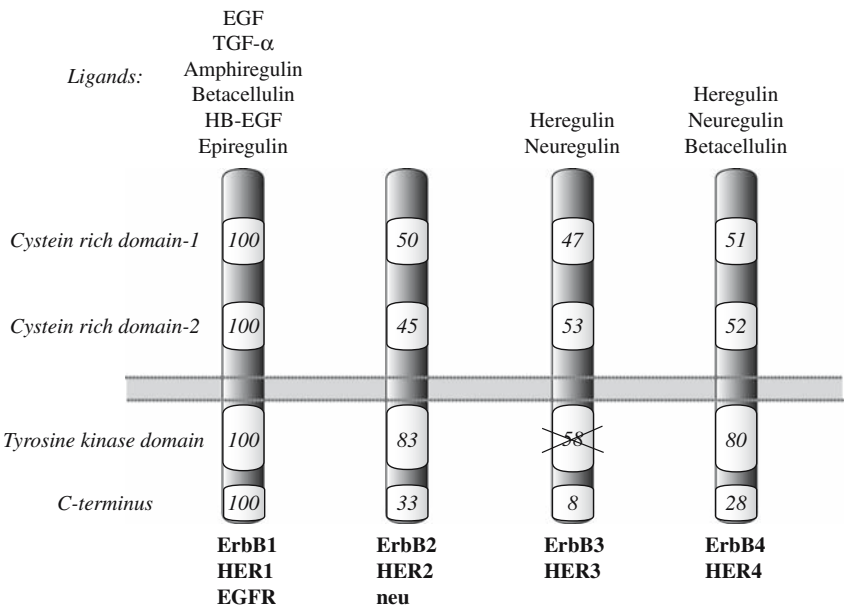

Fig. 1. Diagram demonstrating the ligands, domain structure, degree of homology, and naming convention of ErbB family members. Numbers denote the degree of homology relative to ErbB1/EGFR, expressed as a percentage. The "X" over the tyrosine kinase domain of ErbB3 indicates the lack of tyrosine kinase activity for this monomer. Adapted with permission *(170)*.

2.2. Signaling

Signaling through the EGFR begins when a ligand binds to an ErbB monomer, triggering receptor homodimerization or heterodimerization and subsequent autophosphorylation within the cytoplasmic domain *(8)*. Phosphorylation occurs on specific tyrosine residues and creates binding sites for Src-homology 2 and phosphotyrosine-binding domain-containing proteins, which serve as adaptors of downstream proteins involved in signal transduction. This horizontally and vertically organized network amplifies and integrates the extracellular ligand-binding events, conveying these signals to the nucleus, where genes involved in cellular proliferation and differentiation are regulated *(11,14–16)*. Termination of the ligand-binding-initiated signal can be accomplished by endocytosis of the phosphorylated receptor–ligand complex *(11)*.

The ErbB-signaling network has been conceptualized as a multilayer network consisting of an input layer, a signal-processing layer, and an output layer *(11,16)* (Fig. 2). Ligand–receptor binding is contained within the input layer. Signaling diversity within this layer arises from the different combinations of ligand–receptor interaction as well as the dimerization status (homodimer or heterodimer) of the receptor *(10)*. Heterodimerization leads to stronger mitogenic cues, thought to result from additional phosphotyrosine residues available for receptor stabilization *(17)*. Attenuation of signaling and altered lysosomal degradation also contribute to heterodimer signal diversity *(18,19)*. The formation of homodimers or heterodimers is influenced by the bivalency of ErbB ligands, the differential binding affinities of these ligands, and the pH stability of the ligand–receptor complex *(20–24)*. The signal-processing layer comprises the effector molecules downstream of the receptor. Several important

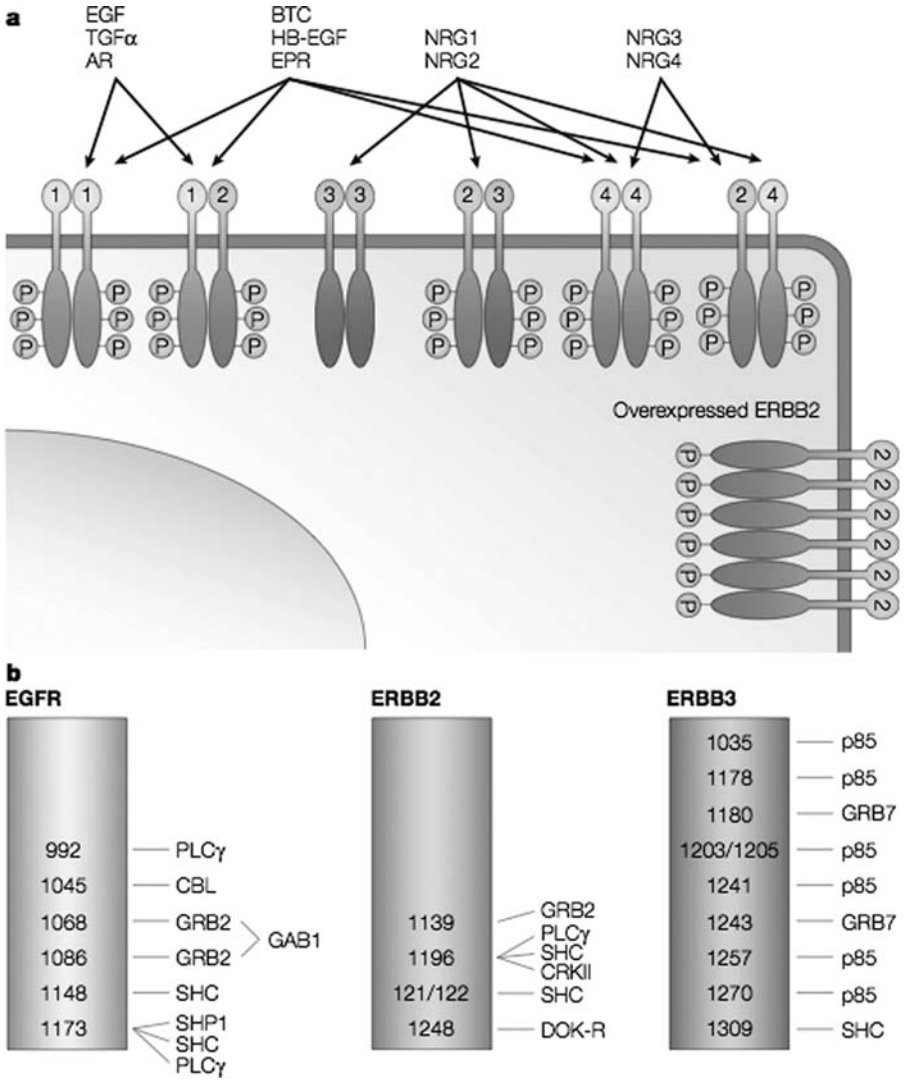

Fig. 2. (A) Epidermal growth factor family members are ligands for ErbB receptors and interact in multiple distinct homodimer and heterodimer configurations. **(B)** Receptor–ligand interaction induces autophosphorylation at a variety of sites within the intracellular receptor domain, leading to activation of multiple downstream signaling pathways. Used with permission *(16)*.

signal amplification pathways are utilized, including the Ras/mitogen-activated protein kinase (MAPK), phospholipase C (PLC)γ/protein kinase C (PKC), phosphatidylinositol 3′-kinase (PI3K)/Akt, and the Jak–Stat pathways *(9,25,26)*. Vertical transmission of signals to the nucleus as well as horizontal crosstalk between pathways amplifies and contextualizes the signal, ultimately leading to activation of nuclear transcription factors. Gene transcription in turn drives the production of proteins that mediate cellular responses including proliferation, migration, adhesion, differentiation, and apoptosis. Collectively, these diverse downstream processes comprise the network's output layer *(11,16)*. An appreciation for the complexity of the EGFR-signaling network in the larger context of growth factor signaling is central to our understanding of organ

(and organism) development. Insights into the function of these networks will continue to yield critical information regarding the molecular pathogenesis of cancer.

3. EGFR—SIGNIFICANCE IN ONCOLOGY

3.1. Pathological Basis of EGFR Signaling During Carcinogenesis

As signaling through the EGFR is required for normal cellular proliferation, survival, adhesion, migration, and differentiation, dysregulation of this pathway can lead to oncogenesis *(9,27,28)*. This may occur by several mechanisms that ultimately lead to hyperactivation of the EGFR and its downstream effectors. Hyperactivation of the EGFR can result from either autocrine or paracrine secretory loops, or through constitutive activation of the receptor regardless of ligand interaction. Secretory loops may become established when the EGFR receptor is overexpressed and ligands are overproduced by either the tumor or the supporting stroma *(27)*. Receptor activation may also result from a mutated receptor, as evidenced by the extracellular-domain truncated receptor EGFRvIII, which displays constitutive tyrosine kinase activity in the absence of ligand binding *(29)*.

Supporting the link between EGFR hyperactivation and cancer, EGFR overexpression, gene amplification, mutation, and rearrangement have been demonstrated in malignant gliomas and several other human malignancies including head and neck, lung, and breast cancers *(30)*. Although the EGFR is normally expressed by an array of cell types, there is variability in the reported incidence of receptor overexpression or dysregulation in human malignancies *(31,32)* (Table 1). In clinical series of epithelial malignancies, the number of tumors with dysregulated EGFR expression ranges from 30 to 50%, variability that may well reflect a lack of standardization in measurement

Table 1
Epidermal Growth Factor Receptor (EGFR) Expression and Potential Prognostic Significance in Human Cancers, Adapted with Permission *(169)*

Tumor type	*Expression (%)*	*Prognostic significance*
NSCLC	40–80	Shorter overall survival and higher metastases
Head and neck	80–100	Shorter disease-free and overall survival
Renal cell	50–90	Poor prognosis, shorter survival in positive tumors
Bladder	31–48	More prevalent in recurrent invasive tumors
Gastric	40	Shorter survival in tumors positive for TGF-α and EGFR
Pancreatic	30–50	Coexpression with ligands associated with reduced survival
Colon	25–77	Higher proportion of tumor cells expressing EGFR associated with poor patient outcome
Breast	14–91	Associated with worse overall survival and steroid receptor-negative tumors
Ovary	35–70	Associated with reduced disease-free survival, overall survival, and drug resistance
Glioma	40–50	Most common tumor exhibiting EGFRvIII mutation

NSCLC, non-small-cell lung cancer; TGF, transforming growth factor.

techniques *(33–35)*. A review of over 20,000 patients across 200 studies for whom EGFR expression data were available determined the prognostic significance of EGFR overexpression was highest in head and neck, ovarian, cervical, bladder and esophageal cancers. More moderate prognostic links were found for gastric, breast, endometrial, and colorectal cancers, whereas non-small-cell lung cancer (NSCLC) prognosis was only weakly associated with EGFR expression levels *(32)*. Cellular response to EGFR inhibitors is also heterogeneous. Preclinical studies with 60 cell lines found that a high level of EGFR expression level alone did not predict well for cellular sensitivity to an array of EGFR inhibitors *(36)*.

The EGFR promotes the establishment and maintenance of the transformed phenotype through several downstream effector pathways (Fig. 3). Activation of the Ras–MAPK pathway ultimately leads to phosphorylation of Erk1/2 and the ribosomal p70-S6 kinase. These in turn activate a variety of transcription factors known to play a role in cellular transformation, including AP1, E2F, and Sp1. The PI3K/Akt pathway activates an array of proteins, primarily through the action of Akt. The proapoptotic protein Bad is a downstream target of the Akt kinase. When Bad is phosphorylated, interaction with Bcl-2 and Bcl-X is impaired, thereby promoting cell survival. Akt furthermore promotes cell cycle progression by downregulating the cyclin-dependent kinase inhibitor $p27^{KIP1}$ and initiates protein translation by activating mammalian target of rapamycin (mTOR) and the initiation factor 4E *(17)*. The PLC/PKC pathway also promotes cellular growth by mediating calcium release from the endoplasmic reticulum, which in turn activates calcium-dependent protein kinases and phosphatases *(17)*.

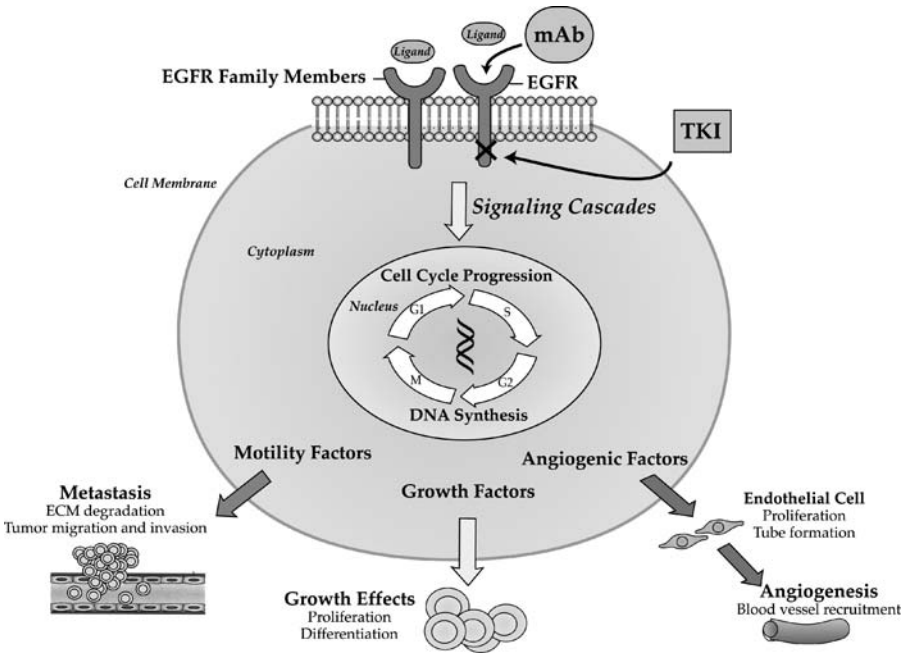

Fig. 3. Schematic of the epidermal growth factor receptor (EGFR) family and downstream pathways. Therapeutic targets for anti-EGFR mAbs and tyrosine kinase inhibitors (TKIs) include motility, growth and angiogenic factors. Adapted with permission *(171)*.

4. PRECLINICAL STUDIES

4.1. Proliferation/Cell Cycle Control

The cellular machinery involved in cell cycle progression offers an attractive therapeutic target for cancer therapy *(37)*. EGF is known to induce cyclin D1 expression, a protein that is required for progression from the G_1 to S phase. Studies of EGFR-signaling inhibition with cetuximab or gefitinib have demonstrated proliferation inhibition in cultured epithelial tumor cells. A primary mechanism of growth arrest is blockade of cell cycle progression at G_1 *(38–45)*. Other mechanisms include accumulation of hypophosphorylated retinoblastoma (Rb) protein and the cyclin-dependent kinase (CDK2) inhibitor $p27^{KIP1}$. Accumulation of these two proteins leads to a reduction in CDK2 activity, which in turn inhibits cell cycle transition from the G_1 to S phase. Further growth arrest occurs by inhibiting CDK2 phosphorylation of Rb, which decreases E2F-mediated transcription *(45–47)*. These results have been corroborated *in vivo* with xenograft studies in which EGFR inhibitor treatment resulted in increased expression of $p27^{KIP1}$ *(44,48)*. Additionally, *in vitro* experiments have demonstrated multiple cyclin-dependent kinases that are inhibited by disruption of EGFR signaling. These include CDK4, CDK6, and cyclin-D3 and lend support to a more widespread involvement of EGF signaling in cell cycle progression *(49)*.

4.2. Apoptosis

Harnessing intrinsic cell death programs for therapeutic advantage represents another promising anticancer strategy. The EGFR-signaling axis is an important target in this regard, as EGF acts as a survival factor by inhibiting apoptosis and promoting tumor growth *(50–52)*. The downstream effectors PI3K/Akt and MEK/Erk appear to be important mediators of EGF-induced cell survival *(53)*. Preclinical studies of targeted EGFR-signaling inhibition demonstrate that this approach can induce or promote apoptosis in human tumor cells. As an example of this effect, treatment of human NSCLC or DiFi (colon cancer) cells in culture induces apoptosis *(46,54,55)*. This observation is not limited to anti-EGFR antibodies, as EGFR TKIs are also effective inducers of apoptosis *(56–58)*. Interestingly, EGFR co-targeting with a combined mAb and TKI enhanced apoptotic cell death over either strategy alone *(59,60)*. Taken together, these findings suggest that in some cancers, EGFR inhibition leads to promotion of apoptosis resulting from activation of proapoptotic pathways, inhibition of antiapoptotic pathways, or a combination of both mechanisms.

In head and neck cancer cell lines, cetuximab triggers a rise in Bax and decrease in *bcl-2* expression *(46)*. In normal keratinocytes, EGFR blockade led to increased expression of the *bcl-2* homolog *Bcl-x(L) (61)*. Subcellular localization of Bax is also impacted by EGFR blockade, with relocation of Bax from the cytosol to the nucleus *(62)*. Co-targeting two proapoptotic molecules, *bcl-2* and protein kinase A, in concert with EGFR-signaling inhibition led to increased apoptosis compared with each individual inhibitor, demonstrating that EGFR-signaling inhibition may be augmented by inhibition of other targets *(63)*. Induction of apoptosis in cancer cell lines by EGFR inhibition strategies is not universal, however, as the prostate cancer cell line DU-145 does not appear to undergo apoptosis after exposure to cetuximab *(43)*. Therefore, although not universally observed, EGFR-signaling inhibition appears to induce or promote apoptosis in a substantial proportion of human tumor cell lines.

4.3. Angiogenesis

The concept that new vessel formation (angiogenesis) is necessary for tumor growth was first proposed in 1971 *(64)*. Angiogenesis has subsequently evolved into an important target for anticancer therapy. The formation of new blood vessels is orchestrated by several angiogenic factors, including basic fibroblast growth factor (bFGF), cytokines such as interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) *(65–69)*. These factors are produced either by tumor cells themselves or by stromal cells including endothelial cells, fibroblasts, smooth muscle cells, or pericytes. Evidence suggests that, in addition to the aforementioned factors, signaling through the EGFR axis plays a role in the regulation of angiogenesis *(48,70–73)*. EGF and TGF-α are known to stimulate angiogenesis, and EGFR is present in vascular endothelial cells *(67,74,75)*. At the cellular level, activation of EGFR by EGF and TGF-α results in increased VEGF expression *(76–79)*. Transcription of VEGF has been linked to TGF-α and is mediated by AP1/AP2 and early growth response gene 1 (EGR-1) *(80)*. Furthermore, inhibitors of EGFR signaling result in the downregulation of VEGF, IL-8, and bFGF in a variety of cancer cell lines *(48,81–83)*. EGFR-blocking strategies, therefore, in addition to their direct antiproliferative and proapoptotic effects, may also inhibit the growth of tumors by limiting tumor vessel formation.

4.4. Metastasis

EGFR expression has been correlated with cellular invasion or metastasis in a variety of model systems. In NSCLC lines, overexpression of EGFR and/or its ligands, as well as several metalloproteinases (MMPs), is associated with an invasive phenotype *(84–88)*. An increase in motility and invasive potential has been demonstrated for EGF, independent of its mitogenic effects *(77,87,89)*. In an *in vitro* model of breast cancer cell motility, macrophages were found to stimulate invasion by paracrine release of EGF *(90)*. Autocrine effects have also been observed for a prostate cancer cell line, with an increase in invasive potential mediated by EGFR-induced urokinase-type plasminogen activator *(91)*. Additionally, EGFR levels have been directly correlated with invasive potential in NSCLC lines *(87)*. These effects are likely mediated through MMPs, as EGFR levels correlate directly with MMP-9 expression and vary inversely with tissue inhibitor of MMP *(87)*. EGFR inhibition strategies diminish tumor cell invasion and downregulate MMP-9, likely by decreasing activation of MAPK and Akt *(72,87,92,93)*. Similar findings have emerged from xenograft models. A model of human transitional cell carcinoma of the bladder treated with cetuximab led to downregulation of MMP-9 mRNA in a dose-dependent manner with complete abrogation of lymph node metastases *(48)*. A combined antimetastasis and antiangiogenic effect was observed in a xenograft model of pancreatic cancer treated with cetuximab alone or in combination with gemcitabine *(94)*.

4.5. Radiation Interactions

A promising role for EGFR-signaling inhibition combined with radiation therapy has emerged over the last several years. Radiation exposure induces cell death by physical means, resulting primarily from an accumulation of lethal DNA-damaging events. Strategies directed at the inhibition of other cellular targets may provide additive or synergistic therapeutic value in combination with radiation. The EGFR pathway

in particular may serve as an important mediator of radiation resistance, as several studies have demonstrated a link between EGFR expression level and cellular resistance to radiation *(95–97)*. Supporting this hypothesis, cancer cell lines transfected with EGFR expression vectors are more radioresistant than their parental lines *(98–100)*. Furthermore, the degree of radioresistance correlates positively with the magnitude of EGFR overexpression *(101)*. EGFR levels and activation status may affect tumor cell survival during radiation, as EGFR and TGF-α are induced following radiation exposure *(102,103)*. Additionally, overexpression of the constitutively active EGFRvIII also leads to enhanced radioresistance *(104)*. Taken together, these preclinical results provide a rationale for EGFR-signaling inhibition during radiotherapy *(83)*.

Several *in vitro* studies of EGFR blockade have demonstrated radiosensitization using a variety of experimental approaches. A broad spectrum of human cancer cells demonstrates radiosensitization with either anti-EGFR mAbs or TKIs *(40,46,82,105–108)*. Co-targeting EGFR signaling and the downstream effectors MEK and PI3K results in synergistic radiosensitization *(109)*. Enhanced radiation-induced apoptosis with EGFR blockade has been observed, suggesting a mechanism of EGFR-mediated radiosensitization *(46,110)*. Data from *in vivo* xenograft models demonstrate a similar positive interaction between EGFR-signaling inhibition and irradiation *(83,111–114)*. Well-established squamous cell carcinoma (SCC) xenografts treated with cetuximab and radiation results in potent regression of tumors in athymic mice over a 100-day follow-up period *(83)*. A threefold enhancement in A431 tumor regression with cetuximab following single-fraction radiation exposure has been demonstrated *(111)*. Similar results have been obtained for both gefitinib and erlotinib. Treatment of head and neck cancer xenografts with gefitinib or erlotinib plus radiation was significantly more effective than either modality alone, potentially reflecting impaired DNA repair capacity in tumors exposed to EGFR inhibitors *(40,115)*. These data lend strong evidence that in selected preclinical tumor models, EGFR-signaling inhibition interacts positively, and perhaps synergistically, with ionizing radiation.

4.6. Chemotherapy Interactions

Considerable evidence exists regarding the capacity for EGFR-signaling inhibition to enhance cytotoxic chemotherapy in both *in vitro* and *in vivo* model systems. Favorable interactions exist for cetuximab and a variety of chemotherapeutic agents, including cisplatin, doxorubicin, paclitaxel, gemcitabine, irinotecan, and topotecan *(94,116–121)*. Complementary data exist for the TKI gefitinib, in which favorable interactions with the aforementioned compounds exist, in addition to carboplatin, oxaliplatin, docetaxel, and etoposide *(81,122,123)*. These favorable effects appear to exist over a broad range of cell and tumor types, including those derived from colon, pancreas, prostate, bladder, lung, head and neck, breast, and vulva *(124–126)*. Erlotinib combined with chemotherapy has been studied in preclinical models of lung, pancreas, and head and neck cancers. When administered in combination with cisplatin and gemcitabine, erlotinib more effectively induced xenograft growth arrest than when either chemotherapy or erlotinib was administered alone *(127–129)*. These promising preclinical data confirm that EGFR-signaling inhibition strategies may serve as clinically useful adjuncts to chemotherapy.

4.7. Biologic Therapy Interactions

Preclinical and clinical models of EGFR-signaling inhibition combined with radiation and chemotherapy have shown considerable promise; however, many patients in clinical trials still do not demonstrate a favorable response. One hypothesis for this observation is that tumors may rely on several aberrant signaling pathways for their growth advantage. This has prompted investigators to combine EGFR-signaling inhibition and other biologically targeted therapies in an effort to increase antitumor efficacy. Targeting both EGFR and HER2 pathways represents one such approach, as both ErbB family members share common downstream signaling mechanisms. Breast cancer cells expressing the estrogen receptor demonstrated growth inhibition with a combined EGFR/HER2 inhibition approach, greater than with either inhibitor alone *(130)*. Similar antiproliferative effects were observed for this combination in hormone-refractory prostate cancer cells treated with radiation *(131)*. Dual EGFR/HER2 targeting has also been validated in colon cancer cell lines, but only lines exhibiting autocrine EGFR release were found to be sensitive to HER2-signaling blockade *(132)*. A dual EGFR/HER2 TKI, lapatinib (GW572016), has demonstrated cytostatic and proapoptotic effects in breast and head and neck cancer cell lines as well as radiosensitizing effects in breast cancer lines *(108,133)*. Dual-agent EGFR inhibition combining an anti-EGFR mAb and TKI has undergone preclinical testing, with enhanced antiproliferative, proapoptotic, and radiosensitizing effects observed for this combination over either agent alone *(59,60)* (Fig. 4). These findings support the hypothesis that single-agent EGFR inhibitor strategies may not be sufficient to control the growth of many complex tumors, providing further rationale for combined molecular inhibitor approaches.

Targeting the EGFR- and additional growth factor-signaling pathways represents an attractive therapeutic strategy, as signaling through parallel pathways may confer resistance to EGFR inhibitor therapy and the disruption of multiple pathways may yield improved antitumor efficacy. One of the most promising dual-pathway approaches combines targeted disruption of EGFR signaling and angiogenesis. As an example, the small molecule AE788 (Novartis, Basel, Switzerland), which inhibits both EGFR and the VEGF receptor (VEGFR), has been shown effective in blocking EGF- and VEGF-mediated proliferation in a variety of cell lines and exhibits antitumor and antiangiogenic effects in xenograft tumor models *(134,135)*. Similar results have been observed with the dual EGFR/VEGFR inhibitor ZD6474 (Astra Zeneca) *(136,137)*. Targeting the platelet-derived growth factor receptor, an important therapy for chronic myelogenous leukemia, has also been combined with EGFR-signaling inhibition in at least one preclinical study. In a model of prostate cancer metastasis, co-targeting both receptors was found to inhibit metastatic bone involvement *(138)*. Dual targeting of the insulin-like growth factor-1 receptor (IGF-1R) and EGFR signaling has also shown promise in a variety of preclinical tumor models *(139,140)*. In conclusion, inhibition of EGFR signaling combined with other targeted therapies is yielding positive preclinical results with promise for eventual clinical application.

In head and neck cancer, co-targeting EGFR signaling and signal transducers and activators of transcription (STAT) is under current investigation. STAT3 has become a particular focus of attention as a direct mediator of EGFR-induced cell cycle progression and apoptosis inhibitor *(141)*. The intracellular domain of EGFR physically interacts with STAT3 in the nucleus, leading to transcriptional activation of inducible

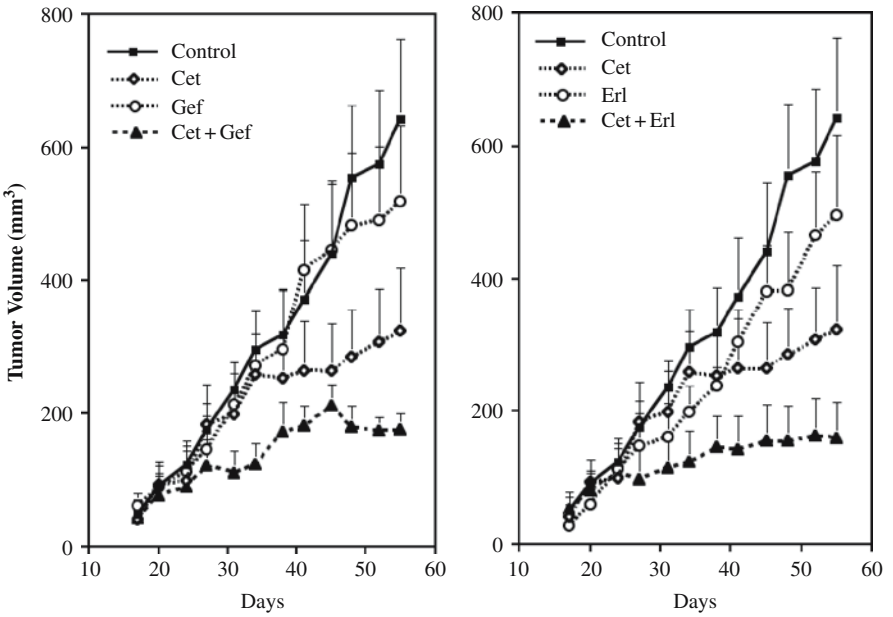

Fig. 4. Antitumor activity of cetuximab in combination with gefitinib or erlotinib in H226 tumor xenografts. After establishment of grafts, animals received either control or experimental treatments twice weekly for a total of eight treatments. Values indicate mean tumor size ($n = 8$ per group). Adapted with permission *(59)*.

nitric oxide synthase (iNOS) *(142)*. Other reports suggest that inhibitors of c-Met and ErbB3 may interact favorably with EGFR inhibition strategies, as these molecules are overexpressed in cell lines resistant to EGFR-signaling inhibition *(143,144)*.

5. CLINICAL STUDIES

The two dominant EGFR inhibition strategies under clinical investigation include the anti-EGFR mAbs and TKIs. Although EGFR-signaling inhibition is successfully accomplished with either class of EGFR inhibitor, several important distinctions between them exist. The pharmacokinetic profiles of mAbs and TKIs vary widely. As they are large macromolecular proteins, mAbs are susceptible to degradation in the gastrointestinal tract, and therefore, oral administration is not effective. mAbs have relatively long half-lives and thus weekly intravenous infusion is the preferred method of clinical administration. The TKIs, by contrast, are small molecules that are effectively absorbed across the gastrointestinal tract; thus oral administration is routine and the short half-life requires daily administration. Specific mechanisms of action for each anti-EGFR class also vary in several subtle but important ways. The mAbs target the extracellular ligand-binding domain of EGFR, whereas the TKIs interact with the catalytic domain in the intracellular compartment. Antibodies binding to the extracellular domain prompt receptor internalization and may stimulate host immune responses to contribute to the therapeutic outcome. TKIs show some degree of cross-reactivity to a variety of tyrosine kinases, which may contribute to their ultimate clinical impact.

Anti-EGFR mAbs and TKIs have been evaluated in clinical trials, both as single agents and in combination with conventional radiation or chemotherapy. Combination strategies are a logical choice due to the non-overlapping action mechanisms and toxicity profiles of targeted EGFR-signaling inhibition and conventional radiation or chemotherapy. The preclinical data summarized above provide strong support for clinical trials examining combination therapies. Selected clinical trials of EGFR inhibitors are briefly reviewed.

5.1. mAb EGFR Inhibitors

5.1.1. Cetuximab

Cetuximab, formerly known as IMC-225 or C225, is a chimeric mouse anti-EGFR mAb that received US Food and Drug Administration (FDA) approval in 2004 for the treatment of irinotecan-refractory colorectal cancer. Cetuximab is the most widely studied anti-EGFR mAb and is the first agent in this class approved for clinical use. Early phase I and I/II trials demonstrated the safety of cetuximab alone or in combination with cytotoxic chemotherapy for patients with metastatic squamous cell carcinoma of the head and neck, colorectal cancer, and NSCLC *(145–148)*.

Efficacy for concomitant cetuximab and chemotherapy was established in a phase II trial in colorectal cancer patients. Patients with metastatic colon cancer refractory to irinotecan ($n = 121$) received concurrent cetuximab and irinotecan, with partial responses observed in 23% and stable disease in 31% *(4)*. A larger European phase II study (the Bowel Oncology with Cetuximab Antibody (BOND) trial) examined the combination of cetuximab and irinotecan ($n = 218$) versus cetuximab alone ($n = 111$) for patients with EGFR-positive irinotecan-refractory metastatic colon cancer *(149)*. Response rates were higher for the combined therapy group (22.9%) versus the cetuximab monotherapy group (10.8%). Median survival was also higher in the combined group (8.6 months versus 6.9 months), although this did not reach statistical significance. Two phase III studies have been initiated in the USA. The Erbitux Plus Irinotecan in Colorectal Cancer (EPIC) trial will examine the combination of cetuximab and irinotecan versus irinotecan monotherapy as second-line treatment for patients with metastatic EGFR-positive colorectal cancer. The Cetuximab plus FOLFOX for Colorectal Cancer (EXPLORE) trial will determine whether patients with EGFR-positive metastatic colorectal cancer will benefit from concurrent cetuximab and 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX) compared with the standard FOLFOX regimen.

Results of an international phase III randomized trial of cetuximab in advanced head and neck cancer have been reported *(3)*. In this study, 424 patients received either definitive radiation therapy alone or the same treatment with concurrent cetuximab. At a median follow-up of 54 months, near doubling of the median survival was observed in patients receiving cetuximab versus patients receiving radiation therapy alone (49 versus 29 months, $p = 0.03$). Statistically significant increases in locoregional control and progression-free survival were also reported for the group receiving cetuximab. This landmark trial provided the first demonstration of clinical efficacy for combined EGFR-signaling inhibition and radiation therapy, providing rationale for further clinical investigation with a variety of anti-EGFR compounds. Furthermore, this is the first study to demonstrate a statistically significant survival benefit for an

anti-EGFR antibody. As a result, in March 2006, the US FDA granted full regulatory approval for the use of cetuximab combined with radiation in the treatment of locoregionally advanced head and neck cancer.

5.1.2. Panitumumab

Panitumumab (rHuMAb-EGFr, Amgen, Thousand Oaks, California), formerly known as ABX-EGF, is a fully human anti-EGFR mAb that has undergone early clinical trials testing in prostate cancer *(150)*. Interestingly, panitumumab appeared to inhibit angiogenesis in this study, validating preclinical models linking EGFR inhibition and angiogenesis. A phase II trial of panitumumab monotherapy in patients with renal cell carcinoma refractory to IL-2/interferon-α demonstrated two objective responses out of 58 patients, with 58% of patients experiencing stable disease. A multicenter phase II trial of panitumumab monotherapy was conducted in 44 patients with metastatic colorectal cancer *(151)*. Stable disease was observed in 52%, whereas 9% had a partial response and 39% experienced disease progression. Phase II trials of panitumumab monotherapy are ongoing in hormone-resistant prostate cancer, colorectal cancer (first line), and NSCLC. A phase III trial of third-line panitumumab in chemotherapy-refractory metastatic colorectal cancer was opened in 2004.

5.1.3. Other mAbs

Several additional anti-EGFR mAbs are currently under development. The majority of these mAbs are fully human, as opposed to the first generation chimeric antibody cetuximab. Advances in antibody-screening technology have made the selection of high-affinity fully human antibodies more feasible, further lessening the potential for a host immune response directed against the mAb. Several bispecific antibodies, co-targeting EGFR and other growth factor receptors, are also in development (Table 2).

5.2. TKI EGFR Inhibitors

5.2.1. Gefitinib

Originally known as ZD1839, gefitinib is approved in the USA for single-agent use in the treatment of chemotherapy-refractory NSCLC. Gefitinib was the first anti-EGFR TKI approved by the FDA for use in cancer therapy. Gefitinib is an effective inhibitor of EGFR kinase activity, with an IC_{50} in the nanomolar range *in vitro*; however,

Table 2
Epidermal Growth Factor Receptor (EGFR) Monoclonal Antibodies in Clinical Trials

Agent	*Type*	*Generic/trade name*	*Institution*
IMC-C225	Chimeric IgG1	cetuximab/Erbitux	ImClone/BMS/Merck
ABX-EGF	Fully human IgG2	panitumumab	Abgenix/Amgen
EMD 72000	Humanized IgG1		EMD Pharms/Merck
MDX-447	Bispecific EGFR/FcRy1	HuMab-Mouse	Medarex/Merck
h-R3	Humanized	TheraCIM	YM Biosciences/CIM
Mab 806	Anti-EGFR VIII		Ludwig Institute

higher *in vivo* concentrations may be required because of competition with intracellular ATP *(152)*. Gefitinib is also known to inhibit the HER2 kinase, albeit at levels 100-fold greater than those required for EGFR-signaling inhibition *(126,152,153)*.

A number of single-agent phase II clinical trials have been carried out using gefitinib in a range of solid tumor malignancies including head and neck cancer, renal cell carcinoma, and advanced breast cancer *(154–159)*. Most notably, the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL-1) and IDEAL-2 trials in NSCLC validated the single-agent efficacy of gefitinib in patients with refractory NSCLC, resulting in FDA approval. Two subsequent phase III trials, Iressa NSCLC Trial Assessing Combination Treatment (INTACT-1) ($n = 1093$) and INTACT-2 ($n = 1037$), failed to demonstrate a significant benefit for the concurrent addition of gefitinib to standard chemotherapy in advanced lung cancer *(160,161)*. The lack of efficacy in these large phase III trials was disappointing in light of the positive results seen in the phase II trials. Some have argued that by not requiring verification of EGFR positivity for entrance into the trial, potential drug effects may have been diluted, even though quantitative EGFR status has not yet been linked to EGFR inhibitor response *(157,162)*.

With the knowledge that a subset of patients with lung cancer exhibited dramatic responses to gefitinib, the existence of EGFR mutations was hypothesized. Two independent groups subsequently demonstrated the presence of multiple in-frame deletions or amino acid substitutions in the EGFR tyrosine kinase domain that strongly predict for positive response to gefitinib *(163,164)*. Interestingly, these mutations were found to be most prevalent in patients who were Japanese, female, and nonsmokers and whose tumor histologies were bronchoalveolar or adenocarcinomas with bronchoalveolar features. Efforts are underway to identify additional EGFR mutations and to determine whether EGFR catalytic domain mutations may ultimately serve as a clinical tool in the selection of patients for EGFR TKI therapy.

5.2.2. Erlotinib

Erlotinib, also known as OSI-774, was the second EGFR TKI approved by the FDA for clinical application. Like gefitinib, erlotinib is a potent inhibitor of EGFR autophosphorylation, with an IC_{50} in the nanomolar range *in vitro*. Similar to gefitinib, erlotinib demonstrates activity against HER2 and has been found to be particularly effective at inhibiting signaling through the mutant EGFRvIII receptor *(165,166)*. Two phase III trials in NSCLC were conducted with a design similar to that of the INTACT-1 and INTACT-2 trials for gefitinib. The Tarceva Lung Cancer Investigation (TALENT) and Tarceva Responses in Conjunction with Paclitaxel and Carboplatin (TRIBUTE) trials examined the efficacy of adding concurrent erlotinib to standard chemotherapy in advanced lung cancer *(167,168)*. As with the INTACT trials, primary survival endpoints were not significantly improved with the addition of erlotinib, although in the TRIBUTE study, the secondary endpoint of time to symptomatic progression was improved. The failure of these trials to demonstrate a benefit for erlotinib in NSCLC parallels findings from the INTACT trials, with similar concerns regarding the difficulty in achieving statistical significance for primary clinical endpoints in the absence of more stringent patient selection.

The National Cancer Institute of Canada Clinical Trials Group recently concluded a 700-patient phase III trial of erlotinib monotherapy versus placebo in second-or third-line metastatic NSCLC. Whereas the INTACT and TALENT/TRIBUTE trials

Table 3
Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitors (TKIs) in Clinical Trials

Agent	*Type*	*Generic/trade name*	*Institution*
ZD1839	erbB1	Gefitinib/Iressa	AstraZeneca
OSI-774	erbB1	Erlotinib/Tarceva	OSI/Genentech/Roche
CI-1033	pan erbB	Canertinib	Pfizer
EKB-569	erbB1/2		Wyeth Ayerst
GW2016	erbB1/2	Lapatinib	GlaxoSmithKline
PKI-166	erbB1/2		Novartis

studied whether adding concurrent gefitinib or erlotinib to chemotherapy was effective in advanced (non-metastatic) NSCLC, the Canadian trial examined erlotinib as monotherapy in the setting of chemotherapy-refractory metastatic disease. Unlike the previous trials, the primary endpoint of overall survival was significantly improved for patients receiving erlotinib compared with those receiving placebo (6.7 versus 4.7 months). Additionally, the secondary endpoints of time to symptomatic progression, progression-free survival and response rate were all statistically significant *(5)*.

5.2.3. Other TKIs

In addition to gefitinib and erlotinib, several additional TKIs are under preclinical and clinical development (Table 3). Generally, these inhibitors have been developed to target the EGFR as well as other ErbB family members, most notably ErbB2. Canertinib (CI-1033, Pfizer Pharmaceuticals, New York, New York) is unique in this regard as it is a pan-ErbB family inhibitor capable of influencing all four ErbB family receptors.

6. CONCLUSION

Molecularly targeted biologic therapy offers the promise of new treatment tools that may be used alone or in combination with conventional cancer therapy modalities. EGFR-signaling inhibition represents one of the emerging strategies within this new cancer treatment paradigm. Our improved understanding of normal and dysregulated EGFR signaling provides sound rationale for targeted disruption of this growth receptor. Dysregulated EGFR signaling impacts a variety of oncogenic processes including proliferation, differentiation, migration, metastasis, apoptosis, and angiogenesis. Disruption of EGFR signaling has gained strong validation in preclinical tumor models and is undergoing extensive clinical testing. The results to date suggest that targeted EGFR inhibition will offer a valuable new therapeutic approach for selected patients with head and neck cancer, colorectal cancer, and NSCLC. The recent identification of EGFR mutations conferring sensitivity to TKIs has set the foundation for continued investigation of more specific molecular predictors of disease response. Ultimately, approaches such as DNA microarray and proteomic analyses may enable tumors to be better characterized at the molecular level, uncovering a "molecular phenotype" of EGFR inhibitor response that may be used to more rationally select patients for anti-EGFR therapies.

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V Established Cancer Therapies

19 Antimetabolites

Janet A. Houghton, PhD

Summary

Cell death has been divided into two main types: programmed cell death (PCD), in which the cell plays an active role, and passive (necrotic) cell death. PCD has been categorized into different subtypes: apoptosis, anoikis, mitotic catastrophe, autophagy, paraptosis, and cytoplasmic (for which the mechanism of molecular activation remains unknown) *(1)*. Senescence arrest, accelerated senescence, and differentiation are also responses that can be induced in response to DNA-damaging agents. Considerable attention has been given to the study of apoptotic responses following DNA damage induced in cancer cells by chemotherapeutic agents. Apoptosis may occur as a primary event following chemotherapy in which genes that regulate apoptosis will influence the outcome of therapy or alternatively as an event secondary to the induction of lethal damage that involves the subsequent processing of cellular damage *(2,3)*. In the former case, apoptosis may occur early within the first few hours of treatment as observed in cells of hematopoietic origin, whereas for solid tumors, the outcome is less clear *(4)*. The particular type of response induced is highly dependent on the agent and dose employed, the type of DNA damage induced, as well as the genetic and cellular phenotypes. It has been proposed that apoptosis may play a lesser role in tumor response to radiation in comparison with the induction of cell death through mitotic catastrophe or a senescence-like irreversible growth arrest *(5)*. However, in comparison with the induction of apoptosis, there is a lack of as much definitive information on other cell death processes that occur in cancer cells in response to chemotherapeutic agents, including antimetabolites. This chapter reviews what is known about these processes at the present time in response to experimental or clinically used agents that are analogs of 5-fluorouracil (FUra), cytidine (Cyd) or purines, hydroxyurea, or that belong to the family of folate antagonists.

Key Words: Antimetabolites; cell death; apoptosis; mitotic catastrophe; necrosis; senescence.

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1. PROGRAMMED CELL DEATH

1.1. Apoptosis (Type I)

It has become widely accepted that DNA-damaging agents, including antimetabolites, induce cell death in neoplastic cells generally by triggering apoptosis. The susceptibility of cells to apoptosis depends upon the status of genes in regulating critical components of the cell death machinery *(6)*, which in turn is influenced by signaling events that result in a complex system of interacting signals that determine the ultimate outcome of the treatment response *(7)*.

1.1.1. FUra, Analogs, and Folate-Based Inhibitors of Thymidylate Synthase

The vast majority of studies with antimetabolites of this class has been conducted with FUra itself, 5-fluorodeoxyuridine (FdUrd, floxuridine), capecitabine (xeloda, a pro-drug for FUra), ralitrexed [tomudex, ZD1694, a folate-based inhibitor of thymidylate synthase (TS)], or ZD9331 (BGC9331). FUra and FdUrd have been widely used in the treatment of colorectal cancer *(8–10)* and are extensively metabolized inside the cell. The metabolite 5-fluorodeoxyuridylate inhibits TS in the presence of 5,10-methylene tetrahydrofolate (CH_2-H_4-PteGlu), resulting in depletion of thymidine-5′-triphosphate (dTTP) as well as elevation in 2′-deoxyadenosine-5′-triphosphate (dATP) *(11)*, induction of DNA damage *(11)*, and S-phase arrest *(12,13)*. DNA-directed effects may also include misincorporation of 2′-deoxyuridine 5′-triphosphate (dUTP) or 5-fluro dUTP (FdUTP) into DNA *(14,15)*. Alternatively, incorporation of 5-fluorouridine triphosphate (FUTP) into RNA interferes with RNA processing and is the primary mechanism by which toxicity of FUra is induced in normal Gastrointestinal (GI) tissues *(16,17)*, in contrast to DNA-directed cytotoxicity that is important in chemotherapeutic response *(15,18,19)*. Apoptosis has been studied extensively in response to TS inhibitors and can be influenced by the status of the p53 tumor-suppressor gene. S-phase arrest occurs rapidly in response to TS inhibition *(13,20)*. In HCT116 cells that express wtp53, cells progress to the G2-phase of the cell cycle, and apoptosis occurs rapidly. In contrast to the presence of mp53, S-phase arrest is maintained during drug exposure, after which time delayed apoptosis is evident *(20)*. Adenoviral transduction of p53 sensitizes cells to FUra-induced apoptosis *(13,21)*. P53 appears to influence the kinetics of apoptosis induction but not overall response as determined by clonogenic survival *(2,13)*. FUra-resistant cell lines have demonstrated reduced apoptosis *(22)*.

The cyclin-dependent kinase inhibitor $p21^{Waf1}$ has demonstrated a significant role in influencing sensitivity to TS inhibitors, influencing both apoptosis *(20,23)* and clonogenic survival *(20)*, in a p53-independent manner. Using isogenic HCT116 cell lines, parental cells were highly sensitive to ZD9331, in contrast to $p21^{Waf1}$ −/− cells that were completely resistant *(20)*. Both cell lines accumulated in S-phase by 24 h after ZD9331 exposure; however, wt cells progressed to G2 and underwent apoptosis prior to mitosis in contrast to $p21^{Waf1}$ −/− cells that re-entered a normal cell cycle. Furthermore, selective induction of $p21^{Waf1}$ in RKO cells was sufficient to induce apoptosis *(20)*. S-phase arrest appears to occur through Chk1-mediated proteolysis of Cdc25A *(24,25)*, leading to inhibition of Cdk2.

Chemotherapeutic agents have generally been considered to activate the intrinsic apoptotic pathway. It has been reported that caspase-8 *(26–28)* and/or caspase-3 *(27,28)* have been directly activated by FUra, and inhibitors of caspase activation can influence the extent of apoptosis induced *(13,29,30)*. However, after co-incubation with the pancaspase inhibitor Z-VAD-fmk, only partial but not complete protection from thymineless stress-induced apoptosis *(31)* or loss in clonogenic survival *(31,32)* was demonstrated in colon carcinoma cells. Caspase inhibitors can block the appearance of the apoptotic phenotype but may delay rather than prevent cell death *(33,34)*. Whether the ensuing death is a form of apoptosis or occurs through an alternative mechanism still remains to be clarified.

Several studies have now demonstrated the involvement of the extrinsic pathway of apoptosis in response to TS inhibitors. The Fas death receptor, belonging to the tumor necrosis factor (TNF) receptor superfamily, is critical in the induction of thymineless stress-induced cell death in TS^- cells *(35)* (Fig. 1), as a component in the mechanism of FUra-induced cell death both *in vitro (36–38)* and in vivo *(39)* or in capecitabine-induced apoptosis *(40)*. Figure 1 demonstrates that in the presence of the NOK-1 antibody that blocks the interaction between Fas and FasL, protection is afforded from the induction of cell death in TS^- cells following thymidine (dThd) deprivation, in contrast to co-incubation with an IgG1 isotype-matched control antibody or control cells. Fas is downregulated in expression in approximately 50% of colon carcinomas *(41,42)*; however, the cytokine interferon-γ upregulates the expression of Fas at the cell surface and sensitizes cells to both Fas-mediated and FUra-induced cell death *(36)*. Agents that target Fas, including the cytolytic anti-Fas antibody CH-11, induce synergistic cytotoxicity (apoptosis and clonogenic survival) when combined with FUra *(36,38,43)*. In the presence of wtp53, FUra induces the expression of Fas *(36,38)*. The cytotoxic ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also belonging to the TNF family of death receptors and their ligands, induces a synergistic interaction in combination with FUra both *in vitro (44,45)* and *in vivo (46)*, which

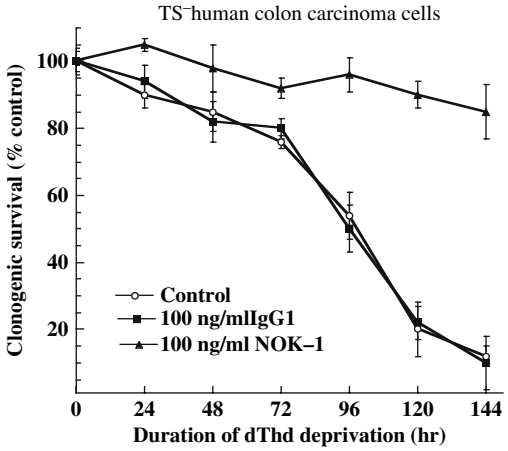

Fig. 1. The extrinsic pathway of apoptosis through the Fas death receptor is involved in the induction of thymineless death in thymidylate synthase $(TS)^-$ cells, determined by clonogenic survival assay *(35)*.

may involve upregulated expression of TRAIL receptors *(47)*, enhanced formation of a death-inducing signaling complex (DISC) *(44,45)*, or downregulated expression of cellular FLICE inhibitory protein (c-FLIP) *(43,44)*. Elimination of the function of DR5 in HCT116 cells conferred resistance to FUra in vivo *(48)*. Genes that are known to directly regulate apoptosis at other sites can influence sensitivity to this class of agent. Drug resistance has been determined in several studies to be influenced by the constitutive expression of nuclear factor NF-kB *(49–51)*. Downregulation of NF-KB *(50–52)* or Bcl-2 *(53)* or overexpression of Bax *(54,55)* sensitized cells to FUra-induced or ralitrexed-induced apoptosis. Furthermore, Bcl-w was found to be an important determinant of FUra sensitivity in intestinal epithelia *(56)*. In human head and neck squamous cell carcinoma xenografts, overexpression of Bax induced apoptosis in response to ralitrexed as well as enhancing tumor regression *in vivo (54)* (Fig. 2). Current data suggest that at least under certain conditions, apoptosis is an important determinant of the cytotoxic activity of TS inhibitors both *in vitro* and *in vivo*, in experimental models.

A third general pathway for the induction of apoptosis originates from the endoplasmic reticulum, resulting in the activation of caspase-12 and caspase-9 *(57–59)*; however, a potential role in antimetabolite-induced apoptosis has not yet been established.

Expression of other genes that are involved in sensitization to TS inhibitor-induced apoptosis includes overexpression of the $p16^{INK4A}$ tumor-suppressor gene *(60)*, oncogenes (HPV16 E6/E7) *(61)* or E2F-1 *(62)*, c-myb (where cells from c-myb−/− mice were hypersensitive to FUra) *(63)*, or treatment with exogenous sphingomyelin *(64)*.

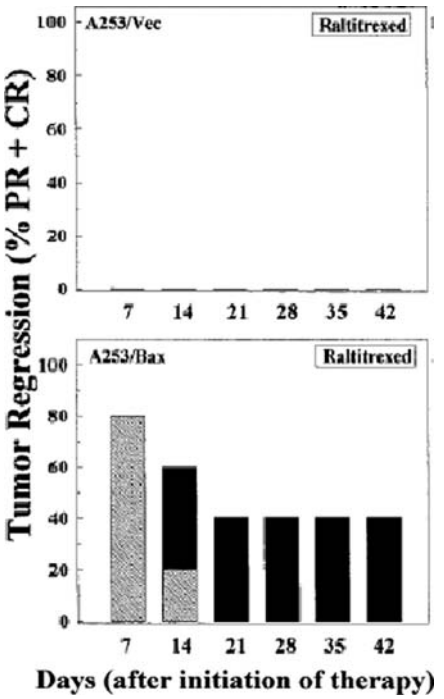

Fig. 2. Tumor responses (partial response+complete response) (PR+CR) in nude mice bearing A253/Vec and A253/Bax xenografts induced by ralitrexed at the maximum tolerated dose (MTD). Each group represents 10–20 animals from two to four independent experiments. CR rates ($p < 0.001$) *(54)*.

In contrast, HaRas overexpression conferred resistance to FUra-induced apoptosis *(65)*, likely mediated through the PI-3K signaling pathway *(66)*, and Cox-2 overexpression has attenuated FUra-induced apoptosis *(67,68)*. DNA mismatch repair (MMR) can detect and initiate responses to many lesions in DNA, including those induced by FUra. MMR-deficient HCT116 cells were significantly more resistant to fluoropyrimidines than MMR-proficient cells, as determined by clonogenic survival, reflecting tolerance to DNA damage *(69,70)*. Although DNA fragmentation was detected and effects were dThd reversible, cytotoxicity did not appear to be reflected in the extent of apoptosis induced in this system.

Clinically, the role of apoptosis regulatory proteins in overall response and survival following treatment with fluoropyrimidines is being evaluated, and this chapter will focus on their expression in colorectal cancer. Few studies have evaluated the correlation between Bcl-2 expression and response of colorectal cancers to chemotherapy. Schneider et al. *(71)* did not find a correlation between Bcl-2 expression and either response to FUra-based therapy or overall survival in advanced colorectal carcinoma. However, Bcl-2-negative lesions were more frequent among patients who reached an objective clinical response when treated with FUra-containing regimens *(72)*. Bax mutations and hence reduced Bax expression occur in colon carcinomas *(73–75)*. In stage III primary colorectal carcinomas, low Bax expression was a negative prognostic factor in these tumors *(76)*, and in patients undergoing surgery for advanced colorectal cancer, patients with Bax^+ primary tumors had significantly better prognosis (5-year survival) than patients with Bax^- tumors *(77)*. Low Bax expression was an independent prognostic marker in patients with metastatic disease of the liver *(78)*. For the IAP family member survivin, expression occurs in 53–61% of adenocarcinomas *(79–81)* and has demonstrated significant correlations with survival rates in colorectal cancers *(79,81)*. High survivin expression has been associated with reduced apoptosis in rectal cancer and may predict for reduced disease-free survival after preoperative radiochemotherapy and surgical resection *(80)*. Elevated survivin/Fas ratios have predicted for recurrent disease in pediatric patients with renal cell tumors *(82)* and for a more aggressive behavioral pattern in neuroblastomas as well as for worse outcome *(83)*. Downregulation of survivin has sensitized tumor cells to chemotherapy *(84)*, although little is currently known concerning survivin as a prognostic factor in response to fluoropyrimidine therapy. Thus, Bax and survivin are examples of genes that are critical in the regulation of apoptosis and clinically may influence treatment response and outcome.

1.1.2. Cyd Analogs

Agents of this class include gemcitabine (2′, 2′-difluorodeoxycytidine, dFdC, Gemzar), Ara-C (cytosine arabinoside, cytarabine), 2-chlorodeoxyadenosine (2-CdA), and 5-aza-2′-deoxycytidine (5-dAzaC, decitabine, DAC). Ara-C is widely used in the treatment of leukemias, the predominant mechanism for cytotoxicity involving incorporation of the drug into DNA during replication following metabolism by dCyd kinase *(85)*. Gemcitabine, used widely in the treatment of lung cancers, is also metabolized intracellularly by dCyd kinase. Decreased dCyd kinase expression is associated with resistance to both agents *(86–88)*, whereas other genes including the M2 subunit of ribonucleotide reductase (RRM2) *(89)*, or the human equilibrative nucleoside transporter 1 *(87)*, are also associated with gemcitabine resistance. Sensitivity to agents of this class is enhanced in the presence of wtp53 *(88,90,91)*.

Cyd analogs appear to utilize predominantly the intrinsic apoptotic pathway requiring mitochondrial involvement in the induction of apoptosis, caspase activation, which is inhibited in the presence of high levels of Bcl-2 or Bcl-xL *(92–95)* or potentiated when these proteins are downregulated *(96–98)* and appears independent of Mcl-1 *(99)*. The ratio of Bax/Bcl-2 has been predictive for the sensitivity of pancreatic carcinoma cell lines to gemcitabine *(100)*. As with TS inhibitors, mitochondrial damage and apoptosis appear to be delayed, but not eliminated, by overexpression of Bcl-2 or Bcl-xL *(94)*. This is consistent with studies that have demonstrated that Bcl-2 can protect cells from apoptosis but not overall loss in clonogenic survival, likely because of the occurrence of dual modes of cell death *(101)*. Z-VAD-fmk has exerted similar effects in drug-induced cytotoxic responses *(102)*.

The intrinsic pathway of apoptosis induced by Cyd analogs is also influenced by secondary signaling pathways that regulate the overall cytotoxic response. Thus, PI-3K *(103–105)*, MAP kinase (MAPK) *(106–108)*, or protein kinase C (PKC) *(109)* influence sensitivity to Cyd analogs. Inhibition of Src tyrosine kinase sensitizes pancreatic carcinoma cells to gemcitabine *(110,111)*. Constitutive activation of NF-KB is linked to drug resistance *(112–114)*, and proteasomal synthesis inhibitors that prevent the function of NF-KB *(115–117)* sensitize cells to these agents. Regarding the role of death receptors in apoptosis induced by Cyd analogs, there is no evidence that these proteins are directly involved *(118,119)*, although TRAIL can cooperate with gemcitabine to amplify apoptotic signaling and apoptosis through the mitochondria *(120)*.

There is little information available from clinical studies on the prognostic value of expression of apoptosis regulatory proteins in response to gemcitabine or Ara-C administered alone. However, in preclinical studies, Colo357-bax tumors overexpressing Bax in SCID mice demonstrated 12-fold greater volume regression than Colo357-wt tumors *(92)*. Gemcitabine combined with the Src tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine (PP2) significantly reduced tumor masses and prevented the onset of metastatic disease in an orthotopic model *(110)*. Furthermore, gemcitabine in combination with the proteasomal synthesis inhibitor Velcade inhibited the growth of non-small cell lung cancer (NSCLC) xenografts *(115)* or in combination with bortezomib inhibited the growth of bladder carcinoma xenografts *(117)*. These studies suggest that *in vitro* approaches that target pathways of apoptosis in response to Cyd analogs have relevance to the overall cytotoxic response in vivo.

An alternate strategy for Cyd analogs is the use of 5-azacytidine or 5-dAzaC as demethylating agent to reverse the documented methylation of critical regions of genes in human cancers, thereby causing the re-expression of these genes after several days *(121)*. To this end, 5-dAzaC appears to be more effective. Clinically, these agents have been used in the treatment of acute myeloid leukemia (AML) and acute myelodysplastic syndromes *(122,123)*. 5-dAzaC has enhanced cellular sensitivity to DNA-damaging agents, including cisplatin, doxorubicin, VP-16, and temozolomide *(124–126)*. Considerable focus has been placed on the ability of 5-dAzaC to induce the re-expression of critical caspases involved in the induction of apoptosis. Cell lines or tumors derived from aggressive neuroblastomas with amplification of *n*-Myc demonstrate deletion or silencing of the caspase-8 gene primarily through methylation of the promoter region, independent of caspase-9 or Apaf-1.

Treatment with 5-dAzaC or re-introduction of caspase-8 to these cells conferred sensitivity to Fas-induced apoptosis, to TRAIL-induced apoptosis, and to chemotherapeutic agents *(127–130)*. Caspase-8 expression is also lost in primitive neuroectodermal tumors *(131)*. 5-dAzaC has also been used to upregulate the expression of caspase-9 in human lung carcinoma cells to enhance p53-induced apoptosis *(132)* and to upregulate caspase-1 expression in renal cancer cells both *in vitro* and *in vivo*, which slows tumor growth indicating that caspase-1 may be involved in oncogenesis *(133)*.

1.1.3. Purine Analogs, Hydroxyurea, and Methotrexate

Three analogs of adenosine including 2′-deoxycoformycin (dCF), 2-CdA, and fludarabine (9-β-D-arabinosyl-2-fluoroadenine) are in current clinical use for the treatment of lymphoproliferative diseases. dCF inhibits the enzyme adenosine deaminase [reviewed in *(134)*], resulting in the cellular accumulation of dAdo and dATP, which induces cytotoxicity. In contrast, 2-CdA and fludarabine are metabolized by dCyd kinase to nucleoside triphosphates, incorporated into DNA, induce S-phase arrest, and are cytotoxic to dividing cells [reviewed in *(135,136)*], influenced by the ratio of dCyd kinase/5′-nucleotidase *(137)*. Both agents also induce cytotoxicity in non-dividing cells, which may involve reduction in RNA synthesis, and depletion of cellular nicotinamide adenine dinucleotide (NAD) and ATP [reviewed in *(138)*]. The induction of apoptosis has been established for both agents in leukemic cells *(139–142)*. Although these agents activate caspases *(143)*, following co-incubation with purine analogs and Z-VAD-fmk, secondary necrosis was detected, and the overall induction of cell death was not inhibited *(144)* (Fig. 3). In cells from patients with B-cell chronic lymphocytic leukemia (B-CLL), 2-CdA induced translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus. In the presence of caspase inhibitors, development of the classic apoptotic phenotype was prevented; however, loss in mitochondrial membrane potential, AIF apoptosis inducing factor translocation, and cell death still proceeded *(145)*.

The S-phase-specific cytotoxicity in dividing ML-1 cells involves activation of Jun N-terminal kinase1 (JNK-1) *(146)*. Cells expressing wtp53 are more sensitive to purine analogs *(147)*, and apoptosis induced by these agents is also influenced by NF-KB *(148)*, Bcl-xL *(149)*, and the ratio of Bax/Bcl-2 *(150)*. Of interest, the ex vivo response to fludarabine of blasts derived from CLL patients correlated with downregulated expression of the survival factor BFL-1 *(151)* but did not correlate with Bax expression levels *(152)*. Furthermore, failure of CLL patients to achieve complete remission correlated with high levels of Mcl-1 expression *(153)*.

The thiopurine antimetabolites, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), are analogs of the purine nucleosides hypoxanthine and guanine, respectively, and are important agents in the treatment of acute lymphoblastic leukemia (ALL) [reviewed in *(154,155)*]. Anabolism to ribonucleotides involves hypoxanthine–guanine phosphoribosyltransferase (HGPRT), or alternatively, 6-MP and 6-TG are S-methylated by thiopurine *S*-methyltransferase to yield nucleobases that are inactive *(156)*. The cytotoxicity of 6-MP and 6-TG is considered to occur through incorporation into DNA resulting in interference with the function of DNA polymerases, ligases, and endonucleases and may involve DNA MMR *(157)*. Thiopurines inhibit de novo purine synthesis *(158)*, and decreased HGPRT determines acquired resistance *(159)*. 6-MP induces apoptosis in activated B lymphocytes *(160)* and in hamster fibrosarcoma cells *(161)*. Apoptosis

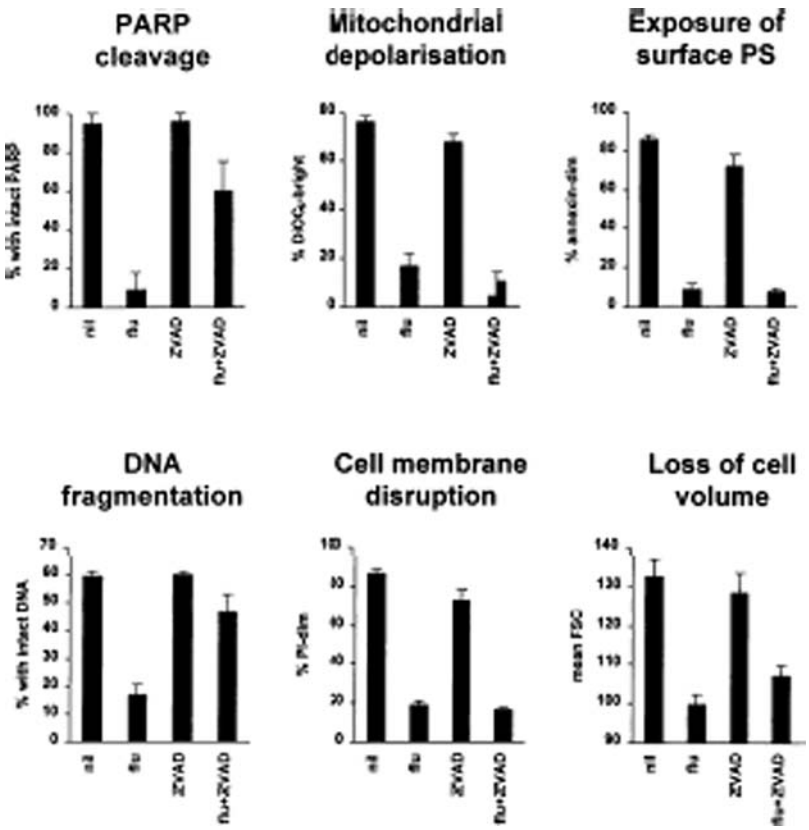

Fig. 3. Effect of Z-VAD-fmk on fludarabine cytotoxicity in lymphocytic leukemia (CLL) cells. Fludarabine induced extensive killing as measured by all six criteria. Poly (ADP-ribose) polymerase (PARP) cleavage and DNA fragmentation indicate that cell death occurred by apoptosis, whereas cell membrane disruption indicates that the majority of apoptotic cells had undergone secondary necrosis. Z-VAD-fmk produced selective inhibition of PARP cleavage and DNA fragmentation *(144)*.

was induced rapidly in activated B cells (within 6 h) and was associated with decreased activity of nitric oxide synthase and decreased Bcl-2/Bax that would otherwise promote cell survival in mature B lymphocytes *(162)*.

Hydroxyurea (HU) is commonly used for the treatment of chronic myeloid leukemia (CML), polycythemia vera, and thrombocytopenia *(163)*. HU depletes dNTP pools by inhibiting RR, thereby inhibiting the conversion of ribonucleotides to deoxyribonucleotides, inducing DNA damage, and preventing DNA replication *(164)*. HU has also been shown to induce apoptosis *(165)*.

Methotrexate (MTX) and its polyglutamates are inhibitors of dihydrofolate reductase (DHFR) and other folate metabolizing enzymes, thereby causing the depletion of reduced folates, dTTP, purines, inhibition of DNA synthesis, and cell death. Intrinsic and acquired resistance mechanisms both *in vitro* and *in vivo* include defective transport, amplification, or altered MTX affinity for DHFR, decreased MTX polyglutamates, or elevated MRP that enhances drug efflux [reviewed in *(166)*]. MTX has been shown to induce cell death through apoptosis in fibrosarcoma cells *(161)*, CHO cells *(167)*, HL60 and Jurkat leukemia cells *(168,169)*, breast cancer cells *(170)*, and intestinal epithelial cells characterized by the sequential activation of caspase-9,

caspase-2, and caspase-3 prior to DNA fragmentation *(171)*. In breast cancer cells, this process may be dependent on caspase-3 but independent of p53 *(170)*. In other cell lines, however, MTX did not appear to induce apoptosis *(169,172)*. Studies suggest that in contrast to FUra and other agents, MTX may not induce a synergistic interaction when combined with TRAIL *(173)* or anti-Fas Abs *(174)*. MTX is used extensively in combination with other agents in the treatment of ALL and in solid tumors.

Although metabolic processes that influence cytotoxicity and drug resistance have been extensively studied for these agents, the induction of apoptosis and the regulatory mechanisms involved in apoptosis are not well documented, although apoptosis has been conclusively demonstrated in certain systems. Furthermore, combination chemotherapy approaches used in clinical therapy make cell death mechanisms in response to individual agents of these groups difficult to determine.

1.2. Anoikis

Anoikis constitutes apoptosis induced in response to lack of adhesion or inappropriate adhesion in cell–matrix interactions and is characterized by all of the features that govern apoptosis *(175)*. It occurs mainly in epithelial cells to prevent cellular shedding and to assure proper developmental positioning of the cells in specialized structures. Anoikis utilizes predominantly the Fas pathway in inducing caspase-dependent apoptosis, FAS-associated death domain (FADD), and also integrin receptors *(176)*. However, the involvement of anoikis in antimetabolite-specific treatment responses has not been well investigated.

1.2.1. Mitotic Catastrophe

An emerging literature suggests that alternative mechanisms of programmed cell death (PCD), independent of caspase activation, can be invoked by chemotherapeutic agents and include mitotic catastrophe, autophagy, or paraptosis. In addition to the mitochondria, lysosomes and the endoplasmic reticulum can play a key role in these processes, resulting in the release of proteases [reviewed in *(177)*].

Mitotic cell death or mitotic catastrophe leads to the formation of interphase cells with multiple micronuclei. This may also occur following aberrant reentry of tumor cells into cycle after prolonged growth arrest. Mitotic catastrophe can occur in apoptosis-competent cells prior to the induction of apoptosis, although apoptosis is not required to elicit cell death. Checkpoint deficiencies in tumor cells promote mitotic catastrophe [reviewed in *(178)*]. In the case of antimetabolites that predominantly inhibit DNA synthesis, this form of cell death could conceivably result from the premature induction of mitosis before the appropriate completion of S-phase or G2-phase as observed for other forms of cellular damage following premature chromatin condensation *(179,180)*. The G2 checkpoint appears critical in preventing mitotic cell death in cells treated with DNA-damaging agents. The vast majority of studies that have determined the induction of mitotic catastrophe in neoplastic cells have utilized the DNA-damaging agents doxorubicin *(181)*, bleomycin *(182)*, or etoposide *(101)* or the microtubule poison taxol *(183)*. Cells that die through mitotic catastrophe do in general not demonstrate DNA ladder formation or DNA strand breaks detectable by TUNEL staining *(178)* and die in the presence of overexpressed Bcl-2 that inhibits apoptosis but not loss in clonogenic survival following drug treatment *(101)*. Very few studies have addressed this form of cell death following treatment with

Table 1
Effects of Different Agents on HTI080 3′SS6 Cells

Agent	ID_{85}	*% SA-β-gal+*	*% micronucleated*	*% sub-G_1*
None		1	1.5	0.4
Doxorubicin	30 nM	79	45	10
Aphidicolin	200 ng/ml	64	45	10
Cisplatin	2.2 μM	55	47	9.3
γ-irradiation	1300 rad	36	63	3.2
Cytarabine	1.5 μM	23	64	7.5
Etoposide	900 nM	15	55	7.5
Taxol	5 ng/ml	9.1	66	7.1
Vincristine	1.5 nM	3.1	62	13

Reproduced from *(181)*.

antimetabolites. In one study, cytarabine (1.5 μM) induced micronucleation in 64% of HT1080 cells in the absence of apoptosis, indicative of mitotic death equivalent to that observed following treatment with taxol or γ-irradiation *(181)* (Table 1). However, following FUra treatment of HeLa or H1299 human lung cancer cells, downregulation of Chk1 expression by Chk1siRNA abrogated FUra-induced S-phase arrest and potentiated FUra-induced cell death following premature chromatin condensation, M-phase progression, and the induction of apoptosis in the presence of a 2N DNA content. This is in contrast to abrogation of the S-phase checkpoint during treatment with CPT-11 that resulted in mitotic catastrophe and cell death in the presence of a 4N DNA content *(25)*. HCT116 cells treated with the TS inhibitor ZD9331 accumulated in S-phase and subsequently progressed to G2 where they underwent apoptosis prior to M, in contrast to HCT116 p21 −/− cells that demonstrated more prolonged accumulation in S-phase followed by cell-cycle re-entry in the absence of apoptosis *(20)*. Although overexpression of Bcl-2 or treatment with Z-VAD-fmk, that inhibit apoptosis, can have less effect on the overall response to TS inhibitors as determined by clonogenic survival assay *(51)*, the contribution of mitotic cell death to this class of agent and other antimetabolites remains to be unequivocally determined.

1.3. Autophagy (Type II) and Paraptosis

Autophagy is characterized by sequestration of bulk cytoplasm and organelles into autophagic vesicles with subsequent degradation by the cellular lysosomal system *(177)*. It can be downregulated by mutation in phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and activation of Akt signaling *(184)*, induced in the presence of DN-Akt and regulated by target of rapamycin (TOR) *(185)*. Autophagy is often observed when apoptosis is suppressed *(186)*. Suppression of autophagy may be involved in the process of tumorigenesis *(187)*. Drugs including vinblastine, rapamycin, and the estrogen antagonist tamoxifen can induce autophagy *(185)*; however, the contribution of this mode of cell death to overall response has yet to be determined, and no studies linking this form of cell death to the cytotoxic response of antimetabolites have yet been reported.

Paraptosis is characterized by progressive swelling of the mitochondria and the endoplasmic reticulum, followed by cytoplasmic vacuolation *(177)*. This form of cell

death is caspase-independent and can be triggered by the TNF receptor family member TAJ/TROY *(188)* and the IGFIR *(189)*; however, its role in cell death induced by DNA-damaging agents including antimetabolites has not yet been established.

2. PASSIVE (NECROSIS) CELL DEATH

Necrosis is characterized by swelling of the cytoplasm and mitochondria, rupture of the plasma membrane, loss of chromatin *(190,191)*, and DNA fragmentation *(191,192)*, and *in vivo* induces an inflammatory response *(176)*. Peters et al. *(30)* demonstrated morphologic evidence of necrosis in the absence of apoptosis in WiDr colon carcinoma cells following treatment with FUra or ZD1694. An additional study employing electron microscopy of MKN45 gastric cancer cells demonstrated mixed patterns of cell death in the population following FUra treatment, including two distinct patterns of necrosis *(191)*. As mentioned earlier, necrosis has been detected as a secondary form of cell death in response to purine analogs *(144)* (Fig. 3). This mechanism of cell death induction has not been well documented in response to antimetabolites, however, when present may contribute to the overall survival of cell populations.

3. SENESCENCE ARREST AND DIFFERENTIATION

Replicative senescence limits the proliferative life span of normal cells *(193)*, whereas accelerated senescence, which is more rapid, can be induced by treatment with DNA-damaging agents *(194)* or introduction of Ras or Raf oncogenic mutants into normal cells *(195,196)*. The phenotype is characterized by enlargement and flattening of cell shape, increased granularity, and expression of senescence-associated β-galactosidase (SA-β-gal). Terminal arrest requires the function of p53, $p21^{Waf1}$, $p16^{INK4A}$, and the shortening of telomeres [reviewed in *(197,198)*].

Earlier studies of differentiation induction by antimetabolites have most frequently involved leukemic cells following treatment with Cyd analogs and are most likely linked to the induction of cellular senescence. Thus, Ara-C induced differentiation of U937 leukemic cells involving $p21^{Cip1}$ *(199)*, and a Cyd deaminase-resistant analog of Ara-C induced differentiation in human myeloid leukemic cell lines *in vitro* and was active in Ara-C-resistant solid tumors in vivo *(200)*. In HT1080 cells, senescence induction was weaker and also independent from mitotic cell death following treatment with Ara-C in the absence of apoptosis *(193)*. In the human B-cell lymphoma cell line BL36, treatment with 5-AzaC decreased cell growth following cell-cycle arrest at the GO/G1-phase, and cells demonstrated phenotypic changes consistent with a differentiated phenotype *(201)*. 5-dAzaC induced re-expression of $p16^{INK4A}$ and the expression of SA-β-gal in hepatocellular carcinoma cells *(202)*. Furthermore, MLL cells were effectively induced to differentiate by all-trans retinoic acid or dihydroxyvitamin D3 in the presence of 5-dAzaC, which effectively induced the expression of $p16^{INK4A}$ *(203)*.

Cellular senescence has also been induced by BrdUrd *(204,205)* or HU *(206)* in several cell lines and by MTX in MCF-7 cells dependent on p53 and caspase function *(170)*. Inhibition of caspase function has switched doxorubicin-induced apoptosis to senescence, although this has not yet been demonstrated for antimetabolites *(207)*. In one study utilizing tumor samples from patients receiving neoadjuvant therapy for breast cancer that involved treatment with cyclophosphamide, doxorubicin, and FUra, SA-β-gal staining was observed in 41% of the specimens and was associated

with low p53 staining (wtp53) and high expression of $p16^{INK4A}$ *(208)*. Hence, it is evident that cell death processes in addition to apoptosis can be induced in the same cell population by antimetabolites and that cellular senescence may play a role in overall response to agents in preclinical model systems and also clinically.

4. CONCLUSIONS

Although the mechanisms that induce cell death following exposure of cancer cells to DNA-damaging agents both *in vitro* and *in vivo* are still being determined, it is evident that the mechanism most studied in response to antimetabolites is the induction of apoptosis. It is clear that apoptosis can be responsible for cell death in cultured cell models and in preclinical models, and clinically, the expression of certain genes that regulates apoptotic responses has yielded prognostic significance in certain studies. However, data generated in the presence of overexpression of Bcl-2 or Bcl-xL, or during co-exposure to inhibitors of caspase activation, indicate that in other systems, the kinetics of induction of cell death may be delayed but not prevented, suggesting that apoptosis may not contribute significantly to the overall survival of a population following drug treatment. Other mechanisms of induction of cell death have been identified in response to antimetabolites, including mitotic catastrophe, necrosis, and senescence, and mixed forms of cell death have also been identified within the same cell population. This field of research is an emerging area in our understanding of the mechanisms by which specific forms of DNA damage kill cells, and a considerable amount is still to be learned about cellular responses of cancer cells induced following DNA damage by the various classes of antimetabolites.

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20 Topoisomerase I Poisons and Apoptotic Topoisomerase I-DNA Complexes

Olivier Sordet, PhD, Yves Pommier, MD, PhD, and Eric Solary, MD, PhD

Summary

Topoisomerase I (Top1), an abundant nuclear enzyme expressed throughout the cell cycle, relaxes DNA supercoiling by forming transient covalent DNA cleavage complexes. We will review three situations leading to enhanced cellular Top1 cleavage complexes. First, Top1 cleavage complexes can be stabilized by camptothecins, which are referred to as Top1 poisons and among the most efficient inducers of apoptosis. The second mechanism is related to exogenous and endogenous DNA lesions that enhance Top1 cleavage complexes. Lastly, Top1 cleavage complexes form during programmed cell death and are then referred to as "apoptotic Top1 cleavage complexes."

Key Words: DNA topoisomerase I; Top1 poisons; Top1 cleavage complexes; camptothecins; apoptosis; mitochondria; arsenic trioxide; staurosporine.

1. INTRODUCTION

DNA topoisomerase I (Top1) is an ubiquitous and essential enzyme as it relaxes DNA supercoiling ahead of replication and transcription complexes *(1–3)*. DNA relaxation proceeds through induction of transient single-strand breaks, thereby allowing rotation of the DNA double helix around the intact phosphodiester bonds opposite to the enzyme-mediated DNA cleavages. Once the DNA is relaxed, Top1 readily religates the break and restores intact duplex DNA. Under normal conditions, the covalent Top1-cleaved DNA intermediates, referred to as "cleavage complexes," are transient. At a given time point, their number remains very low as the DNA religation ("closing") step proceeds much faster than the DNA cleavage ("nicking") step. There are three situations leading to enhanced cellular Top1 cleavage complexes (Fig. 1). First, Top1 cleavage complexes can be stabilized by "Top1 poisons," including camptothecin and

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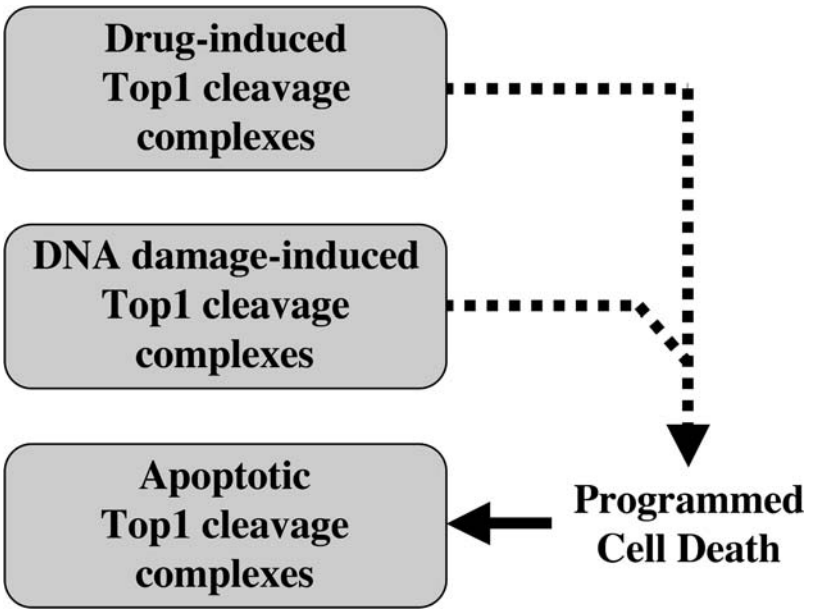

Fig. 1. Induction of cellular Topoisomerase I (Top1) cleavage complexes. Top1 cleavage complexes can be stabilized/induced (i) by cytotoxic drugs, referred to as "Top1 poisons"; (ii) by endogenous and exogenous DNA damage; and (iii) during cell death by apoptosis (apoptotic Top1 cleavage complexes). Drug-induced and DNA damage-induced Top1 cleavage complexes can trigger apoptosis and generate apoptotic Top1 cleavage complexes.

its chemotherapeutic derivatives that bind specifically at the Top1–DNA interface and trap the cleavage complexes by preventing the DNA religation step (for review, see ref. *4*). Secondly, endogenous and exogenous DNA damage, including oxidatized bases, abasic sites, mismatches, strand breaks and carcinogenic adducts *(5–7)*, can interfere with Top1's DNA nicking-closing activities (for review, see ref. *8*). Lastly, Top1 cleavage complexes form during apoptotic cell death that occurs in response to a wide range of stimuli. These complexes, which are likely a general response of cells undergoing apoptosis, are referred to as "apoptotic Top1 cleavage complexes" (for review, see ref. *9*). Connections exist between these three situations as drug-induced and DNA damage-induced Top1 cleavage complexes can trigger apoptosis, thus generating apoptotic Top1 cleavage complexes (Fig. 1). Moreover, apoptotic Top1 cleavage complexes are probably due, at least in part, to oxidative DNA damage.

This chapter focuses first on camptothecins and non-camptothecins Top1 poisons. We will present a common molecular mechanism for the stabilization of Top1–DNA complexes by Top1 poisons, which is referred to as the "5′-end misalignment model." We will describe the apoptotic pathways activated by Top1 poisons. Emphasis will be given to the transmission of the apoptotic signal from the nucleus (DNA lesions) to the cytoplasm (mitochondrial permeabilization). Lastly, we will review the current knowledge on the formation and functional relevance of apoptotic Top1 cleavage complexes. The biochemistry and cellular biology of Top1, the DNA damaging lesions

leading to Top1 cleavage complexes and the DNA repair pathways elicited by Top1 cleavage complexes have been recently reviewed elsewhere and will be only mentioned here *(1–3)*.

2. NOVEL TOPOISOMERASE I POISONS

Camptothecin is a natural compound isolated from *Camptotheca acuminata* (Fig. 2). The sodium salt camptothecin was found to be clinically active but its use was discontinued in the 1970s because of severe side effects *(10)*. In 1985, camptothecin was shown to specifically poison DNA Top1 *(11)*, which generated great interest and provided new opportunities for finding more efficient and less toxic analogs of camptothecin.

2.1. Camptothecin Derivatives

Two water-soluble camptothecin derivatives are currently used for the treatment of human cancers: Irinotecan (CPT-11, Camptosar®) for advanced colorectal carcinomas and Topotecan (Hycamtin®) for ovarian cancers *(12)*. A number of other camptothecin derivatives are in clinical trials: 9-nitrocamptothecin (SuperGen) *(13)*, exatecan mesylate (DX-8951f) *(14)*, Afeletecan® (Bayer AG), CKD-602 (Chong Kun Dang Pharmaceutical Corp.), DRF-1042 (Dr. Reddys Research Foundation), PEG-camptothecin (Prothecan®, Enzon Inc.), MAG-camptothecin (PNU-166148, Pharmacia), ST1481 (Sigma-Tau Healthsci SpA), Homa-copolymer-camptothecin (University of London) and Karenitecin® *(12)*.

Homocamptothecins retain potent Top1 inhibition *(15–18)* and differ from camptothecin by the presence of an additional methylene group in the E-ring, which limits the conversion to the inactive carboxylate. Conversely, the inactive carboxylate of homocamptothecins cannot be converted to the lactone once the E-ring is open *(16)*. The binding of homocamptothecins in the Top1–DNA complex is likely to be similar to the binding of camptothecins as Top1 mutations that confer resistance to camptothecins confer cross-resistance to homocamptothecins *(15)*. However, because of their greater potency, homocamptothecins remain more active in camptothecin-resistant cells *(15)*. The difluorohomocamptothecin derivative BN-80915 (diflomotecan), currently tested in clinical trials, is more potent than SN-38 (active metabolite of CPT-11) and produces more stable cellular cleavage complexes *(19)*.

The camptothecin derivatives presently in the clinic have two major limitations: (i) at physiological pH, the labile alpha hydroxylactone, which is essential for camptothecin activity *(20)*, is in equilibrium with its inactive (carboxylate) form, which binds to

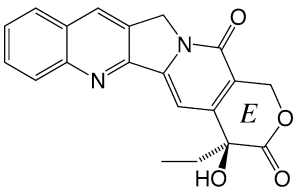

Fig. 2. Structure of camptothecin

serum albumin *(21)*; (ii) the camptothecin-trapped cleavage complexes reverse within minutes after drug removal, which imposes long and/or repeated infusions for cancer treatment.

2.2. Non-Camptothecin Topoisomerase I Poisons: Polyheterocyclic Aromatic Inhibitors

The indolocarbazoles represent the most advanced class of non-camptothecin derivatives in terms of chemotype, clinical development and structure activity *(22–24)*. Among the Top1 inhibitor, indolocarbazole derivatives, NB-506 and J-107088, have recently been selected for clinical trials. Like camptothecins, indolocarbazoles prevent the religation of a subset of Top1 cleavage complexes. By contrast to camptothecin, indolocarbazole generally binds to DNA by intercalation, and the DNA sequence selectivity of these cleavages differ from the pattern of cleavage sites induced by camptothecins *(25–28)*.

A second class of non-camptothecins polyheterocyclic aromatic inhibitors is the indenoisoquinolines. The synthesis of the indenoisoquinoline NSC-314622 was first reported in 1978 *(29)*. Consecutively, a series of indenoisoquinolines were synthesized and found to possess significant anticancer activity *(30,31)*. However, little was known about their anticancer mechanism until recently when a COMPARE analysis of cytotoxicity in the National Cancer Institute (NCI) *in vitro* Anticancer Drug Discovery Screen of 60 cell lines revealed that NSC-314622 was a Top1 inhibitor *(32)*. The patterns of DNA breaks produced by Top1 in the presence of indenoisoquinolines differ from camptothecins. Because of their novel structure, a large number of indenoisoquinolines have been synthesized and tested for Top1 inhibition and antiproliferative activity in the NCI cell screen over the past 3 years *(33–35)*. Generally, the indenoisoquinolines that inhibit Top1 are cytotoxic in the NCI cell lines *(33–35)*. Antitumor activity is also observed for some of these compounds in animal models *(35)*. Indenoisoquinolines are in preclinical development, and current efforts are focusing on testing the antitumor activity of selected indenoisoquinolines (e.g., compound MJ-III-65) in animal models. A co-crystal structure of indenoisoquinolines in the Top1-DNA complex has recently been obtained *(36)*.

The pyrroloquinazolinoquinoline alkaloid luotonin A and its derivatives were reported recently to poison also Top1 *(37)*. Luotonins and camptothecins are structurally related and share the same sequence selectivity of DNA cleavage by Top1. However, luotonins are less efficient than camptothecins at trapping of Top1 cleavage complex and show only weak antiproliferative activity.

Other polyheterocyclic Top1 poisons include nitidine, coralyne, berberine and benzo[a]acridine derivatives. These compounds share a common heterocyclic ring system and generally bind to DNA by intercalation. Although some of them exhibit antiproliferative activity, to the best of our knowledge, they are not in clinical development (for further details, see ref. *22*).

2.3. Non-Camptothecin Topoisomerase I Poisons: Benzimidazoles and Minor Groove Ligands

The bis-benzimidazole dyes, Hoechst 33342, and its parent compound Hoechst 33258 (NSC-32291, pibenzimol) represent a structurally unique class of Top1

poisons. Ho-33342 is commonly used for histochemical staining and flow cytometry analysis of DNA content. Hoechst 33342 and 33258 reversibly trap Top1 cleavage complexes with different sequence selectivity than camptothecins *(38)*. They both bind to Adenine, Thymine (AT)-rich sequences, causing widening of the DNA minor groove *(39)*. However, minor groove binding is not sufficient for Top1 trapping as distamycin, berenil and netropsin do not poison Top1 *(40)*. Hoechst 33342 also disrupts TATA box-binding protein/TATA box element binding *(40)*, suggesting other targets besides Top1.

In recent years, a series of benzimidazoles *(41)*, bibenzimidazoles *(42)* and terbenzimidazole derivatives *(43,44)* have been studied with modifications of the 5-position or 2″-position of terbenzimidazoles. A number of 5-substituted terbenzimidazoles can poison Top1 in biochemical assays. 5-phenyl-terbenzimidazole (5PTB) is the most effective in cell culture assays *(43,44)*. Studies with poly(dA).poly(dT) duplex DNA suggest that 5PTB intercalates and binds in the DNA minor groove *(44)*.

Ecteinascidin-743 (Et-743, NSC-648766) is a potent antitumor agent from the Caribbean tunicate *Ecteinascidia turbinata*. Et-743 is in phase II and III clinical trials, with remarkable activity in soft tissue sarcomas and solid tumors including ovarian carcinoma *(45,46)*. Et-743 traps Top1 cleavage complexes *in vitro* and in cancer cells *(47,48)*. The distribution of the drug-induced Top1 sites is different for Et-743 and camptothecin *(48)*. A derivative of Et-743, phthalascidin (Pt-650), was also found to poison Top1 cleavage complexes *in vitro* and in cells *(47)*. However, Top1 is probably not the primary cellular target of Et-743 as the drug remains cytotoxic in yeast with a deletion in the *Top1* gene *(49)* and in mammalian cells deficient in Top1 *(50)*. Also, Top1 inhibition is only detectable at micromolar concentrations exceeding pharmacologically active concentrations *(51,52)*. Recent studies revealed that Et-743 acts by a novel mechanism of action: poisoning of transcription-coupled nucleotide excision repair *(53)*.

2.4. Top1 Cleavage Complexes Induced by DNA-Damaging Agents

Chemotherapeutic agents that damage DNA can also trap Top1 (for details, see ref. *8*). Incorporation of the nucleoside analogs cytosine arabinoside, gemcitabine (2′-difluorocytosine) and 5-fluorouracile *(54)* immediately downstream from a Top1 cleavage complex prevent the Top1-mediated DNA religation *(55,56)*. Alkylating agents, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, also trap Top1 *(57)*. The resistance of Top1-deficient cells to these drugs *(56,57)* suggests the contribution of Top1 poisoning to their antiproliferative activity. Besides chemotherapeutic agents, Top1 can be trapped by naturally occurring endogenous and carcinogenic DNA lesions, ranging from UV dimers *(58)*, oxidative base lesions, base mismatches and abasic sites *(59)*, DNA strand breaks *(60)*, the carcinogenic adducts, N6-ethenoadenine *(61)*, benzo[*a*]pyrene diol epoxide and formaldehyde adducts *(6,7)* (for review, see ref. *8*). It is not known how frequently such Top1 cleavage complexes form. However, the ubiquitous expression of Tdp1 (tyrosyl-DNA phosphodiesterase), an enzyme specifically involved in the repair of Top1-DNA covalent complexes (for review, see ref. *3*), suggests selective pressure for removing Top1 cleavage complexes, and therefore their natural occurrence.

3. MOLECULAR MODEL FOR TOPOISOMERASE I INHIBITION: MISALIGNMENT OF THE 5′-HYDROXYL END OF THE CLEAVED DNA AND INTERFACIAL INHIBITION

3.1. Binding of Camptothecins and Polycyclic/Heterocyclic Poisons to the Top1–DNA Complex

Camptothecins are non-competitive inhibitors of Top1. They inhibit Top1 by binding both to Top1 and the cleaved DNA *(20,62)*. Consequently, they uncouple the Top1's nicking-closing reaction by preventing the DNA religation ("closing") step. This unique mode of action defines the concept, referred to as "interfacial inhibition" *(63,64)*, that it is possible to interfere with two macromolecules (i.e., Top1 and DNA) to stabilize their interaction. This concept is a paradigm for drug discovery as one of the present objectives in drug development is to interfere with macromolecule interactions. According to this concept, it is conceivable to look for drugs that prevent the dissociation of two macromolecules rather than inhibiting their binding.

Top1 cleavage complexes are not equally trapped by camptothecins. Trapping is most effective at DNA sequences with a T at the 3′-end of the scissile DNA strand, which corresponds to the DNA end covalently linked to Top1 [position −1 (Fig. 3A)],

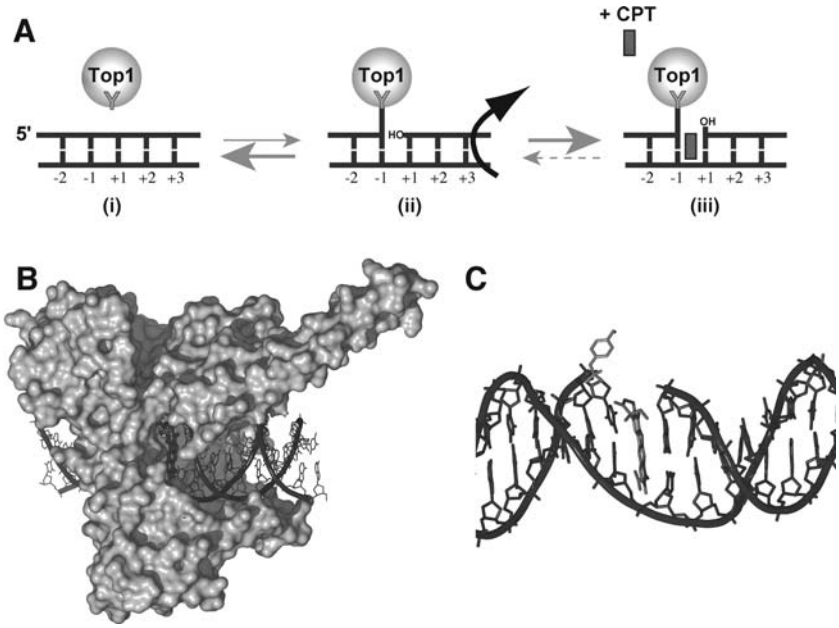

Fig. 3. Interfacial inhibition of Topoisomerase I (Top1) cleavage complex by camptothecins. (A) Schematic representation of the Top1 catalytic cycle with the cleavage complex (panel ii) allowing DNA relaxation by rotation of the cleaved DNA strand around the intact strand at the break site. Camptothecin (CPT) and its clinical derivatives (topotecan) trap the cleavage complex and shift the normal DNA nicking-closing equilibrium. Y = tyrosine. (B, C) Crystal structure of topotecan (or CPT) bound to the Top1 cleavage complex. Top1 is shown surrounding the DNA and topotecan (at the center) is shown at the interface between Top1 and the DNA. In (C), only the tyrosine of Top1 that binds to the broken DNA is shown. Topotecan is shown intercalated between the base pairs flanking the Top1 cleavage site.

and a G at the 5′-end of the broken DNA [position +1 (Fig. 3A)]. It is this DNA sequence dependence that led to the hypothesis that camptothecin formed a ternary complex with Top1 and the DNA by binding at the Top1–DNA interface *(65)*. This hypothesis was further strengthened by the finding that a derivative of camptothecin with an alkylating group at position 7 can form an adduct with the +1 guanine (at the N3 position) in the presence of active Top1 *(66)*.

It has now been shown that camptothecins stabilize Top1 cleavage complexes by forming a ternary complex, Top1–DNA–drug. In the topotecan co-crystal with an irreversible Top1 cleavage complex *(67)* (Fig. 3B and C) and in the proposed models, the camptothecin polycyclic rings intercalate at the Top1–DNA interface between the bases that flank the DNA cleavage site generated by Top1 *(20,65,66,68–70)*. Camptothecin prevents therefore the DNA religation by keeping the 5′-end of the broken DNA out of alignment with the Top1–DNA phosphotyrosyl bond that needs to be attacked by the 5′-hydroxyl of the broken DNA during religation (Fig. 3).

Recently, experiments with intercalating ligands demonstrated position-specific trapping of Top1 cleavage complexes by polycyclic hydrocarbons (benzo[*a*]pyrene diol epoxide adducts) intercalated between the bases that flank the Top1 cleavage site or that are immediately downstream from the cleavage site *(5,71)*. A unifying model is that the polycyclic aromatics (camptothecins, indolocarbazoles, indenoisoquinolines, luotonins, coralyne, berberine and nitidine derivatives) bind to a common site in the Top1–DNA complex by stacking (intercalating) either on the 5′-side or the 3′-side of the base pair immediately downstream [position +1 (Fig. 3A)] from the Top1 cleavage site *(70)*. The differences in DNA cleavage patterns (i.e., differential intensity of cleavage at any given site) between compounds might be due to specific interactions between particular drugs and the bases flanking the Top1 cleavage site *(70)*.

A potential exception to this model has been proposed for nogalamycin *(72)*, which traps Top1 cleavage complexes by intercalating away from the Top1 cleavage site and by inducing a local bent downstream from the Top1 cleavage, which interferes with DNA religation. Thus, nogalamycin bound to a Top1–DNA complex may act similarly to minor groove ligands.

3.2. General Model for Top1 Poisoning: "5′-Terminus Misalignment"

Minor groove binding drugs (such as benzimidazoles and Et-743) poison Top1 by binding immediately downstream (3′) from the cleaved DNA strand. This would alter the structure of the DNA downstream from the cleavage site, preventing DNA religation by Top1. Trapping of Top1 by base modifications at position +1 (for review, see ref. *8*) results probably also in structural modifications of the broken end downstream from the Top1 cleavage site. Together, the molecular observations presented above lead to a relatively simple and general mechanism for the trapping of Top1 cleavage complexes: presence of a ligand that either intercalates or binds to the minor groove, or presence of DNA modifications that result in a misalignment of the 5′-hydroxyl DNA terminus, and interfere with the religation of Top1 cleavage complexes. As indicated at the beginning of this section, the inhibitors act in a non-competitive manner by preventing the dissociation of Top1–DNA complex.

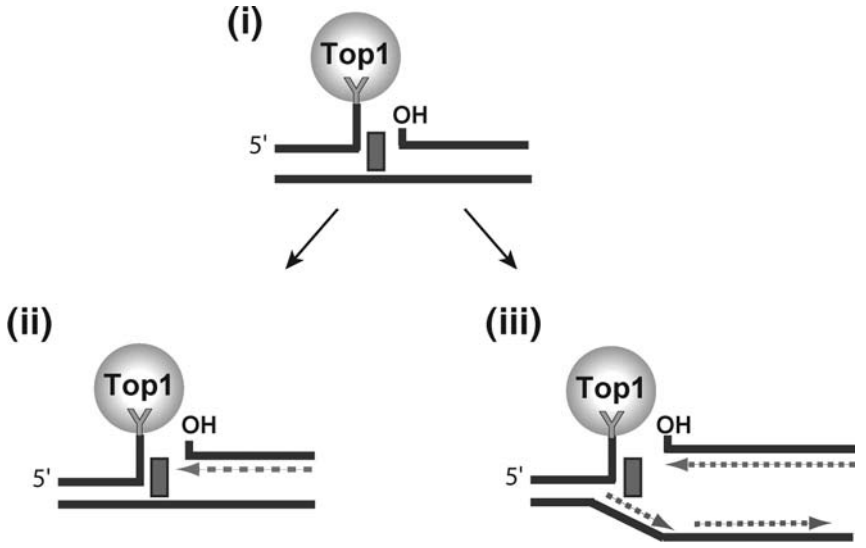

Fig. 4. Conversion of Topoisomerase I (Top1) cleavage complexes into DNA damage during replication and transcription. (i) Schematic representation of a Top1 cleavage complex trapped by camptothecin (rectangle). Top1 is covalently bound to the 3′-end of the broken DNA. The other end is a 5′-hydroxyl (OH). (ii) Conversion of the cleavage complex into an irreversible covalent Top1–DNA complex by a colliding transcription complex (left dashed arrow represents RNA). (iii) Conversion of the cleavage complex on the leading strand into an irreversible covalent Top1–DNA complex associated with a replication DNA double-strand break by a colliding replication fork (left dashed arrow represents leading replication; right dashed arrows represent lagging replication).

4. CELLULAR LESIONS INDUCED BY TOPOISOMERASE I POISONS

Top1 cleavage complexes are readily reversible after camptothecin removal, and short exposures to camptothecin (for less than 1 h) are relatively non-cytotoxic. Persistent drug exposure is required to induce apoptosis, as Top1 cleavage complexes are converted into irreversible DNA lesions by cellular metabolism (Fig. 4). DNA double-strand breaks (DSBs) are generated during replication *(73)*. During transcription, collision of the elongating RNA polymerase II with a Top1 cleavage complexes on the leading strand can induce a duplex DNA–RNA and generates in all cases an irreversible DNA lesions *(74)*. Transcription-dependent apoptosis appears mainly mediated by p53 (for review, see ref. *75*).

Although in most cancer cells, the cytotoxicity of camptothecin appears primarily related to replication-mediated DSBs *(11,76)*, the protection of cells by the DNA polymerase inhibitor aphidicolin is generally limited. A replication-independent cytotoxicity can be observed in non-replicating cells such as neurons *(77)* and lymphocytes (Sordet and Pommier, unpublished).

5. SENSING DNA DAMAGE INDUCED BY TOPOISOMERASE I POISONS: ATM, ATR AND DNA-PK

Proteins and protein complexes, called "DNA sensors," bind to (or at the proximity of) DNA lesions and activate checkpoint and repair proteins. ATR, ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) are serine/threonine kinases known to be activated by Top1 poisons-mediated DNA damage.

ATM responds preferentially to DSBs *(78)*. However, the activation of ATM does not strictly require its physical interaction with DNA breaks. Alteration of chromatin structures induced by interruptions of DNA integrity might generate the initial signal for ATM activation *(79)*. Such changes in chromatin topology induce trans-phosphorylation (on S1981), which dissociates ATM dimers into active ATM monomers *(79)*. Recent reports suggest that the protein phosphatase-5 and phosphatase-2A might be involved in ATM activation *(80,81)*. Activated monomeric ATM phosphorylates nucleoplasmic substrates such as p53 *(79,82)*. Nbs1, Brca1 and other BRCT proteins are believed to recruit ATM substrates, including Chk2. ATM can also phosphorylate the BRCT proteins *(83)* such as Nbs1, Brca1, Smc1, 53BP1 and Mdc1.

ATR (ataxia-telangiectasia and RAD3 related) is activated by replication-associated DNA damage *(84)*. According to the current model, the binding of ATR to stressed replication forks is mediated by replication protein A (RPA). The persistent and longer stretches of single-stranded DNA associated with stalled replication forks are coated by RPA polymers that serve as a template for the recruitment of the ATR-interacting protein–ATR complex *(85)*. The concomitant (but ATR-independent) loading of RAD17, the 9-1-1 complex (Hus1, Rad1 and Rad9) and claspin cooperates for full activation of ATR.

DNA-PK consists of the Ku heterodimer (Ku70/80) and the catalytic serine/threonine kinase subunit (DNA-PKcs). Whether DNA-PKcs requires the binding of Ku proteins to the DNA ends for activation is still a matter of debate. Autophosphorylation of DNA-PKcs also seems to be required for efficient DNA repair, in spite of a loss of kinase activity *(86)*. In addition, Ku70 has been recently involved in apoptosis (see Section 7.2.).

The above-mentioned DNA sensors activate cellular effectors leading to cell cycle arrest, DNA repair and/or apoptosis. The following sections focus on the apoptotic pathways elicited by Top1 poisons-mediated DNA damage. The DNA repair and checkpoint pathways have been described elsewhere *(3)*.

6. MITOCHONDRIAL AND PLASMA MEMBRANE RECEPTOR PATHWAYS ARE ACTIVATED BY TOPOISOMERASE I POISONS

Camptothecins are among the most efficient inducers of apoptosis. They activate the mitochondrial "intrinsic" pathway and, in some cell types, the transmembrane "extrinsic" pathway. Both pathways lead to full caspase activation. Caspases belong to two groups: initiator and effector caspases. Initiator caspases are activated as they bind to adapter molecules, after which, they activate effector caspases. The initiator caspase for the mitochondrial pathway is caspase-9 (and possibly caspase-2), whereas the initiator caspases for the transmembrane pathways are caspase-8 and caspase-10. Both pathways share the effector caspases (caspase-3, caspase-6 and caspase-7). These latter enzymes cleave intracellular targets, leading to biochemical and morphological changes that characterize apoptosis.

6.1. The Mitochondrial "Intrinsic" Pathway

Most chemotherapeutic drugs, including Top1 poisons, increase the permeability of the outer mitochondrial membrane as they induce apoptosis *(87)*. This permeabilization causes bioenergetic failure and permits the release of soluble molecules from the intermembrane space of the mitochondria to the cytosol. These molecules

include apoptosis-inducing factor (AIF) and endonuclease G that migrate to the nucleus to condense chromatin and cleave DNA, respectively. Other mitochondrial proteins cooperate in the cytosol to activate a cascade of caspases (cytochrome c, Smac/DIABLO and HtrA2/Omi) *(87)*. Cytochrome c induces oligomerization of apoptosis protease activating factor-1 (Apaf-1) in the presence of ATP *(89)*. Apaf-1 oligomers recruit procaspase-9 in a complex called the "apoptosome" where juxtaposition of procaspase-9 molecules results in autoactivation *(90,91)*. Mature caspase-9 activates additional caspase-9 molecules as well as caspase-3 and caspase-7. In turn, caspase-3 activates downstream caspases in a proteolytic cascade *(92)*. Smac/DIABLO and HtrA2/Omi, released simultaneously with cytochrome c, neutralize proteins of the inhibitor of apoptosis protein (IAP) family. Some of these IAPs associate to procaspase-9, caspase-3 and caspase-7 to inhibit their proteolytic activity, possibly by triggering their proteosomal degradation after ubiquitinylation *(93)*.

The Bcl-2 family of proteins controls, at least in part, the release of the above-mentioned mitochondrial proteins *(94,95)*. This family includes more than 30 anti-apoptotic and pro-apoptotic molecules characterized by the presence of 1–4 conserved Bcl-2 homology (BH) domains (BH1–BH4). Schematically, the anti-apoptotic proteins of the family such as Bcl-2 itself and Bcl-xL inhibit mitochondrial permeabilization, whereas the pro-apoptotic "multidomains," mainly Bax and Bak, exert the opposite effect. The BH3-only proteins (including Bid, Bad, Bim, Noxa, Puma and many others) act as sensors of cellular damage. Each of them is activated by a specific cell damage and translocates to mitochondria to promote their permeabilization by activating Bax and Bak proteins and/or by inhibiting Bcl-2 and Bcl-xL *(96)*.

6.2. The Transmembrane "Extrinsic" Pathway

In some cell types, apoptotic response to Top1 poisons implicates the plasma membrane receptor Fas (APO-1/CD95). Crosslinking of Fas by its natural ligand (FasL) induces clustering of Fas, which in turn recruits the adapter Fas-associated death domain (FADD) *(97)* and procaspase-8 *(98)* to form the death-inducing signaling complex (DISC). When oligomerized in the DISC, procaspase-8 auto-activates and releases mature caspase-8 *(99)*, which induces apoptosis by two different pathways depending on the cell type *(100)*. In type I cells, large amounts of active caspase-8 formed at the DISC induce direct cleavage/activation of procaspase-3 and the downstream caspase cascade independently of mitochondria. In type II cells, small amounts of DISC and active caspase-8 are insufficient to directly activate procaspase-3 but sufficient to cleave the "BH3-only protein" Bid, generating an active fragment (tBid) that migrates to the mitochondria and activates the mitochondrial death pathway *(101,102)*.

Top1 poisons are among the various anticancer drugs that activate the death receptor pathway by enhancing the expression of Fas and FasL *(103–105)*. At least in some cell types, this up-regulation is transcription dependent and implicates p53 *(106)*. Interaction of FasL with Fas at the cell surface defines an autocrine/paracrine pathway similar to that observed in activation-induced cell death in T lymphocytes. However, the role of FasL in drug-induced apoptosis is probably not essential because antagonist antibodies or molecules that prevent FasL interaction with Fas *(103,107)* do not suppress apoptosis. Anticancer drugs can induce Fas clustering at the cell surface of tumor cells in the absence of FasL *(108)*. This clustering could take place in lipid rafts at the plasma membrane *(109)*. However, apoptosis induced by chemotherapeutic

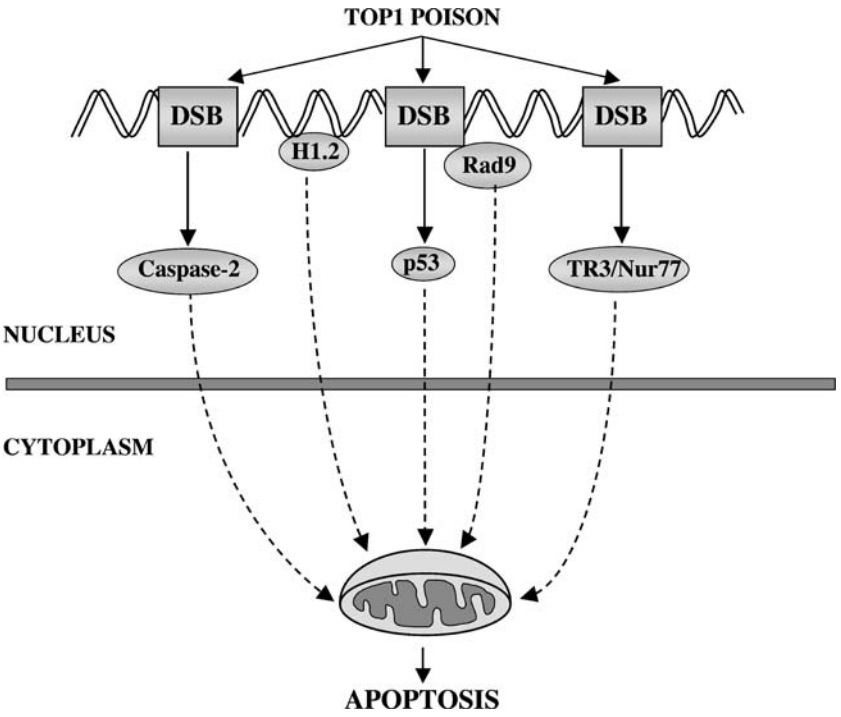

Fig. 5. Overview of the transduction of Topoisomerase I (Top1)-mediated DNA damage signals from the nucleus to mitochondria. Top1-mediated DNA damage induces the nucleo-cytoplasmic redistribution of caspase-2, histone H1.2, p53, Rad9 and TR3/Nur77, which promote mitochondrial permeability and apoptosis. See text for further details. Dashed lines represent nucleo-cytoplasmic redistribution.

drugs is not altered in embryonic fibroblasts from FADD *(110)* and caspase-8 *(111)* knockout mice indicating only a partial role for the death receptor pathway in response to chemotherapeutic agents.

In summary, Top1 poisons generally induce apoptosis by the mitochondrial pathway. This pathway can be amplified, at least in some cell types, by the transmembrane pathway. To the best of our knowledge, Top1 poisons and other genotoxic agents (e.g., ionizing radiation) activate the same transductional pathways to permeabilize mitochondria during apoptosis and have been therefore extensively reviewed *(112,113)*. The following section will provide examples of molecules that transmit apoptotic signals from the nucleus (DSBs) to the cytoplasm (mitochondria) (Fig. 5).

7. FROM DNA DAMAGE IN THE NUCLEUS TO APOPTOSIS IN THE CYTOPLASM

As we discussed in Section 5., ATM (and also ATR and DNA-PK) responds preferentially to DSBs. Top1 poisons generate DSBs during replication. Four known substrates of ATM are implicated in apoptosis: p53, Chk2, E2F1 and c-Abl (http://discover.nci.nih.gov/mim/) (see also ref. *87*). ATM phosphorylates/activates directly p53 and E2F1 thereby enhancing their stability and transcriptional activity.

ATM can also activate p53 and E2F1 indirectly by phosphorylating/activating the checkpoint kinase Chk2. The third Chk2 substrate implicated in apoptosis is promyelocytic leukemia (PML). ATM also phosphorylates/activates c-Abl. PML-mediated, c-Abl-mediated and E2F1-mediated apoptosis can occur through both p53-dependent and p53-independent pathways. Thus, apoptosis induced by DNA damage can schematically be divided into a p53-dependent manner and/or p53-independent manner.

7.1. p53-Dependent Apoptosis Induced by DNA Damage

p53 is a transcription factor (for review, see ref. *114*) for a set of pro-apoptotic proteins from the Bcl-2 family (Bax, Bid, Noxa and Puma) that promote mitochondrial permeabilization, and thereby the release of cytochrome c. p53 also induces ASC (apoptosis-associated speck-like protein) that relocalizes Bax to mitochondria *(115)*. Several other mitochondria-targeting proteins are induced by p53. Some of these, such as ferrodoxin reductase, are involved in the production of reactive oxygen species (ROS), while others permeabilize mitochondria either directly or indirectly by less understood mechanisms. These proteins include p53AIP (p53-regulated apoptosis-inducing protein-1), mitochondrial chloride intracellular channel-4 *(116)*, PIDD (p53-induced protein with a death domain; implicated in caspase-2 activation; see below), and histone H1.2 (see below). p53 also transactivates components of the transmembrane "extrinsic" pathway (e.g., Fas/CD95, DR5 and PERP), proteins located to the endoplasmic reticulum (see ref. *117*), caspase-6 and the procaspase-9 adapter Apaf-1. p53 also represses the transcription of the anti-apoptotic proteins Bcl-2 and survivin (an IAP that prevents caspase activity and regulates cell cycle).

p53 also induces apoptosis in a transcription-independent manner by directly targeting mitochondria (Fig. 5). After DNA damage, a fraction of p53 is exported from the nucleus and binds to the outer mitochondrial membrane *(118)*. Mitochondrial p53 binds to Bcl-2 and Bcl-xL and neutralizes their inhibitory effect on Bak (and Bax), resulting in Bak oligomerization and subsequent mitochondrial permeabilization *(118)*. Bcl-2 and Bcl-xL interact with the DNA-binding region of p53, the same region that harbors the vast majority of "hot spots" mutations in human cancer cells. Thus, some p53 mutations that interfere with DNA binding also interfere with p53 binding to Bcl-2 and Bcl-xL.

It has been proposed that p53 triggers a rapid first wave (within minutes) of transcription-independent apoptosis that precedes a second slower/delayed (within hours) transcription-dependent wave. The importance of p53 mitochondrial translocation in the response to DNA damage is controversial. The direct mitochondrial effect of p53 is cell type dependent, as wild-type p53 in primary fibroblasts does not translocate to mitochondria whereas wild-type p53 in thymocytes does. The molecular basis of this difference is not known. In addition, p53 itself can bind to DNA strand breaks *(119)* and might therefore be involved also in DNA damage detection/repair.

Histone H1.2 also relocalizes to mitochondria following DSBs (Fig. 5). This process requires p53 but is independent of p53 transcriptionnal activity *(120,121)*. H1 is the histone subunit that binds the chromatin linker region between the nucleosomes. Humans and mice have eight histone *H*1 genes. Among them, all of the somatic H1s (H1.1–H1.5) are ubiquitously expressed in all tissues throughout development. After DSBs, all nuclear histone H1 variants relocate partially to the cytoplasm. However, only H1.2 is able to trigger cytochrome c release from mitochondria. The mechanism for

the nucleo-cytoplasmic redistribution of H1.2 is unclear as H1.2 does not undergo any obvious post-translational modification, neither change in expression. DSBs themselves or the subsequent p53-dependent repair process may cause H1.2 release from chromatin to the nucleoplasm and subsequently in the cytoplasm. At the mitochondrial level, H1.2 induces conformational activation and oligomerization of Bak. In mitochondria isolated from Bak-deficient cells, H1.2 fails to induce cytochrome c release. The mechanism for H1.2 to cause the conformational change of Bak and the subsequent cytochrome c release remains to be determined. H1.2 appears to respond specifically to DSB-induced apoptosis as down-regulation of H1.2 by antisense RNA or small interfering RNA (siRNA) reduces apoptosis induced by X-rays or the Top2 inhibitor etoposide, but not by tumor necrosis factor-α or UV radiations. Also, thymocytes and cells in the small intestine from H1.2-deficient mice show strong resistance to X-ray-induced apoptosis.

Procaspase-2 might also link DNA damage and mitochondria *(122)* (Fig. 5). The "PIDDosome," a molecular complex containing PIDD, whose expression is induced by p53, and RAIDD/CRAIDD, an adaptor protein with a death domain, activate procaspase-2 in the nucleus *(123)*. Increased PIDD expression results in spontaneous activation of procaspase-2 and sensitization to apoptosis by genotoxic stimuli *(123)*. In the nucleus, juxtaposition of procaspase-2 molecules (dimerization) results in auto-cleavage/activation *(124)*, a similar mechanism of activation to the initiator procaspase-8 and procaspase-9 (see Section 6.). Release of mature caspase-2 can stimulate directly mitochondrial release of cytochrome c. This process requires the processing of procaspase-2 but not its enzymatic activity and is also independent of Bax, Bak and Bcl-2 *(125)*.

p53 can activate multiple pathways besides apoptosis, including cell cycle arrest/checkpoint, DNA repair, senescence and angiogenesis. Both cell cycle arrest and apoptosis prevent replication of damaged DNA and represent a coherent response for p53 as "the guardian of the genome." However, the p53-mediated cell cycle arrest response might in some cases antagonize p53-mediated apoptotic response. For instance, activation of $p21^{CIP1}$, which was among the first isolated p53-dependent genes, induces both cell cycle arrest in response to low doses of camptothecin and blocks DNA damage-induced apoptosis *(126,127)*. p53 may selectively induce apoptosis in cells with elevated E2F1 activity, such as pRb-deficient cells. p53 binds to the cyclin A box of E2F1, and this complex induces apoptosis when cyclin A is low *(128)*. E2F1, like p53, is negatively regulated by Mdm2, and both E2F1 and p53 are up-regulated in response to DNA damage *(129)*. p53 may also specifically promote apoptosis when it is transcribed as a N-terminal truncated variant, designated p53/47. In contrast to p53, p53/47 lacks the Mdm2-binding domain. Thus Mdm2 expression increases the ratio p53/47 to p53. p53/47 has a different gene expression profile: up-regulation of Bax and down-regulation of p21 *(130)*. It is therefore possible that specific modifications of p53 might selectively activate apoptotic or cell cycle arrest genes. Depending on the cellular context, p53 might selectively activate one set of genes or the other. The pleiotropic regulation of p53 (for details, see ref. *131*) could allow fine tuned adjustments of p53 levels and p53 phosphorylation (depending on the intensity of DNA damage), which could account for the selectivity of p53 for transactivating cell cycle arrest and/or apoptotic genes.

7.2. p53-Independent Apoptosis Induced by DNA Damage

In spite of the apparent pivotal role of p53 in apoptosis *(132,133)*, p53-null cells such as HL60 and U937 human leukemic cells undergo apoptosis readily in response to DNA-damaging agents *(134–136)*. In these cells, the p53-related protein p73 does not compensate the lack of p53 as apoptosis is also transcription independent. Moreover, more than 50% of human tumors contain mutated and defective p53. Although such tumors might be defective in their apoptotic response *in vivo*, experiments performed in cell cultures demonstrate that these tumors can undergo apoptosis in response to Top1 poisons *(137)*. p53-independent apoptosis involves the receptor TR3 and the checkpoint kinase Rad9 that target directly mitochondria in response to DNA damage and also the DNA repair protein Ku70. Other p53-independent pathways include Chk2, E2F1, PML and c-Abl (for review, see ref. *87*).

The orphan receptor TR3 (also called Nur77 or nerve growth factor-induced protein B) is a transcription factor of the steroid/thyroid receptor superfamily. TR3 is involved in promoting cell proliferation *(138)*. It is also a critical inducer of apoptosis. *TR3* gene is rapidly induced by different apoptosis-inducing agents, and overexpression of a dominant-negative TR3 protein *(139)* or inhibition of TR3 expression by antisense TR3 mRNA *(140)* inhibits apoptosis. By contrast, constitutive expression of TR3 induces apoptosis *(141)*. Although the mitogenic effect of TR3 occurs in the nucleus through target gene regulation, the pro-apoptotic effect of TR3 occurs in the cytoplasm independently of its transactivating activity. In response to apoptotic stimuli (including DSBs), TR3 translocates from the nucleus to mitochondria where it induces the mitochondrial release of cytochrome c *(142)* (Fig. 5). Despite lacking classical mitochondria-targeting sequences, TR3 relocates to mitochondria by binding to the Bcl-2 N-terminal loop region. The TR3-Bcl-2 interaction induces a Bcl-2 conformational change that exposes its BH3 domain *(143)*. TR3 could therefore act on mitochondria by converting Bcl-2 from an anti-apoptotic to a pro-apoptotic member of the Bcl-2 family.

Another molecule that directly targets mitochondria is the cell cycle checkpoint Rad9 (Fig. 5). Rad9 is loaded as a complex with Hus1 and Rad1 (known as 9-1-1 complex) onto damaged chromatin by a clamp loader consisting of Rad17 and replication factor C *(144)*. The 9-1-1 complex promotes the phosphorylation/activation of Chk1 by ATR, which in turn induces cell cycle arrest. Following DNA damage, Rad9 can also migrate from the nucleus to mitochondria. The BH3-like domain of Rad9 interacts with Bcl-2 and Bcl-xL, which induces apoptosis *(145)*. Dual phosphorylation of Rad9 by c-Abl *(146)* (which is itself activated by ATM) and PKCδ *(147)* may be required for Rad9 to bind Bcl-2 and Bcl-xL. In summary, Rad9 seems to have two opposite functions: (i) cell survival by activating Chk1 and cell cycle arrest and (ii) cell death by activating the pro-apoptotic Bcl-2 proteins and mitochondrial pathway. The predominant role of Rad9 is likely to promote cell survival after Top1 poison-mediated DNA damage as *Rad9–/–* ES cells are hypersensitive to camptothecin *(148)*.

Ku70 is another molecule involved in both DNA repair and apoptosis. In addition to its nuclear localization, Ku70 is present in the cytoplasm where it binds to Bax, preventing Bax mitochondrial localization and pro-apoptotic activity *(149,150)*. Bax-mediated apoptosis is suppressed by overexpression of Ku70. By contrast, Bax-mediated apoptosis is enhanced by down-regulation of Ku70. Upon apoptotic stimuli, Bax is released from Ku70 *(150)*. Acetylation of Ku70 by p300/CBP and PCAF/GCN5 may induce this dissociation *(151)*. As a result, the N-terminus region of Bax is

exposed, allowing Bax to form a large complex, called the "baxosome," containing Bax itself, BH3-only proteins, cardiolipin, and probably other unidentified proteins. In the baxosome, Bax undergoes conformational changes, allowing its insertion into the outer membrane mitochondrial, where it promotes the release of cytochrome c and other pro-apoptotic molecules. Because Ku70 accumulates following DSBs, it is possible that Ku70 would prevent Bax from inducing a premature apoptosis (if the cell can still repair). However, beyond a certain threshold of DNA damage, Ku70 would release Bax to induce apoptosis.

The relative importance of these pathways in p53-deficient cells is not known. It will be interesting to use genetically altered cells and/or selective pharmacological inhibitors to determine the relative contribution of each of these pathways.

8. FORMATION OF TOP1 CLEAVAGE COMPLEXES DURING APOPTOSIS

Besides their role in the initiation of apoptosis (see Section 7.), stabilization of Top1 cleavage complexes occurs in cells undergoing apoptosis *(152)*. "Apoptotic Top1 cleavage complexes" have been detected in different human cell lines exposed to a wide range of stimuli, which by themselves do not act directly as Top1 inhibitors. Such stimuli include arsenic trioxide (As_2O_3) *(153)*, staurosporine *(154)*, etoposide, Fas-L (CD95-L, Apo-1L) or TRAIL (Apo-2L) (Sordet and Pommier, unpublished), indicating that formation of Top1 cleavage complexes is likely a general response of cells undergoing programmed cell death.

The formation of apoptotic Top1 cleavage complexes is, at least in part, related to DNA alterations that occur during apoptosis and interfere with Top1's nicking-closing activities (see Section 2.4.). Indeed, As_2O_3 and staurosporine induce oxidative DNA lesions as a result of ROS production, and modulation of ROS levels was directly correlated with the formation of Top1 cleavage complexes *(153,154)*. Accordingly, cellular exposure to H_2O_2 also induces Top1 cleavage complexes *(155)*. Thus, As_2O_3 and staurosporine (as well as a wide range of apoptotic stimuli) induce the generation of ROS that damage DNA (oxidized bases and abasic sites), thereby generating Top1 cleavage complexes in apoptotic cells (Fig. 6). It is also possible that some of the DNA breaks produced by apoptotic nucleases such as DFF40/CAD *(156)*, endonuclease G *(157)*, or Ape1 *(158)* contribute to the trapping of Top1 cleavage complexes *(60)*.

Mitochondria are likely to participate in the production of the ROS and the Top1 cleavage complexes during apoptosis as Bcl-2 overexpression prevents their formation *(153)*. Bcl-2 prevents the permeabilization of the outer mitochondrial membrane, and therefore, the release of cytochrome c and the downstream activation of caspases (see Section 6.1.). Activated caspase-3 feeds back on permeabilized mitochondria, which further dissipates the mitochondrial transmembrane potential ($\Delta\psi m$) and induces the further accumulation of intracellular ROS *(159,160)*. Caspase activation is involved in the generation of the apoptotic cleavage complexes as the general caspase inhibitor z-VAD-fmk prevents $As_2O_3^-$ and staurosporine-induced ROS and Top1 cleavage complexes *(153,154)*. Activation of caspases could therefore generate the ROS that lead to Top1 cleavage complexes during apoptosis (Fig. 6).

A critical question arising from these studies centers on defining the role of apoptotic Top1 cleavage complexes. Top1 down-regulation by siRNA in human cells

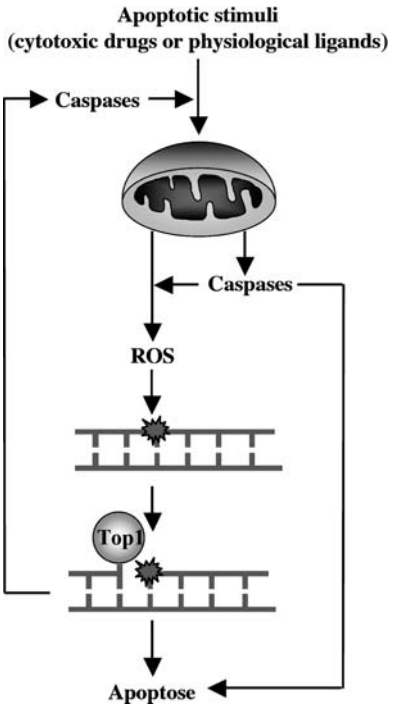

Fig. 6. Formation of apoptotic Topoisomerase I (Top1) cleavage complexes. Apoptotic stimuli (cytotoxic drugs or physiological ligands) induce the generation of reactive oxygen species (ROS) from mitochondria. ROS produce oxidative DNA lesions, which in turn generate Top1 cleavage complexes. These cleavage complexes might participate in apoptosis by generating DNA strand breaks and degrading chromatin. The Top1 cleavage complexes could also engage the apoptotic machinery in trans by activating caspases.

reduces DNA fragmentation induced by As_2O_3 and staurosporine *(153,154)*. Also, mouse P388/CPT45 cells lacking Top1 show a reduction in apoptotic DNA fragmentation when exposed to As_2O_3 or staurosporine as compared to P388 cells expressing Top1 *(153,154)*. Lastly, ectopic expression of Top1 in *Saccharomyces cerevisiae* confers sensitivity to H_2O_2 *(155)*. Thus, apoptotic Top1 cleavage complexes are likely to contribute to a programmed nuclear dismantling.

These findings raise the possibility that Top1, which is abundant and essential in mammalian cells, could also participate in apoptosis by engaging the apoptotic machinery in trans, as trapping of Top1 (for instance, by camptothecins) is among the most efficient inducers of apoptosis *(87)* (http://discover.nci.nih.gov/pommier/pommier.htm). Thus, apoptotic Top1 cleavage complexes could serve to amplify the apoptotic process initiated by chemotherapeutic drugs and possibly by physiological ligands such as Fas-L and TRAIL.

9. CONCLUSION

Cellular responses to Top1 poisons include DNA repair, cell cycle arrest and/or apoptosis and thus determine cell survival or cell death. It is becoming increasingly clear that cell cycle checkpoint and DNA repair pathways such as the ATM/Chk2,

Rad9 (9.1.1) and Ku70 pathways are also connected to the apoptotic pathways. A more complete molecular interaction network connecting these pathways will emerge in the near future. One of the challenge is to understand how these pathways are integrated and how in the presence of extensive DNA damage, the same DNA damage sensors and checkpoints that stop cell cycle progression and promote DNA repair can activate apoptosis. Thus, a promising new area of research is the elucidation of the relationships between specific DNA lesions, sensor proteins, checkpoints, DNA repair and apoptosis. Integration of these pathways in comprehensive molecular interaction maps *(87,131, 161–168)* (see also http://discover.nci.nih.gov/mim) should reveal the interplay between the cellular determinants of cellular response to topoisomerase inhibitors and other types of DNA damages. They should also provide opportunities to develop novel therapeutic strategies and markers to better predict and follow tumor responses to therapeutic agents.

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21 Perturbations of Cellular Functions by Topoisomerase II Inhibitors

All Roads Lead to Cell Death?

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Summary

DNA topoisomerase II inhibitors such as doxorubicin, daunorubicin, and etoposide are among the most widely used anticancer agents for treatment of both solid tumors and hematological malignancies. Clinically active topoisomerase II inhibitors are able to induce different types of cell death/growth arrest including apoptosis, mitotic catastrophe, and necrosis as well as drug-induced pseudo-senescence (DIPS), autophagy, and differentiation. Topoisomerase II may itself modulate the cell death processes induced by other classes of anticancer agents. Topoisomerase II is involved in the formation of high-molecular-weight apoptotic DNA fragments both directly and indirectly through its interaction with the caspase-activated DNase (CAD) nuclease. Topoisomerase II can also regulate the occurrence of mitotic catastrophe through its capacity to modulate mitotic entry and cell cycle progression in early mitosis.

Key Words: Topoisomerase II; anticancer agents; doxorubicin; etoposide; cell death; growth arrest; apoptosis; necrosis; mitotic catastrophe; drug-induced pseudo-senescence; autophagy.

1. DRUG-INDUCED CELL DEATH

Our understanding of the different types of cell death/proliferation arrest induced by anticancer agents and other cellular stress has substantially improved during recent years. However, the experimental evidence has also contributed to an increasing realization of the complexity of these cellular processes. Before reviewing our current knowledge about the mechanisms of cell death/proliferation arrest induced by topoisomerase II inhibitors, we like to discuss a number of factors that play an important role in the cellular response to anticancer agents. This includes tissue and cell type,

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the identity of the cytotoxic agent, and pharmacological factors such as dose and exposure time.

Cells can die or become growth-arrested by a number of different mechanisms including apoptosis, necrosis, mitotic catastrophe, drug-induced pseudo-senescence (DIPS), autophagy, and differentiation. However, all these processes are not necessarily operational in all cells and tissue types. Even a given cell death process induced by the same agent may not occur the same way in different tissues. For example, early work on apoptosis focused on thymocytes or cell lines like the widely used HL-60 human leukemia cell model, in which extensive endonucleosomal degradation could be observed within a few hours after drug exposure. By contrast, for solid tumor cell lines, even massive apoptosis is usually a much slower process appearing 12–24 h after drug exposure and which may not necessarily be accompanied by endonucleosomal DNA fragmentation.

Although most of the current literature classifies alkylating agents, antimetabolites, topoisomerase inhibitors, and even irradiation under the global term "DNA-damaging agents," there are substantial differences between the different classes of agents as well as between agents of the same class. As an example, the topoisomerase II inhibitor doxorubicin is able to induce several types of cell death as well as DIPS and differentiation. By contrast, amsacrine, another topoisomerase II inhibitor, is principally a strong inducer of apoptotic cell death. In this respect, it is interesting that doxorubicin shows major activity toward different types of solid tumors including breast cancer whereas the clinical activity of amsacrine is basically limited to leukemias.

An often neglected parameter in the literature is the importance of pharmacological factors such as dose and exposure time. It is well established that a short-term exposure to a high concentration of an anticancer agent may not necessarily result in the same biological effects as a sustained exposure to lower doses of the same drug, as observed clinically for the antimetabolite 5-fluorouracil. Furthermore, at comparable exposure times, a given drug may induce different types of cell death dependent on the drug concentration. For example, in HL-60 myeloid leukemia cells, daunorubicin induced rapid apoptosis at 0.5–1 μM but mitotic catastrophe and delayed apoptotic cell death at 0.1 μM. These concentrations are all within the clinical dose range *(1)*. This concept is further illustrated in Fig. 1.

At low drug concentrations, exposure to topoisomerase II inhibitors is accompanied by reversible cell cycle arrest, by DNA repair, or, if the DNA lesions are incompletely repaired, by mutagenesis. At higher drug concentrations, we may observe delayed

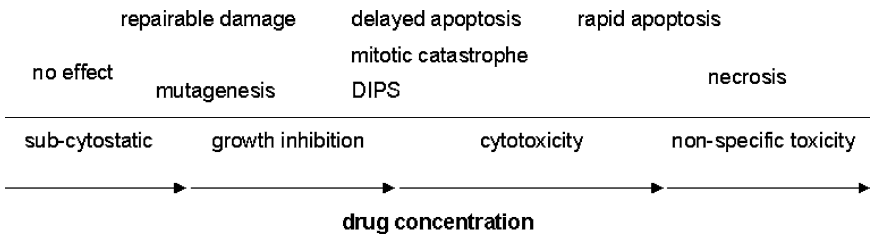

Fig. 1. Different cellular effects induced by topoisomerase II inhibitors as a function of drug concentration.

apoptosis, mitotic catastrophe, and DIPS. Finally, high drug concentrations usually lead to rapid apoptosis or necrosis. Taken together, we can conclude that the cellular outcome of exposure to genotoxic anticancer agents such as topoisomerase II inhibitors depends on both the genetic and epigenetic background (cell or tissue type, oncogenic alterations) and on pharmacological parameters such as the nature of the drug, dose, and exposure time.

2. TOPOISOMERASE II AND ITS INHIBITORS

The nuclear enzyme DNA topoisomerase II regulates DNA topology and chromatin organization in living cells *(2)*. In higher eukaryotes, there are two isoforms of topoisomerase II, alpha and beta, that are coded by different genes and have partially different cellular functions. However, both isoforms of the enzyme are able to relieve torsional stress in DNA by controlled breakage-reunion of both DNA strands and a passage of a DNA segment though the strand break. Topoisomerase II is absolutely essential for chromatin condensation and separation of sister chromatids during mitosis (for recent reviews, see *3* and *4*).

2.1. Inhibition of Topoisomerase II Leads to DNA Damage

All topoisomerase II-directed agents interfere with at least one step of the catalytic cycle and are thus classified as topoisomerase II inhibitors. DNA topoisomerase II inhibitors are among the most widely used anticancer agents and show clinical activity toward both solid tumors and hematological malignancies. Clinically used topoisomerase II inhibitors principally act by stabilizing a covalent DNA–topoisomerase II complex (the so-called cleavable complex) with nuclear DNA. These compounds are often classified as topoisomerase II poisons. Although the cleavable complexes themselves are reversible and disappear when the drug is removed, collision between the complexes and ongoing macromolecular synthesis can give rise to secondary DNA damage like single- and double-strand breaks that trigger diverse cellular responses such as cell cycle arrest, DNA repair, cell death pathways, and altered transcription (reviewed in *5*). It is currently believed that the cytotoxic and antitumor effects of topoisomerase II-directed agents are principally mediated by the induction of cleavable complexes and are further influenced by three factors: where in the genome the complexes are formed, how many complexes are present, and how long they last. It has long been debated whether topoisomerase II inhibitors preferentially induce cleavable complexes and/or secondary DNA damage in specific regions of the genome, such as Matrix Associated Region (MAR) sequences *(6)*. Quantitative and qualitative differences in DNA damage may also be a result of the capacity of the drug to inhibit either one or both isoforms of DNA topoisomerase II *(7)*. Specific cleavage at defined sites of the DNA could explain why different topoisomerase II inhibitors results in differential effects on the DNA transcription patterns.

A second group of topoisomerase II inhibitors is the so-called catalytic inhibitors. This group is a heterogeneous group of compounds that are able to inhibit the catalytic activity of topoisomerase II by a variety of mechanisms other than cleavable complex formation. This class of compounds has been extensively reviewed recently *(8)*. The most widely studied catalytic inhibitor is ICRF-187 (dexrazoxane) and structurally

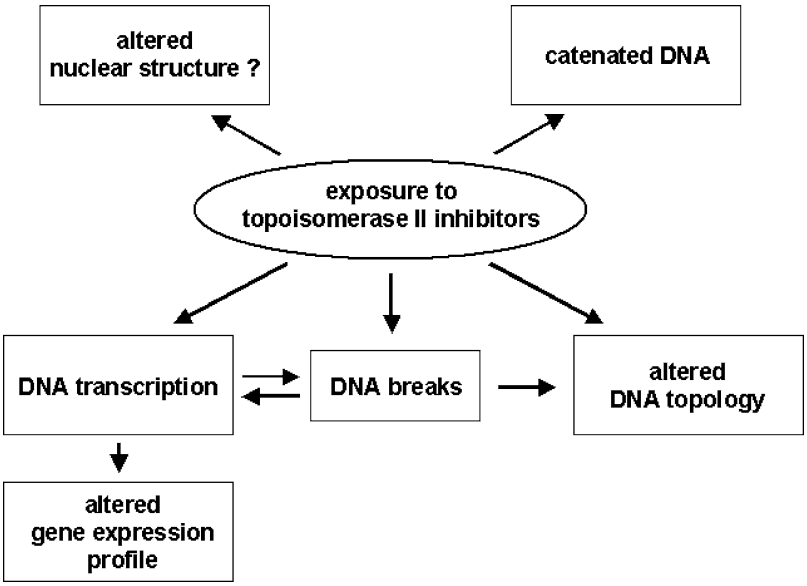

Fig. 2. Changes in DNA structure and function in cells exposed to topoisomerase II inhibitors.

related bisdioxopiperazine derivatives that are able to stabilize non-covalent topoisomerase II complexes on DNA *(9)*. Although less genotoxic than the topoisomerase II poisons, the ICRF compounds are also able to induce DNA damage and chromosomal aberrations either as a result of secondary damage following collision with macromolecular synthesis machineries or as a result of aberrant mitosis *(10)*.

2.2. Interference with Biological Functions of Topoisomerase II

In addition to inducing DNA damage, topoisomerase II inhibitors are likely to interfere with the normal biological functions of topoisomerase II as outlined in Fig. 2. These principally include its role as a transcriptional regulator, its contribution to the DNA damage response, and its mitotic activities.

2.2.1. Transcriptional Modulation

When immobilized on the DNA, the drug-associated topoisomerase II is no longer able to relax or decatenate DNA, which results in alterations of local topology and nuclear architecture. The role of DNA topology on transcription is incompletely understood in higher eukaryotes but has been extensively characterized in yeast and bacteria, in which it plays a major role in the expression of certain genes *(11)*. Importantly, this group includes genes coding for proteins involved in the stress response to external factors such as changes in pH, oxygen, and osmolarity, as well as gene products required for invasion. Exposure of mammalian cells to the topoisomerase II inhibitor etoposide stimulates the expression of interferon regulatory factor-7 (IRF-7), which plays an important role in the development and maturation of the immune system *(12)*, and of molecules involved in cell death and survival signaling. For example, although etoposide treatment of human HL-60 leukemia cells resulted in practically unchanged expression of cell death receptor-3 (DR-3), DR-4,

and FAS, the expression of silencers of death domain (SODD) and FADD-like-il converting enzyme (caspase-8) (FLICE) proteins, which serve as inhibitors of the cell death receptor signaling pathways, decreased substantially *(13)*. This was accompanied by aggregation of DR3 and DR4, but not FAS, into high-molecular-weight death complexes at the cell surface *(13)*. In glioma cells, etoposide was reported to upregulate the expression of DR-5 and downregulate the caspase-8 inhibitors Cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIPs) *(14)*.

2.2.2. Induction of the DNA Damage Response

Topoisomerase II is able to form molecular complexes with a number of proteins involved in cell cycle and survival signaling. These include the p53 oncogene suppressor, the BRCA1 C-terminus (BRCT) containing protein TopBP1, 14-3-3ε, the mitotic protein kinase CDK1 (also called Cdc2 kinase), and the survival protein kinase CK2 (reviewed in *3*). Topoisomerase II is involved in chromatin condensation during apoptotic execution which, in part, is mediated by its interaction with the caspase-activated DNase (CAD) *(15)*. Topoisomerase II alpha serves as a nuclear scaffold for CAD during apoptosis, thereby concentrating CAD at specific regions of the chromatin, most likely at the base of the chromatin loops. At the same time, the DNA cleavage activities of topoisomerase II are activated by the physical interaction with CAD, and simultaneous inhibition of topoisomerase II alpha and caspases was reported to completely abolish apoptotic chromatin condensation in isolated HeLa cell nuclei *(15)*.

2.2.3. Mitotic Functions of Topoisomerase II

Topoisomerase II is known to play several essential roles during late G2 and early mitosis. First, topoisomerase II is required for premitotic chromosome individualization, the process by which different chromosomes become untangled and converted into individual units *(16)*. The protein also plays a direct role in early chromosome condensation by a so far poorly understood mechanism that, at least in part, seems to rely on protein–protein and DNA–protein interactions *(17)*.

In addition, topoisomerase II alpha is needed for recruitment of CDK1 to the chromatin of mitotic chromosomes *(18)* and thus for mitotic phosphorylation of other chromatin-associated proteins. Finally, topoisomerase II is required for the separation of intertwined sister chromatids in prometaphase *(19,20)*. Recently, different genetic models have shown that the alpha isoform of topoisomerase II directly influences the cell cycle progression in G2 and during early mitosis, because downregulation of topoisomerase II alpha, but not topoisomerase II beta, protein levels results in delayed G2 transit and prolonged early mitosis *(21,22)*. These studies strongly suggest that topoisomerase II might, at least in part, control the onset of mitotic catastrophe by regulating the fraction of cells entering mitosis.

3. CELL DEATH AND CELL GROWTH ARREST

3.1. Morphology of Tumor Cells Treated with Topoisomerase II Inhibitors

The morphology associated with different types of cell death and growth arrest in tumor cells following exposure to topoisomerase II inhibitors is illustrated in Fig. 3. After treatment with topoisomerase II inhibitors, cells (Fig. 3A) may die by

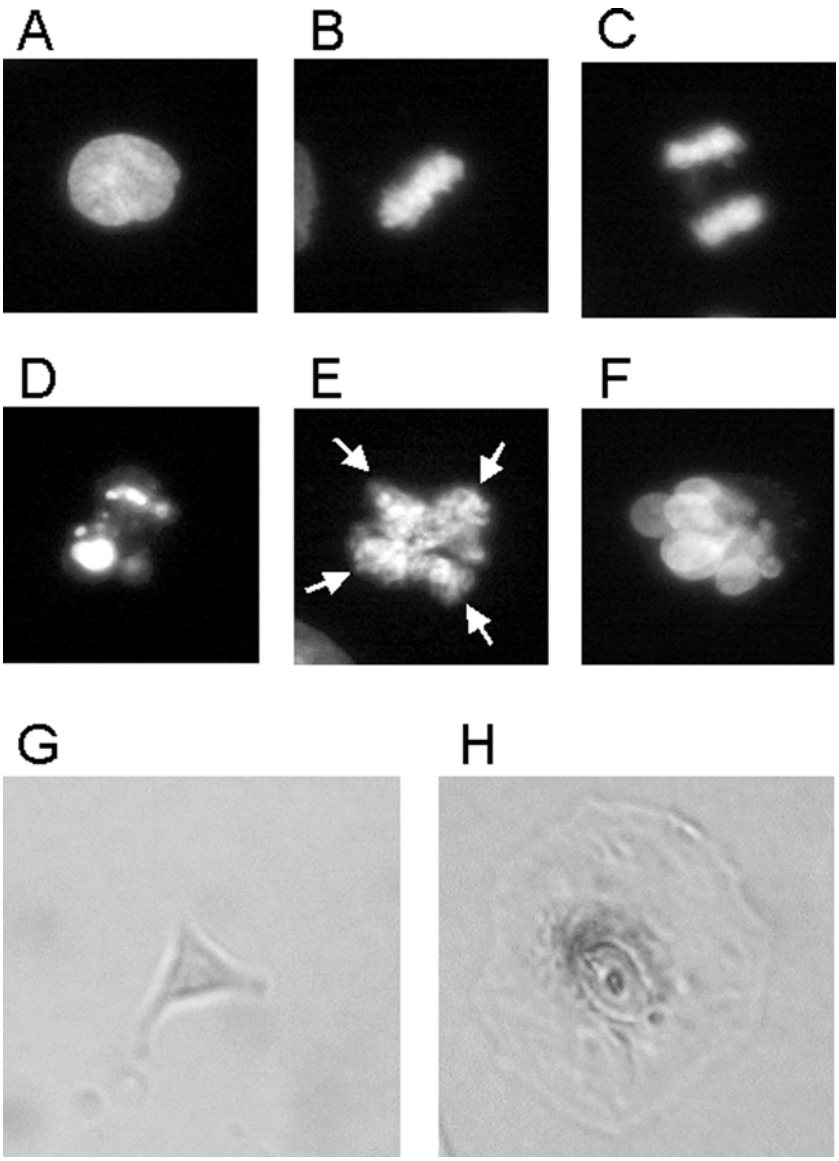

Fig. 3. Nuclear morphology of untreated A549 carcinoma cells (panels A–C). A549 cells treated with the topoisomerase II inhibitor triazoloacridone c-1305 (panels D–F). After prolonged drug exposure, cells (G) become enlarged and flat and express senescence-associated-B-galactsidase (H). Original magnifications ×200 (A–F) and ×50 (G and H).

apoptosis, characterized by fragmentation of the nucleus into discrete fragments of highly condensed chromatin (Fig. 3D). Damaged cells may also progress into mitosis (for comparison, normal metaphase and anaphase are shown in Fig. 3B and C, respectively) and undergo mitotic catastrophe that is characterized by the presence of highly entangled and non-segregated chromosomes (Fig. 3E), frequently with attempted segregation into multiple poles (arrows) and appearance of cells with segmented nuclei (Fig. 3F). After prolonged drug exposure, some cells (Fig. 3G) become very big and

flat with an extended lysosomal compartment and biochemical markers of cellular senescence such as senescence-associated-β-galactosidase (SA-β-Gal) with optimum activity at pH 6 (Fig. 3H). This phenotype corresponds to DIPS cells. Original magnifications are ×200 (pictures Fig. 3A–F) and ×50 (Fig. 3G and H).

3.2. Death by Apoptosis and Necrosis

As discussed above (See Section 1), treatment of tumor cells with topoisomerase II inhibitors may result in different cellular effects depending on the dose that is applied (Fig. 1). At sufficiently high drug concentrations, cells mostly die by activation of an endogenous cell death program, characterized by the presence of distinct morphological and biochemical alterations that collectively are called apoptosis *(23–25)*. Apoptosis can be a rapid process occurring a few hours after drug treatment or a delayed process happening after one or more cell divisions. Biochemical changes that are associated with apoptosis include activation of specific proteases (caspases), DNA fragmentation, and cleavage of a subset of proteins with important cellular functions, such as DNA repair [e.g., poly(ADP)-ribose)polymerase (PARP) and DNA-dependent protein kinase (DNA-PK)], or structural proteins (e.g., lamins, topoisomerase II alpha or actin). These biochemical changes are accompanied by specific morphological features such as chromatin condensation, nuclear fragmentation, cellular shrinking, and formation of cell fragments called apoptotic bodies that *in vivo* are engulfed by neighboring cells or macrophages to prevent local inflammation. Apoptosis is a highly controlled process, and it is increasingly evident that the final outcome of drug treatment depends on a delicate balance between pro- and anti-apoptotic signaling *(26)*. Therefore, the susceptibility of tumor cells to either undergo apoptosis or escape apoptotic death pathways is an important determinant in the response to antitumor agents.

It should be stressed that although induction of apoptosis by topoisomerase II inhibitors has been particularly well documented in the literature, other cell death pathways are also activated including mitotic catastrophe, autophagy (discussed in sections 3.3 and 3.5), and necrosis. To add to the complexity, some of the biochemical and morphological features of apoptotic cells have also been described for other types of cell death. For example, non-random DNA fragmentation and caspase activation have been observed in cells undergoing mitotic catastrophe *(27,28)*. Likewise, rapid and massive apoptosis may appear indistinguishable from necrosis because of the lack of healthy neighboring cells and local macrophages that can engulf the apoptotic bodies of the dying cells.

Interestingly, the choice between apoptotic and necrotic cell death is partly determined by the intracellular levels of available ATP. It has been reported that etoposide treatment was accompanied by necrosis under ATP-depleting conditions whereas apoptosis was induced at higher ATP levels *(29)*. Interestingly, modulation of cellular ATP levels by 2-deoxy-D-glucose, an inhibitor of the glycolytic ATP production, in etoposide-treated human glioma and squamous carcinoma cells was accompanied by enhanced cytotoxicity suggesting that necrosis may, at least in some situations, be an efficient mediator of etoposide toxicity *(30)*. Classically, necrosis is considered as a non-specific death mechanism, in contrast to programmed apoptotic cell death. However, increasing evidence suggest that necrosis may also be governed by an intrinsic cell death program *(31,32)*.

PARP appears as a crucial player in the choice between the apoptotic and the necrotic cell death program. Topoisomerase-mediated DNA strand breaks could lead to activation of PARP and subsequent depletion of intracellular ATP due to production of energy-consuming ADP-ribose oligomers at the expense of the PARP substrate, NAD^+ (*33* and references therein). In support of this hypothesis, it has been reported that cells with low PARP activity are resistant to etoposide *(34,35)*. Interestingly, the influence of PARP is closely monitored in apoptotic cells where PARP is inactivated by caspase-specific cleavage, thus maintaining cellular ATP levels sufficiently high to carry out the energy-dependent apoptotic process.

3.3. Mitotic Catastrophe

3.3.1. Definitions and Mechanisms

Several different definitions of mitotic catastrophe are used in the literature. Usually, it refers to a type of cell death that occurs during mitosis, most frequently during the metaphase to anaphase transition *(36)*, and this is the definition that we use here. However, some define mitotic catastrophe as a case of aborted mitosis resulting in aneuploidy *(28)*. Mitotic catastrophe may also be defined as aberrant chromosome segregation and/or cytokinesis resulting in the formation of G1 cells with a tetraploid DNA content *(37)*.

Mitotic cell death is due to activation of the apoptotic program by either caspase-independent mechanisms *(38)* or by a classical apoptotic pathway associated with caspase-2/3 activation and/or mitochondrial membrane permeabilization *(39)*. In the presence of strong survival signaling, cells will not die immediately but may stay viable for prolonged periods of time in a multinucleated, aneuploid or polyploid state. It is unclear what eventually happens with cells that survive abnormal chromosome segregation, but the presence of polyploid cells is often followed by the appearance of long-term survivors and is thus associated with drug resistance *(1,40)*. Interestingly, at least in some situations, the polyploid cells are able to restitute mitosis and return to the normal diploid state by the process of de-polyploidization *(41)* or by an unusual process of cell division called neosis, which is characterized by karyokinesis through nuclear budding followed by asymmetric, intracellular cytokinesis *(42)*.

Classical topoisomerase II inhibitors (etoposide, daunorubicin, and doxorubicin) have been shown to induce mitotic catastrophe in human tumor cells, in which apoptosis was inhibited by overexpression of Bcl-2 *(43)*, in cells overexpressing P-glycoprotein *(1)* or when the functioning of the DNA damage checkpoint has been perturbed by checkpoint abrogators such as caffeine *(44)* or by genetic modifications, such as inactivation of 14-3-3σ *(45)*. Recent data have shown that the imidazoacridone C-1311, a topoisomerase II inhibitor associated with the induction of very low levels of cleavable complexes, produced mitotic cell death in human colon carcinoma HT-29 cells *(46)*. Other studies have shown that inhibition of topoisomerase II in Ptk1 cells by the catalytic inhibitor ICRF-187 had dramatic effect on chromosome condensation and chromosome distribution during mitosis *(47)*. Massive chromosome bridges were formed in cells treated with ICRF-187, and the chromatin mass was unable to segregate during metaphase/anaphase transition resulting in unequal distribution of DNA between daughter cells. Interestingly, in many cases, all DNA was transferred to one nucleus, which caused production of polyploid cells with a 4N DNA content. It was concluded

that accumulation of cells with a 4N DNA content following treatment with ICRF compounds may reflect either a G2 arrest or incomplete mitotic chromosome segregation. It should be stressed that mitotic catastrophe is not restricted to topoisomerase II inhibitors but is also induced by other DNA-damaging agents such as irradiation and cisplatin *(48–50)* and by microtubule inhibitors *(51,52)*.

3.3.2. Roles of Checkpoint and Survival Signaling

It is evident that a critical aspect of mitotic catastrophe is checkpoint failure in G2 or during early mitosis, which permits cells with damaged DNA to reach metaphase. In agreement, exposure of etoposide-treated cells with G2 abrogators such as caffeine led to mitotic death and increased cytotoxicity *(44)*. However, once damaged cells reach metaphase, the remaining mitotic checkpoints most likely enforce the mitotic catastrophe. Strong metaphase checkpoints including the classical spindle assembly checkpoint and the mitotic DNA damage checkpoint *(53)* result in prolonged mitotic arrest of DNA-damaged cells that subsequently die directly out of metaphase. In agreement, abrogation of the spindle checkpoint of DNA damaged cells was accompanied by escape from mitotic cell death and abnormal mitosis *(54)*. Recent results suggest the presence of an additional checkpoint controlling mitotic exit by preventing cytokinesis. This checkpoint would favor mitotic cell death by preventing mitotic exit *(55)*.

Besides checkpoint function, survival signaling seems to be important for cells to survive abnormal chromosome segregation. This explains why overexpression of Bcl2 or survivin may rescue cells from mitotic cell death following treatment with topoisomerase II inhibitors despite abnormal chromosome segregation *(56)*.

The role of oncogene suppressors such as p53 in mitotic catastrophe is much less clear although it is often reported that mitotic catastrophe preferentially occurs in cells with non-functional p53 *(36,57)*. However, a recently identified suppressor of mitotic catastrophe is the *Xenopus* ortholog of 53BP1, a BRCT protein previously identified in humans through its ability to bind the p53 *(58)*. Furthermore, mitotic catastrophe due to Chk1 deficiency seems to be mediated by a p53-dependent pathway that involves both the ataxia-telangiectasia-mutated (ATM) and the ATM- and Rad3-related (ATR) checkpoint kinases *(59)*. Whether the same mechanisms are operative following DNA damage induced by topoisomerase II inhibitors remains to be established.

In conclusion, the survival of cells with damaged DNA during mitosis depends on many factors including (i) the different checkpoints and adaptation processes controlling the entry, transit, and exit from mitosis (generally, early mitotic checkpoints will tend to prevent mitotic catastrophe whereas late mitotic checkpoints will facilitate the mitotic catastrophe), (ii) proteins such as topoisomerase II that are needed for normal mitotic functions, and (iii) the expression of survival factors and survival signaling that can inhibit the induction and execution of apoptosis during mitosis.

3.4. Pseudo-Senescence (DIPS) Induced by Topoisomerase II Inhibitors

3.4.1. Definitions and Markers

Multicellular organisms have evolved different mechanisms to prevent the development of cancer originating in cells with deleterious DNA mutations. There are basically two ways of eliminating such cells, of which the best understood is the

induction of cell death. The second pathway is the induction of cellular senescence (irreversible growth arrest) after a well-defined number of cell population doublings before cells actually become cancerous.

The terms "cellular senescence" and "replicative senescence" refer to the state where normal somatic cells stop proliferating due to critical telomere shortening. Importantly, DNA damage induced by oxidative stress, UV irradiation, DNA-alkylating agents, histone deacetylase inhibitors, or DNA topoisomerase II inhibitors may also lead to irreversible growth arrest with a phenotype resembling cellular senescence *(60)*. In this review, we refer to this process as DIPS. DIPS principally occurs in tumor cells, usually happens after only one or two cell divisions, and, in striking contrast to replicative senescence, is independent of telomere length. It is currently not known whether DIPS is controlled by pathways that overlap with those of replicative senescence or, alternatively, is mechanistically different from replicative senescence despite the morphological and biochemical similarities.

Many anticancer agents including the topoisomerase II inhibitors doxorubicin, daunorubicin, and ICRF-193 are able to induce DIPS at relatively low doses that do not lead to apoptosis *(36,61)*. This is observed not only for tumor cells *in vitro* but also in animal models *(36)* and in cancer patients *(62)*. Importantly, studies with the alkylating agent cyclophosphamide have shown animals with tumors able to undergo drug-induced senescence have a much better prognosis following chemotherapy than those harboring tumors with senescence defects *(63)*. These findings suggest that DIPS may be an important factor in the tumor response to chemotherapy. However, this has been difficult to verify in the clinic, because the most frequently used biochemical assay, SA-β-Gal staining (with optimum pH of 6) is not always reliable, especially in tissue samples *(62)*.

DIPS induced by topoisomerase II inhibitors, such as doxorubicin, is observed both in cells with functional and non-functional p53 *(50)*, although the senescence phenotype is attenuated in p53 and p21 null cells *(36)*. This suggests that DIPS is favored by functional p53 and/or p21 although the two gene products are not absolutely required. DIPS may also be favored by the presence of functional P16, because SA-β-Gal-positive breast cancers cells treated with doxorubicin *in vitro* or *in vivo* were characterized by high levels of p16 *(62)*. However, many cell lines that are used for studies on DIPS *in vitro* and *in vivo* have inactivated P16, usually by hypermethylation of the promoter region. Thus, it seems that p16 is not required for induction of DIPS by antitumor agents although it might still be necessary for the maintenance of DIPS-associated growth arrest. Together, these findings suggest that even modest changes in the expression of genes such as p53, p21, and p16 may determine whether the drug-treated cells are directed toward apoptosis, mitotic catastrophe, or DIPS.

The relationship between cell death and DIPS is unclear. Most likely, DIPS is able to suppress the apoptotic program. Recent studies have shown that caspase inhibitors may switch drug-induced apoptosis to an alternative type of "default death" which resembles DIPS. Treatment of human neuroblastoma cells with combinations of doxorubicin and caspase-3 inhibitors led to increased p21/WAF1 expression and enhanced SA-β-Gal *(64)*. Moreover, usually prolonged growth arrest following treatment with DNA damaging agents is accompanied by cell death. Because cells undergoing DIPS can stay viable for extended time periods, there must be a mechanism which blocks pro-apoptotic signaling or provides survival signals to these cells.

Another important question is whether antitumor agents are able to induce DIPS in both normal and tumor cells. Although DIPS can be observed in both normal and transformed cells *in vitro*, analysis of tissue samples from cancer patients treated with the CAF regimen (cyclophosphamide, doxorubicin, 5-fluorouracil) showed exclusively tumor-specific SA-β-Gal staining with no detectable staining of neighboring normal cells *(62)*. This suggests that *in vivo*, DIPS preferentially occurs in tumor cells.

3.4.2. DIPS-Associated Pathways

Although DIPS typically is induced by DNA-damaging agents, it is not clear which type of DNA damage (single-stranded or double-stranded DNA breaks, chromosome breaks) triggers DIPS. It has been reported that permanent growth arrest of human fibroblasts is limited to agents creating double-strand DNA breaks *(65)*. This is in line with data obtained for senescing human cells that accumulated unrepaired DNA double-strand breaks *(66)*. Interestingly, DNA damage foci in senescent cells co-localize with telomeric DNA *(67)*. Thus it is possible that although cells undergoing DIPS show unchanged telomere length, DNA damage induced by antitumor drugs (such as topoisomerase II inhibitors) may lead to inactivation (uncapping) of telomeres. This could in turn activate a permanent DNA damage response state leading to growth arrest with markers of senescent cells.

Another fascinating possibility is that DIPS might be associated with changes in adenine thymidine (AT)-rich nuclear scaffold sequences that are preferential binding sites for topoisomerase II. It has been reported that 5-bromodeoxyuridine induces a senescence-like phenotype in HeLa cells, which is strongly potentiated by AT-binding ligands such as Hoechst 33258 *(68)*. Concomitant gene expression analysis by DNA microarray technology showed that the genes most affected by AT-binding ligands were located on or near Giemsa-dark bands (i.e., heterochromatin) of human chromosomes. Interestingly, it has been postulated that packaging of proliferation-promoting genes into repressive heterochromatin is a key step in cellular senescence *(69)*. Topoisomerase II may thus be directly involved in turning off proliferation-promoting genes and/or switching on senescence-associated genes by mediating the chromatin condensation and matrix association.

Finally, it should be recalled that at least some topoisomerase II inhibitors, including doxorubicin, produce oxidative stress in tumor cells that could lead to indirect DNA damage *(70)*. It is well established that oxidative stress can lead to stress-induced premature senescence (SIPS) in normal cells *(71)*. Although the relation between DIPS and SIPS is not known, it is likely that doxorubicin-induced DIPS is, at least in part, mediated by the induction of oxidative stress. Furthermore, hydrogen peroxide, which is produced during oxidative stress, has been shown to be potent inhibitor of topoisomerase II *(72)*. Although speculative, we suggest that the potency of doxorubicin as a DIPS-inducing agent may be linked to its ability to induce formation of covalent DNA–topoisomerase II complexes both directly and indirectly through formation of hydrogen peroxide.

3.4.3. Reversibility of DIPS

One of the current dogmas is that once cells become senescent, they never return to the proliferative state or, in other words, that cellular senescence is irreversible. Although this appears to be true for replicative senescence, this issue has only

recently been addressed for DIPS following exposure to two different topoisomerase II inhibitors, ICRF-193 *(73)* and doxorubicin *(74)*. Studies with human embryonic fibroblast cells showed that ICRF-193-treated cells resumed proliferation after an initial growth arrest *(73)*. More recent data revealed that treatment of MCF-7 human breast carcinoma cells with a clinically relevant dose of doxorubicin was accompanied by the appearance of senescence-resistant cell clones, which express high levels of CDK1 *(74)*. This study as well as others *(75)* suggest a new mechanism of tumor cell resistance to topoisomerase II inhibitors and probably also to other DNA-damaging agents, by avoiding drug-induced senescence associated with the maintenance of high CDK1 levels/activity. These results show that at least in some cases, drug-induced senescence may be followed by resumption of cellular proliferation. This issue is particularly important because induction of DIPS has been proposed as an alternative approach to treat human cancers.

3.5. Induction of Autophagy by Topoisomerase II Inhibitors

Autophagy is a process during which a cell degrades its cytoplasmic material, including organelles, in their lysosomes (for recent review, see 76). This unusual pathway is activated during stress conditions such as nutrient- or amino acid-deprivation and likely serves to provide substrates for energy metabolism and protein synthesis by degradation of the cytoplasmic components. In this way, autophagy provides a short-term survival mechanisms under unfavorable growth conditions. Recent results suggested a dual role for autophagy including both protection from apoptotic cell death and execution of alternative ways of cell death *(77)*. Autophagic cell death was observed after treatment of cells with inactivated Bax and Bak genes with etoposide *(78)*. Interestingly, drug-induced autophagy in these cells was not simply a consequence of the lack of apoptosis as no autophagy was detected in cells deficient in two other pro-apoptotic factors, Apaf-1 and caspase 9, nor in cells treated with etoposide and the pan-caspase inhibitor zVAD *(78)*. Cell death with signs of autophagy was also observed in epirubicin-treated breast cancer cells and was further enhanced by addition of medroxyprogesterone acetate *(79)*. These results show that at least in some situations, autophagy may substitute for apoptosis in drug-treated tumor cells. Much work is still needed to clarify the role and regulation of autophagy during drug-induced cell death, and in particular the relationship between autophagy and apoptotic pathways. It can be proposed that autophagy initiates the death pathway but requires the apoptotic machinery to finalize the cell kill. In this respect, it is interesting that some of the proteins that regulate autophagy have also been shown to interact with pro-apoptotic factors including FADD and Atg5 *(80)* as well as Bcl-2/Bcl-X_L and Beclin-1 *(81)*. Autophagy could benefit cellular survival by removal of damaged cell components following DNA damage *(82)*, and from this perspective, manipulation of autophagy has the potential to improve anticancer therapeutics.

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22 The Significance of Poly-Targeting in Apoptosis Induction by Alkylating Agents and Platinum Drugs

Jan M. Woynarowski, PhD, and Barbara A. Woynarowska, PhD

Summary

Apoptosis is believed to be an important aspect of the anticancer potency of alkylating agents (AAs) and platinum (Pt) complexes. Despite the high clinical utility of these classes of drugs, the nature and determinants of the apoptotic sensitivity/resistance of cancer cells to these agents are not completely understood. One underappreciated aspect is the wide and variable spectrum of cellular targets of AAs and Pt drugs and the complexity of the responses to poly-targeted insults. This chapter discusses the heterogeneity of targeting profiles for diverse drug types and the interdependence of apoptotic routes elicited by the damage to various cellular targets. Although many of these agents target DNA, DNA damage is not the only cause of their apoptotic effects. Drugs that alkylate proteins are strongly apoptotic, even if they do not react with DNA. The ability of alkylating and Pt drugs to damage and inactivate specific proteins and to globally distort the state of the proteome needs to be considered as a self-standing apoptotic stimulus and a factor that enhances lethal responses to DNA damage. Particular emphasis is placed on the significance of drug effects on redox-regulating proteins of the thioredoxin family. Disruption of protein redox homeostasis is likely to be critical for death/survival in response to poly-targeted alkylating and Pt drugs. Differential distortion of redox regulation is suggested as a molecular basis underlying the demonstrated potential of specific drugs such as irofulven and oxaliplatin to promote apoptosis in cancer cells while sparing normal cells.

Key Words: Alkylating drugs; platinum drugs; DNA adducts; protein alkylation; protein targeting; apoptosis; redox regulation.

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1. INTRODUCTION

Alkylating agents (AAs) and platinum compounds (Pt drugs) are among the most useful clinical anticancer drugs (Fig. 1, Table 1). These categories include such widely used drugs as nitrogen mustards, nitrosoureas, mitomycins, cisplatin and the newer Pt analogs. All the main clinical AA/Pt drugs share the ability to damage cellular DNA. DNA lesions are widely accepted as the critical factor in the ability of AA/Pt drugs to kill cancer cells, including specifically the proapoptotic potential *(1,2)*.

Apoptosis is thought to represent a pharmacologically relevant outcome for a majority, if not all, of the anticancer AA/Pt drugs *(3–6)*. Yet, even a cursory review of the literature reveals a multitude of disparate, sometimes seemingly incongruent observations on apoptotic potency of these drugs in cancer cells. The magnitude of apoptotic responses to AA/Pt agents and their timing strongly depend on a specific drug, cell type, culture conditions and cell position in the cell cycle *(7)*. Although a

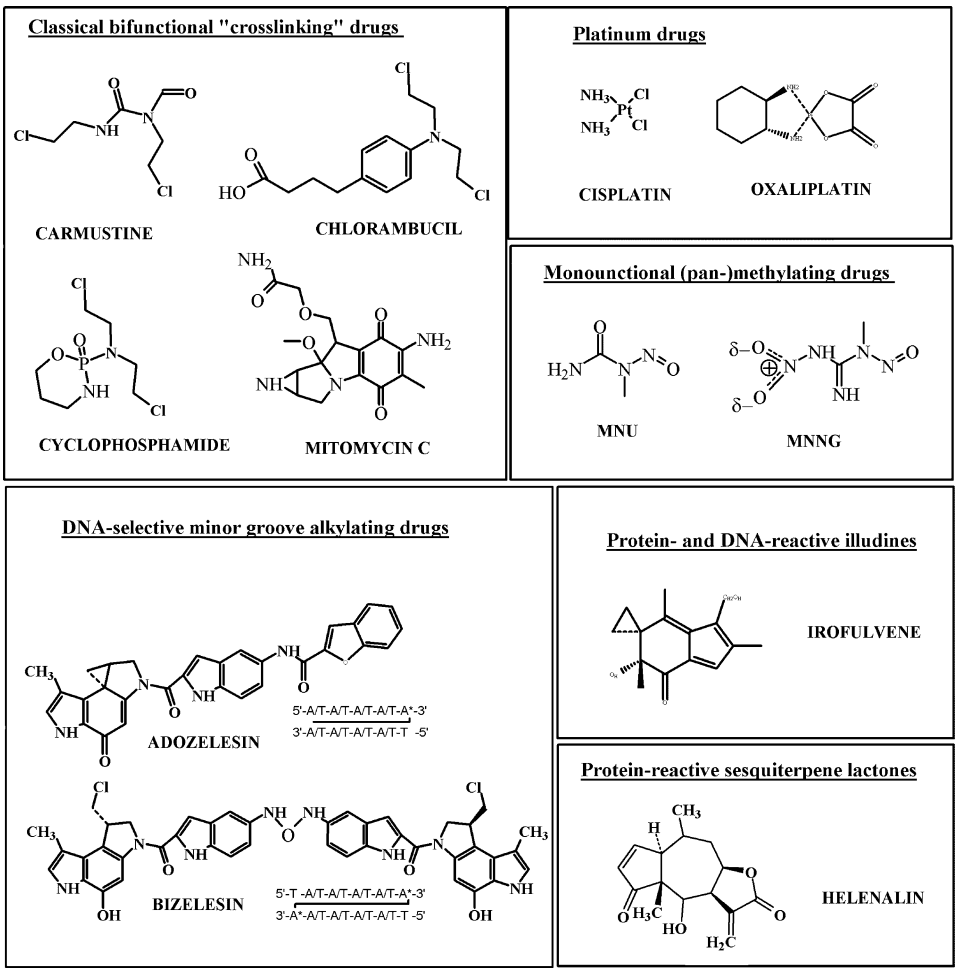

Fig. 1. The structures of compounds representing different classes of alkylating agents and platinum drugs.

Table 1
Alkylating Agents and Platinum Drugs and Their Targeting Preferences

Type/clinical status	*Specific Targeting Attributes*[a]	*Examples*	*References*
Bifunctional alkylating agents	**DNA +**	**Nitrogen mustards:** melphalan and chlorambucil	*17–22*
• Broad targeting: Proteins +SMNs >> DNA +RNA	Monoadducts>> inter-strand crosslinks ≅ DNA–protein crosslinks	**Quinone antibiotics:** mitomycin C **Nitrosoureas:** carmustine and CCNU	
• DNA crosslinks	Preference for N^7G (up to 90% of DNA adducts), although O^6G adducts thought important for some drugs	**Cyclophosphamides:** cyclophosphamide and iphosphamide	
• Used in clinic	Marginal sequence preference for G clusters Genome distribution: nearly random **RNA +** **Proteins +++++** Specificity: some binding preferences possible Small nucleophiles +++++		
Anticancer platinum drugs	**DNA +**	Cisplatin, oxaliplatin and carboplatin	*9,18,23–33*
• Broad targeting: proteins +SMNs >> DNA +RNA	Intrastrand crosslinks+monoadducts>> inter-strand crosslinks ≅ DNA–protein crosslinks. Marginal sequence preference for G clusters		
• DNA crosslinks	Genome distribution: nearly random		

(Continued)

Table 1
(Continued)

Type/clinical status	*Specific Targeting Attributes*[a]	*Examples*	*References*
• Used in clinic	**RNA +** **Proteins +++++** Specificity: some binding preferences possible (thioredoxin reductase) **Small nucleophiles**[b] **+++++**		
Monofunctional alkylating (methylating) agents	**DNA +**	Methylnitrosourea, methylnitronitrosoguanidine, methylmethanesulfonate, temozolomide and dacarbazine	*4,34–36*
• Broad targeting: proteins +SMNs >> DNA +RNA	Monoadducts at various sites, activity attributed to the formation of O^6G adducts		
• Carcinogenic	Sequence specificity: none		
• Some clinical utility	Genome distribution: random **RNA +** **Proteins +++++** Specificity: unlikely **Small nucleophiles**[a] **+++++**		
Minor groove alkylating drugs	**DNA ++++**	**CPIs:** bizelesin, adozelesin and duocarmycins	*37–45*
• DNA selective	Selective reactivity: only with double-stranded DNA (at N^3A)	**Non-covalent minor groove binders with alkylating moieties:** tallimustine	
• Highly cytotoxic	Sequence-specific monoadducts (adozelesin, duocarmycin and tallimustine) or interstrand crosslinks (bizelesin). Strong non-covalent duplex stabilizing interaction		

• In clinical development	High sequence specificity for discrete AT-rich motifs (which depend on individual drugs) Genome distribution: some CPI drugs target defined genomic regions (AT islands) **RNA –** **Proteins –** **Small nucleophiles –**		
Illudines • Protein subset >> DNA +RNA • Promising anticancer properties (irofulven) • In clinical development	**DNA +**(monoadducts) **RNA +** **Proteins +++++** Specificity: some selectivity (thioredoxin) **Small nucleophiles +**	Irofulven and illudin S	*46–52*
Sesquiterpene lactones • Protein subset/SMNs • Numerous compounds from medicinal plants with anticancer activity • Preclinical development	**DNA –** (helenalin) **RNA –** **Proteins +++**(some selectivity, p65 subunit of NF-κB, glutaredoxin and thioredoxin) **Small nucleophiles +++**	Helenalin and parthenolide	*53–62*

CPI, cyclopropylindoline; SMN, small molecule nucleophile.

[a] Symbols ('+' and '−') denote relative drug affinity to individual types of targets.

[b] SMNs, such as cysteine and glutathione.

range of pathways and specific biomolecules have been implicated in pharmacological apoptosis *(8)*, no universal markers have emerged thus far that would consistently predict apoptosis by AA/Pt drugs. Sensitivity or resistance to some drugs in specific cellular models can be attributed to differences in the formation and repair of DNA damage, but predictive generalizations are hard to make, as exceptions and discrepancies are common. Importantly, certain types of AA drugs are proapoptotic, even though they do not react with DNA at all. These seemingly inconsistent properties lead to several questions. Is DNA damage invariably the sole or decisive factor in apoptosis by AA/Pt drugs? If DNA damage plays a non-exclusive role, what other mechanisms may participate in cell death induced by AA/Pt drugs? Is the classic DNA damage-initiated and caspase-mediated apoptosis the only and/or always essential route for AA/Pt drugs? Finally, the ultimate goal of all cancer strategies is to eradicate cancer cells without causing excessive harm to normal cells. Can then normal cells be spared from apoptosis by AA/Pt drugs? What factors may predict such differential responses?

Complete answers to these pressing questions may not be available at present, despite the vast literature on apoptotic responses to AA/Pt drugs. Generalizations are particularly difficult, given the variability of responses. Therefore, it would be a daunting task to comprehensively review all aspects of apoptosis induction by AA/Pt drugs. Several key areas, such as the roles of DNA damage, repair mechanisms and DNA damage-related apoptotic signaling, are already the topics of recent in-depth review articles *(9–13)*. It seems worthwhile, however, to critically evaluate those aspects of lethal effects of AA/Pt drugs that are potentially important yet have received less attention and/or are a potential source of confusion or misinterpretations. This chapter attempts to streamline some of such understudied issues by focusing on the targeting diversity of various types of AA/Pt drugs and, in particular, underscoring non-DNA targets as factors contributing to apoptosis.

2. TARGETING PROFILES OF AA AND PT DRUGS

Cellular targets obviously represent the most fundamental level in the action of any drug and are the origin of all the downstream responses, including apoptosis. Still, the recognition of the primary cellular targets of AA/Pt drugs and, consequently, the interpretations of downstream signaling induced by these compounds are often subject to oversimplifications. AA/Pt anticancer drugs differ profoundly in their structures, chemistry and biological properties (Fig. 1). Whereas DNA is undoubtedly an important target for many AA/Pt drugs, referring to such drugs as "DNA-reactive agents" may be misleading without additional qualifiers. All AA/Pt drugs share electrophilic nature that makes them inherently reactive toward a spectrum of cellular nucleophiles rather than toward a single type of biomolecules. Irrespective of how important DNA reactivity might be, it reflects only one side of the multifaceted and complex molecular pharmacology of these compounds. Electrophilic drugs tend to react much more readily with sulfhydryl groups than with nucleophilic groups in DNA [the general order of reactivity with nucleophiles is thiols $\gg$ amino groups $>$ phosphates $>$ hydroxyls *(14)*]. Therefore, typically 80–90% of the total macromolecular adducts of conventional AA/Pt compounds in the cell are formed with proteins (some remarkable exceptions are discussed in Section 2.3.). In addition to protein targeting, most AA/Pt drugs show substantial reactivity with small molecule nucleophiles (SMNs) such as

glutathione (GSH). Consistent with multiple targets, these drugs elicit profoundly complex networks of cellular responses *(15)*.

With researchers' attention focused on DNA damage, these "other" reactivities are too often ignored in the interpretation of cell responses to AA/Pt drugs or dismissed by labeling these agents as "non-specific DNA alkylators." However, the viewpoint that an ideal antitumor drug would have only one target is at variance with the fact that AA/Pt drugs are among the most efficacious and beneficial mainstream drugs in the clinic, whereas various narrowly targeted strategies are plagued by the insufficient ability to eradicate cancer cells *(16)*. An alternative perspective is to properly recognize AA/Pt drugs as poly-targeted agents and make every effort to better understand their complex molecular pharmacology. Examples of AA/Pt drugs of diverse targeting profiles are listed in Table 1 and discussed in the subsequent sections.

2.1. Multiple Cellular Targets of Conventional Bifunctional AA and Pt Drugs

2.1.1. DNA Binding—Assorted Lesions of Low-Sequence Specificity

Clinically relevant conventional bifunctional AA/Pt agents share several key mechanistic characteristics despite differences in their chemistry. Both bifunctional AA and Pt drugs form irreversible (covalent) adducts with nuclear DNA, reacting mainly with N7 position in guanines *(17,18)*. The biological activity of bifunctional AA drugs is thought to depend mainly on the formation of bi-functional adducts, specifically interstrand crosslinks. Interstrand crosslinks are also formed by Pt drugs, although intrastrand crosslinks represent the prevailing type of Pt-derived bifunctional lesion.

Interstrand crosslinks are regarded as one of the most lethal types of DNA damage. These lesions cannot be readily by-passed by replication/transcription machinery, and their removal requires complex repair mechanisms *(10)*. Hence, drugs such as nitrogen mustards, mitomycins, nitrosoureas and Pt compounds are often referred to as "DNA-crosslinking agents." However, these drugs also induce a spectrum of non-crosslinked DNA lesions. For example, interstrand crosslinks correspond to only 1–10% of all DNA adducts formed by bifunctional AA drugs and 5–20% of Pt–DNA adducts *(17,18,29)*. Another type of bifunctional lesion formed by both AA and Pt drugs, DNA–protein crosslinks, comprise a similar fraction of total adducts. Monoadducts, which constitute a majority of DNA lesions formed by these nominally "crosslinking" agents, are, in principle, less lethal and easier to repair *(18,64)*. Thus, monoalkylating compounds are expected to be inferior to their bifunctional, crosslink-capable counterparts. Still, there are exceptions to this rule that cannot be readily explained in terms of DNA targeting. For instance, the crosslinking mitomycin C is not more cytotoxic than its non-crosslinking (monoalkylating) derivative *(65)*. Low lethality of monoadducts makes these lesions highly mutagenic. Accordingly, monoadducts in normal tissues, formed in large numbers also by bifunctional AA/Pt drugs, are linked to secondary, treatment-induced tumors *(17,66,67)*.

Conventional AA/Pt drugs lack the ability to target specific genes or defined functional regions. Reflecting the preference for G clusters, GC-rich regions in cellular DNA are somewhat more affected by drugs such as cisplatin or oxaliplatin than AT-rich regions *(30)*. However, G clusters or crosslinking motifs GC and CG are ubiquitous

(39). Therefore, at the genomic level, DNA adducts by the conventional AA/Pt drugs are nearly randomly distributed *(30,39,68)*.

2.1.2. RNA—an Ignored Unknown

Little is known about RNA adducts formed by "DNA-reactive" AA/Pt drugs. Levels of RNA adducts are comparable *(23,69)* or even several times higher than the levels of DNA adducts *(70)*. RNA alkylation, for example, by nitrogen mustard, interferes with protein synthesis on the adducted RNA template *(71)* and may elicit RNA repair processes *(36)*. Although the significance of drug–RNA adducts remains virtually unknown, it is reasonable to expect that even a partial and "non-specific" interference with the functions of various classes of RNA would contribute to cell responses, synergizing with the consequences of DNA damage. For example, drug effects on RNA functions might affect transcriptional responses. Yet, changes in transcription of specific genes in cells exposed to AA/Pt drugs are routinely interpreted solely in terms of responses to DNA damage.

2.1.3. Proteins and SMNs—Likely Contributing Targets

The ability of bifunctional AA drugs and Pt agents to react with proteins and with SMNs is well known *(14,17,18,23)*. Although AA agents bind to sulfhydryls in cysteine residues, Pt drugs are also reactive with methionine residues *(25–28,72,73)*. Drugs of the cyclophosphamide group can form adducts with proteins and SMNs through their principal active form (phosphoramide mustard) and also through acrolein that is released as a by-product of drug activation *(22,74)*.

Bifunctional AA/Pt drugs react extensively with proteins *(75–78)*. For instance, as much as 90% of macromolecular adducts of Pt drugs are formed with cellular proteins *(23)*, while the nitrogen mustard drug chlorambucil binds profoundly to proteins in a variety of cellular structures *(79)*. Unfortunately, systematic investigations of protein targeting in the mechanistic context are rather uncommon. This bias is illustrated by the stark contrast between approximately a dozen of reports devoted to protein reactivity of Pt drugs and many thousands of publications on DNA damage and DNA adducts formed by these agents. Precisely, the opposite bias is evident in the reactivity of AA/Pt drug—in the presence of both DNA and thiol compounds, cisplatin reacts almost exclusively with biological thiols, whereas the formation of Pt–DNA adducts is marginal *(24)*. Moreover, other studies have ruled out the possibility that Pt–protein adducts might form transiently, serving as a "reservoir" of drug for DNA binding *(25,80)*. Various drugs, including cisplatin, may have an even greater affinity to SMNs than to proteins *(25,80)*. Other drugs may favor proteins. For example, the hydrophobic cyclohexane moiety of oxaliplatin may facilitate oxaliplatin docking in hydrophobic pockets of targeted proteins *(29,32)*. Protein targeting by oxaliplatin is further enhanced, relative to cisplatin, by a greater reactivity of oxaliplatin with sulfhydryls and methionine residues *(72,81)*.

Various bifunctional AA and Pt drugs probably react with a multitude of proteins. Solid evidence exists that at least some of the affected proteins are highly relevant cancer targets. Notable examples include the inhibition of the activity of important redox-regulating proteins: thioredoxin reductase (TrxR), GSH reductase, glutaredoxin (Grx) and, to lesser degree, Trx by bifunctional AAs (such as carmustine) and Pt drugs *(82–87)*.

2.2. Omni-Reactive and Non-Specific Monofunctional Alkylating Drugs

Methylating agents represent an extreme case of non-specific targeting by AA drugs. Lethal effects of compounds such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate are often attributed to the massive formation of O^6 guanine adducts, although N-methylations by such drugs tend to outnumber O-methylations *(4,34–36,88)*. Although both types of methylations are proapoptotic, these unremarkable DNA lesions are relatively readily repaired, profoundly less lethal and more mutagenic than bifunctional DNA lesions. Also, methylating compounds broadly affect proteins and SMNs *(89)*. It is puzzling that such non-specific agents might still be able to differentiate cancer from normal cells, as implied by their clinical utility.

2.3. Selective DNA Targeting by Sequence-Specific Minor Groove Binding DNA Alkylators

The only known class of AA drugs that are truly and inherently selective for DNA comprises some agents that combine the alkylation of DNA bases with a tight fit in the minor groove allowing for strong non-covalent interaction. These unique agents include cyclopropylindolines (CPI), such as bizelesin and adozelesin, and their subgroup of cyclopropylbenzoindolines (CBI) (Fig. 1, Table 1) *(37,40,41,90)*. DNA alkylation by CPI/CBI drugs (at N3 position of adenine residues) is preceded by the induced fit-activation that absolutely requires double-stranded DNA *(38)*. Because of the need for this unique mode of in situ activation, CPI/CBI drugs do not react with single-stranded nucleic acids (DNA or RNA), proteins and SMNs.

CPI/CBI drugs are capable of remarkably high sequence specificity in their interactions with DNA. For example, bizelesin binds to DNA occupying 6 bp sites with a very high preference for $T(A/T)_4A$ (Fig. 1). CPI drugs with a single reactive center (e.g., adozelesin) form monoadducts with DNA, whereas those with two centers (bizelesin) form interstrand crosslinks *(40,42)*. However, the combination of covalent monoadducts and non-covalent groove interaction (e.g., by adozelesin) affects DNA duplex in a way that is functionally equivalent to the effects of a covalent interstrand crosslink. Thus, adozelesin–DNA monoadducts can be regarded as covalent/non-covalent crosslinks.

The CPI–DNA adducts fitting tightly in the minor groove induce relatively little distortion to DNA structure, unlike bulky and distortive N^7-G adducts of classical AA/Pt drugs. Consequently, DNA adducts formed by CPI compounds (crosslinks and monoadducts alike) are inefficiently recognized by repair mechanisms *(91)*. Finally, the remarkable sequence specificity of some of the CPI drugs translates into their ability to preferentially target regions of repetitive AT-rich DNA in the genome (termed AT islands) *(40,41,92,93)*. CPI drugs are among the most potent anticancer compounds ever discovered inhibiting cell growth in the low picomolar concentrations. Yet, puzzlingly, these compounds are poorly apoptotic *(94,95)*.

Like CPI/CBI drugs, various other sequence-specific alkylating drugs combine an alkylating "warhead," such as a nitrogen mustard, with a backbone of non-covalent minor groove binders (distamycin, netropsin, bisbenzimide or poly-amides capable of "reading" DNA sequence) [for review, see refs *96–98*]. Tallimustine, a benzoyl nitrogen mustard derivative of distamycin A, is one such compound that, unlike conventional untethered poly-targeted nitrogen mustards, seems to react only with DNA *(68,99)*.

Collectively, alkylating drugs capable of composite covalent/non-covalent interaction in the DNA minor groove offer the potential for sequence-specific and region-specific DNA damage combined with non-reactivity toward nucleophiles other than DNA. Therefore, minor groove binding AA drugs are qualitatively different from conventional AA and Pt drugs.

2.4. Nucleic Acids and Protein Targeting by Novel "Dual-Action" Alkylating Drugs

Another category of novel alkylating drugs comprises sesquiterpenoids of the illudins family *(100–102)*. Irofulven (hydroxymethylacylfulvene) is a semi-synthetic illudin with demonstrated clinical efficacy *(103–106)* and broad activity against solid tumors. Like the conventional AAs, irofulven reacts with nucleic acids, forming under pharmacologically relevant conditions several million DNA adducts/cell, which probably reflects monoalkylation events *(47,49,50)*. However, the levels of irofulven adducts to proteins exceed 4-fold to 10-fold the level of DNA adducts *(47,107)*.

Irofulven binds to a range of cellular proteins but in a distinctly non-random fashion *(107)*. The key redox-regulating protein, cytoplasmic Trx1, was identified as the most adducted protein in cytoplasmic extracts from cells exposed to [^{14}C]irofulven *(107)*. Irofulven binding to the purified Trx1 and its partner TrxR1 have also been demonstrated *(107)*. In reaction with Trx, the drug alkylates only one of five cysteine residues *(107)*. In addition, irofulven has decisively greater affinity for protein thiols, such as those in Trx1, over SMNs *(107)*, unlike other electrophilic agents that tend to react more readily with SMNs. Targeting and inactivation of Trx and other redox-regulating proteins seem to play major roles in the potent apoptotic properties of irofulven and other dual-action drugs (as discussed in Sections 5. and 6.).

2.5. Protein Targeting by DNA Unreactive (Single Action) Alkylating Drugs

A distinct targeting pattern is exhibited by sesquiterpene lactones (SLs), natural AAs that have been repeatedly identified as biologically active components of various medicinal plants *(108,109)*. SLs with promising antitumor activity, such as helenalin and parthenolide, resemble illudins in their reactivity toward thiols and in their potent proapoptotic properties. However, these SLs fundamentally differ not only from the illudins but also from all the conventional clinical AAs, in that helenalin and parthenolide do not react with DNA.

Although SLs react with many cellular proteins, studies on parthenolide and helenalin have centered in recent years on their ability to inhibit nuclear factor (NF)-κB, an important transcriptional regulator implicated in survival pathways and inflammatory processes. These SLs, however, are not specific NF-κB inhibitors *(60,110)*. For example, helenalin inhibits the activities of multiple enzymes in each of the following categories: energy metabolism, enzymes of DNA synthesis and redox-regulating proteins (Trx, Grx, glutathione-*S*-transferase and several mixed function oxidases) *(56,57,111)*. Although SLs rapidly react also with SMNs (notably GSH) *(57,58)*, this reaction is reversible so that a fraction of drug molecules is always available for reaction with protein targets *(58)*.

3. GENERAL ATTRIBUTES OF CELL DEATH INDUCED BY AA AND PT DRUGS

Apoptosis reflects not a single feature that can be measured and quantified in a universal way but rather a multifaceted phenomenon that is defined based on a variety of biochemical markers and morphological changes characterized by a specific spatio-temporal order *(6,112–114)*. It is thus not easy to identify a universal set of attributes that would (i) adequately and uniformly define cell death in response to AA/Pt drugs and (ii) would allow for prediction of such responses. Poorly understood poly-targeting diversity of specific AA/Pt drugs blurs our understanding of these apoptotic responses. The interpretations are further complicated by the profound dependence of responses to AA/Pt drugs on treatment conditions and intricate temporal and causative relationships among various molecular markers of the apoptotic process. Even in the same cell population, individual apoptotic markers peak at different times and show significant variability in their magnitudes *(115,116)*. Even the underlying mechanistic nature of specific events that serve as apoptotic markers is subject to change over time. Another layer of complexity is added by the diversity of biological models that respond very differently to the same insult. Therefore, attempts to generalize disparate literature observations by cross-comparing data extracted from separate studies are difficult, at best, and are further complicated by fundamental conceptual differences among researchers on what constitutes apoptosis.

A widespread notion equates apoptosis with a caspase-mediated cell death involving either the intrinsic pathway mediated by mitochondrial dysfunction or the extrinsic pathway mediated by death receptors, or the interplay of both routes *(117,118)*. However, inhibition of the caspase pathway frequently fails to protect cells from death, resulting merely in a phenotypic shift. It is well established that some agents, in addition to caspase-mediated pathways, activate parallel caspase-independent death routes *(112,119,120)*. The term "caspase-independent apoptosis" is often used to denote specifically cell death commencing by the leakage from mitochondria of apoptosis-inducing factor (AIF) and endonuclease G, although various caspase-independent mechanisms of cell demise are likely to exist *(119)*.

Both caspase-independent and caspase-mediated death pathways tend to result in early reorganization of phospholipids in the cell membrane *(119)*, which offers a characteristic and widely used marker of early apoptosis. The caspase-mediated route ultimately leads to extensive and progressive cleavage of nuclear chromatin producing a range of fragments (~2–50 kbp, sometimes oligonucleosomal length) *(121)*. By contrast, AIF-mediated apoptosis is associated only with infrequent long-range DNA cleavage *(120,122)*. In both caspase-mediated and AIF-mediated routes, the loss of cell membrane integrity is a late event, which is one of the characteristic attributes of apoptosis as opposed to rapid loss of membrane integrity in necrosis.

For the purpose of this chapter, the term apoptosis is used in a broad sense as an irreversible cascade of cell de-organization initiated "from inside" and resulting in cell demise. This definition emphasizes the following universal aspects of apoptosis: (i) self-accelerating de-organization and degradation of cellular components signifies the irreversible phase when the cell becomes destined to die, (ii) caspase cascade may coexist with (or be supplanted by) other types of degradative processes and (iii) the irreversible degradation commences when affected cells still maintain basic morphological structures and remain metabolically active.

This broad definition fits not only both caspase-dependent and caspase-independent routes but also other morphologically identifiable manifestations of cell death that might escape the classical strict differentiation as either apoptosis or necrosis, such as apoptosis-like necrosis or necrosis-like apoptosis *(112,119)*. It needs to be underscored that for anticancer drugs, diverse manifestations of cell death (i) stem from the common insult, (ii) are often present concurrently, and (iii) cumulatively contribute to the irreversible eradication of cancer cells. The last attribute is by far the most relevant in the pharmacological context.

4. IS THE TARGETING OF DNA ALONE ALWAYS SUFFICIENT FOR APOPTOSIS BY AA?

The subsequent sections address the characteristics of apoptotic responses associated with specific targeting profiles of AA/Pt drugs. Targeting DNA will be compared and contrasted to single-action protein targeting and to dual actions on DNA and proteins.

4.1. The Classical Model of DNA Damage-Induced Apoptosis

In principle, DNA adducts interfering with DNA template properties are sufficient for the inhibition of cell proliferation, albeit not necessarily for cell death *(2)*. In addition to direct effects on DNA function, however, DNA lesions initiate a cascade of responses that involve DNA damage recognition and repair systems, chromatin remodeling, cell cycle checkpoints, transcriptional reprogramming and downstream signaling through routes that are often redundant, highly branched, interdependent and cell type specific (recently reviewed in refs *10,12* and *36*). In this web of responses, DNA lesions that remain unrepaired (or incompletely repaired) shift the balance toward pathways that lead to apoptotic cell death *(10,12,88)*.

Our understanding of the role of DNA damage in apoptosis stems largely from the investigations on ionizing radiation (IR). In the IR-based model, unrepaired DNA damage initiates signaling in which a pivotal role is played by tumor suppressor p53. Transcriptional transactivation by p53 promotes death signaling, which often involves the apoptosis signal-regulating kinase 1 (ASK1)/MAPK/c-Jun N-terminal kinase (JNK) pathway *(11)*. Non-transcriptional effects of p53 are thought to mediate the activation of death receptor pathways *(123)*. Importantly, wild-type p53 participates in the repair of DNA damage, which may enhance survival. Although mutations or inactivation of p53 are, in principle, expected to attenuate apoptotic responses to IR-induced DNA damage, this is not always the case *(124,125)*. The complex roles of p53 in sensitivity/resistance to IR *(126,127)* underscore how elusive are the connections between DNA damage and apoptosis even for IR, which is not only the most extensively investigated DNA damaging agent but is also mechanistically incomparably simpler than multifaceted AA/Pt drugs.

DNA damage by IR as well as by drugs that act as relatively "pure" DNA-damaging agents (such as topoisomerase poison etoposide) results in a shift in the balance of proapoptotic and antiapoptotic members of the Bcl-2 family, mitochondrial dysfunction (but no early release of AIF) and the activation of the initiator caspase-9 and the executioner caspase-3 *(61,128–131)*. Consequently, cell death commences essentially through the caspase-dependent apoptosis *(132)*, even though some AIF release may occur as a secondary effect caused by caspase activation *(133)*.

The IR-based model of apoptosis is, in part, applicable to cell death induced by DNA-damaging AA/Pt drugs, albeit with two important caveats. First, at the clinically relevant IR doses that are sufficient to induce potent "reproductive cell death," IR is a relatively poor inducer of apoptosis *(134–136)*. In addition, apoptotic effects caused by the clinically relevant IR doses reflect mainly post-mitotic "secondary" apoptosis that is delayed by several days and triggered by mitotic catastrophe, rather than by direct apoptotic signaling from residual DNA damage *(134,137,138)*. By contrast, apoptosis induced by various AA/Pt drugs is characterized by much more extensive magnitude and a markedly faster progression. Moreover, unlike the post-mitotic effects of IR, drug effects may reflect direct, pre-mitotic apoptosis *(139)*. The second caveat stems from the complicated targeting profiles of AA/Pt drugs. Although biological effects of IR are mainly derived from DNA damage, the poly-targeted nature of many AA/Pt drugs makes it difficult to unambiguously isolate (experimentally as well as conceptually) the responses to DNA damage from the contribution of drug effects on other targets.

4.2. Low Apoptotic Potency of Single-Action DNA-Selective Alkylating Drugs

Agents that solely target DNA (such as bizelesin and adozelesin) are poorly apoptotic despite potent inhibition of cell proliferation in the picomolar range of concentrations *(68,94)*. For example, bizelesin-inhibited acute lymphoblastic leukemia CEM cells remain irreversibly arrested in G2 but show apoptosis only at drug concentrations exceeding by two orders of magnitude cell growth inhibitory concentrations *(91,140, 141)*. Only at $100 \times IC_{80}$, apoptotic DNA fragmentation became noticeable, albeit still at a relatively low level *(140)*. The less-specific adozelesin, but not bizelesin, induced modest apoptotic responses (albeit still at high drug concentrations) in colon carcinoma HCT116 cells *(142)*. Interestingly, p21-deficient HCT116 cells responding to either adozelesin or bizelesin showed a markedly weaker G2/M block accompanied by enhanced apoptotic indices compared with wild-type cells *(142)*. As the apoptotic responses in p21-deficient HCT116 cells were delayed (by 48 h), it is possible that they corresponded not to direct drug effects but rather to "generic" secondary apoptosis triggered by the mitotic catastrophe, like post-mitotic apoptosis in response to the clinically relevant doses of IR.

A plausible explanation for the poor apoptosis in response to bizelesin is that the extremely lethal DNA lesions induced by this drug are simply too infrequent (estimated at <10/cell at cell growth inhibitory concentrations) *(39,94)* to elicit significant apoptotic signaling. Somewhat more noticeable apoptosis by adozelesin parallels a markedly higher number of DNA adducts formed by this drug *(94)*. Data for other DNA-selective alkylating minor groove binders lend support to the possibility that apoptosis may be inversely proportional to the lethality of DNA lesions. A modest apoptosis only at supralethal concentrations was observed for duocarmycin A, a CBI compound that is reminiscent of adozelesin in terms of cytotoxic potency *(143)*. By contrast, substantial apoptosis was observed at pharmacologically relevant levels of an achiral-*seco* CBI analog that acts at micromolar rather than picomolar concentrations *(45)* and most likely forms substantially more DNA adducts than equitoxic levels of adozelesin.

Furthermore, tallimustine, another alkylating minor groove binder, forms two orders of magnitude more DNA adducts than bizelesin at equitoxic concentrations *(68)*.

Accordingly, unlike bizelesin, tallimustine does promote some apoptosis at pharmacologically relevant concentrations, but these effects are still modest compared to equitoxic concentrations of conventional AA drugs that form orders of magnitude more adducts than tallimustine *(144,145)*. On the other hand, a tallimustine congener, brostallicin, which is nominally less "specific" because it has additional targets besides DNA, is markedly more apoptotic (M. Broggini, personal communication).

4.2.1. Conclusion

The mechanisms of cell death promoted by pharmacologically relevant levels of AA/Pt drugs are probably much more complex than the general model of DNA damage-induced apoptosis that is derived largely from responses to supra-lethal levels of IR. Solely targeting DNA may not result in significant apoptosis, when relatively few DNA lesions of high lethality are produced. A similar idea has been proposed for IR that DNA lesions must exceed certain threshold level for the activation of DNA damage signaling and apoptotic responses *(146)*. This threshold of DNA damage is likely to be modulated by the cellular milieu and drug effects on other cellular targets.

5. PROTEIN DAMAGE IN CELL DEATH—THE PROTEOME AS A COLLECTIVE APOPTOTIC TARGET

Extensive evidence from studies using various protein-reactive agents leaves no doubt that protein damage is sufficient to produce substantial apoptosis in the absence of DNA adducts or any other apoptotic stimulus *(141,147–151)*. As discussed above (Section 2.5.), protein binding of sulfhydryl-reactive AA drugs, such as SLs (helenalin and parthenolide), functionally inactivates the adducted molecules *(56,60,108,110,111)*. It does not mean that the activities of those specific proteins in the cell are, or need to be, completely abrogated. As adducts formed by a majority of protein alkylators are distributed among many proteins, only a proportion of any specific protein type is likely to become directly inactivated. It is essential, however, to realize that all the drug–protein adducts together shift the equilibrium between the reduced sulfhydryl forms of cellular proteins (Prot-SH) and the oxidized disulfide forms (Prot-S-S). Re-equilibration of Prot-SH/Prot-S-S balance across the proteome also affects those protein molecules that are not directly adducted. Both direct inactivation and all of the oxidative changes in the entire proteome collectively affect the redox state, and their consequences are amplified by the redox-sensing systems.

5.1. Redox Distortion—Achilles Heel of Cancer Cells

Understanding the mechanisms of cellular redox regulation may help one to appreciate how important the distortion of this regulation could be for apoptotic responses to AA/Pt drugs in cancer cells and their potential to spare normal cells. One anticipated uniform consequence of protein targeting by AA/Pt drugs (either single action or poly-targeted) is oxidative distortion of redox homeostasis. Aberrant redox status of the cell is one of the critical determinants of cell death *(152,153)*. Normally functioning cells maintain reductive conditions that are manifested by the abundance of reducing equivalents and the proper reduced status of cellular proteins *(154,155)*. In cancer cells, however, adaptive metabolic changes to a high glucose-utilizing phenotype (the

"Warburg effect"—the well-known hallmark of malignant transition) *(156)* result in a shift toward a more oxidized state *(154,157)*. Vast literature corroborates the abnormal redox environment and increased sensitivity to oxidative stress of human cancers compared with normal tissues *(158–160)*.

Whereas oxidative stress is often associated with reactive oxygen species (ROS) generation, ROS formation is only one of the possible symptoms of abnormal redox status. Oxidative distortion of the sulfhydryl-disulfide balance at the protein level and depletion of NADPH, the primary cellular source of reducing equivalents, may not trigger immediate ROS generation. Not only is abnormal Prot-SH/Prot-SS balance alone proapoptotic, but it can also facilitate and enhance death responses to DNA damage.

5.2. Apoptotic Effects of Single-Action Protein-Reactive AAs

Single-action protein targeting alkylators, such as helenalin and parthenolide, are potent apoptotic stimuli. Pharmacologically relevant levels of these agents are known to trigger massive apoptosis as a direct result of the decrease in the ratio of Prot-SH to Prot-S-S (i.e., the pro-oxidative distortion of the proteome) *(148,151,161)*. As these drugs react also with SMNs, the effects on the proteome are enhanced by the distortion of the redox balance at the level of small molecules. The latter effects reflect the depletion of cellular GSH and the generation of ROS *(54,161–164)*. Apoptotic effects of parthenolide involve the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) *(54)*. The resulting cell death is associated with the activation of caspase-7, caspase-8 and caspase-9. In parthenolide-treated colorectal cancer cells, caspase-8 activation preceded cleavage of Bid, a proapoptotic member of the Bcl-2 family, Bax translocation to the mitochondria and release of cytochrome c to cytoplasm *(165)*. Apoptotic effects of parthenolide are abrogated by an antioxidant *N*-acetyl cysteine (NAC) and potentiated by a treatment promoting oxidative stress *(164)*. Rapid induction of apoptosis by parthenolide in leukemic cells involves an atypical pattern of responses *(53,166)*. Specifically, although some cells showed activation of caspases followed by a rather rapid loss of plasma membrane integrity, other cells (observed concurrently) underwent a caspase-independent death, assessed as atypical necrosis *(53,166)*. Atypical apoptosis involving rapid loss of plasma membrane integrity in response to parthenolide was also observed in breast cancer cells *(167)*. These effects were linked to targeting of the plasma membrane proteins by parthenolide *(166)*. Interestingly, although parthenolide induces potent apoptosis in leukemic cells, normal hematopoietic cells are relatively resistant *(164)*. This differential apoptosis was mainly attributed to the increased oxidative state of cancer cells.

Parthenolide-induced activation of JNK was found to lead to massive apoptosis in cancer cell lines resistant to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and TNF-α *(167,168)*. These effects involved phosphorylation of Bid and increased Bid cleavage *(167)*. In addition, parthenolide was able to promote apoptosis in antiestrogen-resistant breast cancer cells (derived from MCF-7 cells) *(169)* and to sensitize breast cancer cells to paclitaxel *(170)*. In part, these effects were attributed to suppressed NF-κB activation *(168–170)*. However, NF-κB inhibition alone does not sufficiently explain apoptotic potency of parthenolide, for example, in cells with inactive NF-κB *(53,164,166)*. Collectively, several lines of evidence implicates

distorted redox status as a major factor in the antiproliferative and apoptotic effects of parthenolide *(54,164)*.

Redox distortion plays a role also in proapoptotic effects of helenalin *(162,171,172)*. The key event in helenalin-induced apoptosis is mitochondrial dysfunction, including $\Delta\Psi_m$ collapse and the release of cytochrome c *(60–62,151)* followed by the activation of caspase-3 and caspase-8, phosphatidylserine externalization and the "classical" apoptotic nuclear morphology. Both phosphatidylserine translocation and apoptotic DNA fragmentation in helenalin-treated leukemic cells were abrogated by the pan-caspase inhibitor Z-VAD-fmk [benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone] *(62)*. However, apoptotic responses induced by helenalin were not attenuated by the overexpression of the antiapoptotic proteins Bcl-x(L) or Bcl-2 *(61,62)*. In addition, caspase inactivation had no effect on the cytotoxic effects of helenalin in prostate cancer cells (Woynarowska and Woynarowski, unpublished data). These results suggest that cell death caused by helenalin has Bcl-2-independent and caspase-independent components.

Bcl-2-independent mitochondrial dysfunction and caspase-independent apoptosis might well be a hallmark response to protein-alkylators in general. Bax/Bcl-2-independent $\Delta\Psi_m$ collapse and the release of AIF are relatively well-understood consequences of the oxidation of proteins that control the integrity of the inner mitochondrial membrane *(120,122,133,150)*. AIF translocates to the nucleus, where it promotes a long-range disruption of the nuclear chromatin. The AIF-mediated route represents an "alternative" apoptotic pathway that coexists with the Bax-mediated mitochondrial dysfunction in responses to agents that oxidize critical thiol groups in mitochondrial membrane proteins. AIF-mediated caspase-independent apoptosis is a characteristic feature of cell death induced by the protein oxidant diamide *(122)*. As discussed below (Section 6.3.), Bcl-2-independent and caspase-independent effects are implicated in cellular responses to protein-reactive AA/Pt drugs that also target DNA (dual-action agents).

5.2.1. Conclusion

Reactions of single-action AAs with proteins distort protein redox balance, which leads to potent apoptotic effects. These effects may be enhanced by the oxidative distortion at the level of small molecules and ROS generation. Unlike apoptosis originating from DNA damage, apoptosis reflecting protein damage/ROS generation is marginally affected by antiapoptotic members of the Bcl-2 family. Apoptosis resulting from targeting proteins (or, more precisely, the proteome as a collective target) may involve caspase-independent routes, in addition to caspase-dependent components.

5.3. At the Crossroads of Apoptotic Signaling—the Trx System

Whether or not oxidative insult (such as exposure to thiol-reactive AA/Pt drugs) results in apoptosis depends on the compensatory capacity of the endogenous redox buffers. The redox status of the cell is controlled by two major interacting systems—the GSH system and the Trx system. Although the GSH system buffers oxidative changes affecting small molecules, the independently regulated Trx system controls the redox status at the protein level *(173–175)*. The Trx system is universally and in multiple ways involved in cellular responses to protein-damaging agents and in modulation of responses to DNA damage (Fig. 2).

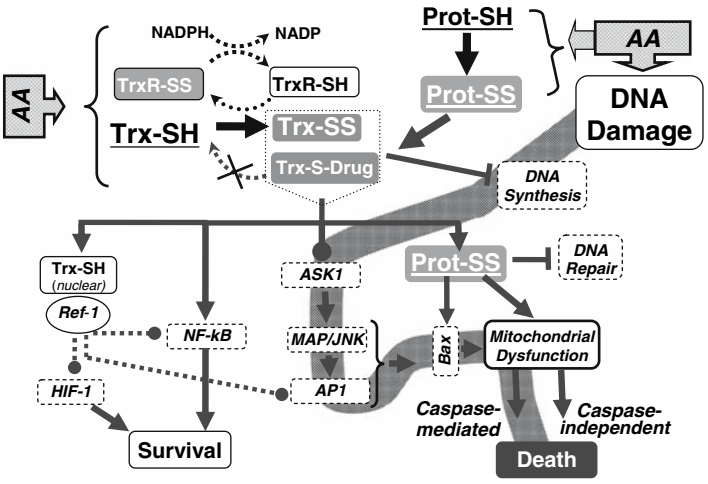

Fig. 2. Distortion of protein redox balance and targeting of the thioredoxin system in the interplay of death and survival responses to protein-damaging and DNA-damaging agents. The thioredoxin (Trx) system acts as a sensor and "nano-switch" regulating protein redox status. Depletion of reduced Trx (Trx-SH) by oxidation (reversible) and by drug adduction (irreversible) affects the global protein thiol-disulfide status. Reciprocally, a pro-oxidative shift in global protein redox balance (such as that caused by massive protein adduction by poly-targeted AA/Pt drugs) affects the status of the Trx system. The pathways of protein damage processing and the resulting signaling are marked with black arrows. Protein damage directly leads to apoptosis through caspase-dependent and independent routes, but also facilitates the execution of apoptosis and enhances death responses originating from DNA damage (the latter responses are symbolized by the gray ribbon).

Catalytically active (reduced) Trx acts as electron donor to numerous proteins that require the reduced sulfhydryl groups for their proper folding and function *(176)* restoring the reduced status of oxidatively inactivated proteins. Trx also plays an essential role in the repair of drug-adducted proteins by removing drug molecules in a trans-alkylation reaction. In either case, having re-activated another protein, Trx itself becomes inactivated. Oxidized Trx can be regenerated by the NADPH-dependent flavoenzyme @ thioredoxin reductare (TrxR) *(177)*. As the regeneration reaction consumes NADPH, its efficiency depends on the global balance of cellular redox equivalents. Thus, under conditions of oxidative stress, a proportion of oxidized (inactive) Trx molecules increases. Moreover, either Trx or TrxR can be irreversibly inactivated by direct reaction with AA/Pt drugs.

The Trx system has been proposed to act as a molecular "nano-switch" *(178)*. The "on" or "off" states, determined by the redox status of Trx/TrxR redox couple, are critically linked to proliferation, differentiation and apoptosis. Both the global non-specific protein damage/oxidation and specific targeting of Trx and/or TrxR proteins compromise the redox status and, thereby, the vital functions of the Trx system (Fig. 2). Even slight changes in the available reduced Trx (Trx-SH) are rapidly amplified in a cascade of responses.

Trx-SH is essential for the maintenance/restoration of the proper reduced status of many functionally critical proteins *(173)*. Therefore, even partial dysfunction of the Trx system has detrimental consequences that can propagate into more severe oxidative changes (Fig. 2). Irreversible cell degradation commences when such oxidative changes

progress far enough to disrupt the regulation of the redox status of mitochondrial proteins. Thus, oxidation of proteins that control the integrity of the inner mitochondrial membrane leads to the collapse of mitochondrial membrane potential ($\Delta\Psi_m$) and the leakage of apoptogenic components *(174,178)*. The released factors activate not only the caspase cascade but also caspase-independent death route. As Trx-SH acts as a negative regulator of ASK1, oxidized Trx facilitates the activation of ASK1/JNK/MAPK death signaling pathways (which play important roles in transducing DNA damage signaling, Fig. 2), leading to mitochondrial dysfunction and caspase-mediated apoptosis *(179,180)*.

Distortion to the Trx redox status is magnified by the cascades of redox-dependent transcriptional reprogramming *(177,181–183)*. Trx oxidation in cytoplasm may initially promote the nuclear translocation of such redox-dependent effectors as NF-κB and AP-1. However, the transactivation of these effectors [as well as hypoxia-inducible factor-1 (HIF-1)] to the transcriptionally competent forms in the nucleus depends on reduced redox effector factor-1 (Ref-1), whose reduction in turn requires reduced nuclear Trx (Fig. 2). As Ref-1 also functions as abasic site endonuclease (APE) in base excision repair *(88)*, its oxidized status may enhance cell death by adversely affecting the repair of DNA damage. Moreover, Trx/Ref-1 couple is also involved in the redox-mediated control of p53 protein conformation and p53-dependent signaling *(184,185)*. Thus, even though redox distortion may initiate pro-survival signaling, this signaling is likely to be futile under conditions of persistent redox imbalance and impeded Trx activity.

Tumor cells that function under abnormal oxidative conditions may need to accelerate the turnover of Trx and TrxR to maintain the redox integrity of their proteomes. Both Trx and TrxR proteins are highly inducible under stress conditions *(173,186)*. Accordingly, increased aggressiveness and radioresistance and chemoresistance of a large proportion of clinical tumors are attributed to elevated levels of Trx and/or TrxR *(187–189)*. The increased dependence of cancer cells on the Trx system, however, constitutes also their Achilles heel by opening a window of opportunity for agents targeting Trx and other redox-regulating proteins *(190)*.

5.3.1. Conclusion

The state of the Trx system and redox-dependent signaling in cancer cells could be critical for cell responses to all AA/Pt drugs that react with proteins. The oxidative shift in protein sulfhydryl/disulfide balance elicits potent apoptotic signaling and fundamentally changes cellular milieu. The distortion affects several vital pathways of signal transduction, including those involved in responses to DNA damage.

6. ENHANCED APOPTOSIS BY COMBINED TARGETING OF DNA AND PROTEIN REDOX STATUS

If DNA damage alone and protein damage alone are proapoptotic, it is reasonable to expect that a co-induction of both types of damage would lead to cumulative apoptotic effects *(141)*. Can the "dual action" amount to more than the sum of its components in terms of apoptosis induction? What is more important—DNA damage or protein damage? These questions have not yet been fully answered, as it is inherently difficult to dissect the interplay between signal transduction initiated by damage to nuclear DNA and protein damage-activated redox signaling. It may well be that some

AA drugs promote apoptosis mainly through protein damage, with DNA damage (if induced) providing an additional boost. A reversed situation is also plausible (e.g., for Pt drugs) that DNA damage can be essential, although protein damage would still substantially enhance lethal consequences of DNA lesions. In either scenario, poly-targeting, including proapoptotic consequences of protein damage, may help equalize apoptotic responses across diverse biological models.

As illustrated by the promising properties of irofulven and oxaliplatin, both variants of dual action may offer pharmacological advantages, as both drugs consistently induce apoptosis in tumor cells, including those models that are resistant to the classical DNA damage-promoted apoptosis. Moreover, irofulven and oxaliplatin serve as a paradigm that apoptosis induced by dual-action drugs may spare normal cells.

6.1. Irofulven—Interplay Between Redox Distortion from Protein Damage and Nuclear Signaling from DNA Damage

Irofulven at concentrations promoting significant apoptosis in cancer cells forms approximately 10^8 drug–protein adducts/cell, a significant fraction of which affect Trx *(107)*. Although irofulven also induces numerous DNA lesions, they are less frequent than drug–protein adducts (~10-fold difference in prostate cancer cells) and do not include crosslinks that typify highly lethal DNA damage. Thus, the balance of lethal effects of irofulven might be skewed toward a significant contribution from protein damage and, in particular, from targeted redox-regulating proteins.

6.1.1. Distortion of Redox Homeostasis by Irofulven

Consistent with a role of protein damage and Trx targeting, irofulven distorts protein redox homeostasis *(107)*. As found in prostate cancer cells, early redox-mediated responses include transient induction of Trx expression and DNA binding activation of redox-regulated transcription factors NF-κB and AP-1. Importantly, however, even at the peak of Trx protein level, *Trx activity* remains below the level observed in untreated cells *(107)*. This inhibition of Trx activity progressed markedly with time and was followed by symptoms of a global pro-oxidative distortion of redox homeostasis, that is, elevated protein carbonyls and decreased protein sulfhydryls. In addition to Trx, irofulven inhibited, albeit to a lesser degree, intracellular TrxR and another redox-regulating protein, glutathione S-transferase (GST) *(107,191)*. Irofulven cytotoxicity and apoptosis are inversely correlated with basal levels of Trx protein in prostate cancer LNCaP cells and its two genetically related sublines LNCaP-Pro5 and LNCaP-LN3 *(48)* (Woynarowska and co-workers, unpublished results).

Noteworthy, ROS generation was modest and delayed relative to changes in Trx expression and its activity *(107,140)*. In addition, several ROS scavengers tested were unable to block apoptotic effects of irofulven (Woynarowska and Woynarowski, unpublished data). Thus, ROS formation seems to reflect a consequence rather than a cause in irofulven-induced apoptosis, in agreement with other data suggesting that irofulven reacts more readily with proteins than SMNs, unlike SLs, helenalin and parthenolide. Accordingly, although cytotoxicity of ROS-inducing SLs depends on GSH levels, GSH depletion has marginal effect on irofulven cytotoxicity *(192)* (Woynarowska, unpublished data).

6.1.2. Oxidatively Distorted Environment and DNA Damage Signaling—Caveats and Opportunities

Irofulven is consistently cytotoxic and is a potent inducer of apoptosis in cellular models that are deficient in specific aspects of the classical DNA damage signaling *(48,49,193,194)*. Likewise, forced overexpression of Bcl-2 has little effect on irofulven-induced caspase-mediated DNA fragmentation and virtually no effect on the decrease in cell viability and growth inhibition *(61)*. Thus, DNA damage and resulting signaling might not be a decisive factor in responses to irofulven. Nevertheless, irofulven–DNA adducts are probably not insignificant. By interfering with DNA functions and activating partially non-overlapping apoptotic routes, DNA lesions may accelerate cell death and/or increase the proportion of irreversibly inactivated cells. DNA damage located in transcribed genes seems important for cell survival after exposure to irofulven, as impediments to transcription-coupled repair machinery sensitizes cells to the drug *(195,196)*. By contrast, the integrity of the global repair is not vital *(195,196)*, which also suggests that a large proportion of irofulven-induced DNA lesions may be relatively inconsequential.

Importantly, DNA damage signaling promoted by drugs such as irofulven is complicated by overlapping effects on the proteome. Responses that are usually presumed to originate from DNA damage may be profoundly modulated by the oxidatively distorted environment. Possible contribution of protein redox distortion needs to be considered in such aspects as (i) the activities of DNA damage recognition and processing proteins, (ii) the status of DNA damage signaling pathways, and (iii) cell-cycle redistribution (Fig. 2). For instance, compromised redox homeostasis is known to impede DNA damage recognition and repair by oxidative inactivation of cysteine-rich proteins involved in these processes *(173,197)*. In fact, one of such proteins, ataxia telangiectasia mutated (ATM), which is a major transducer in responses to DNA damage, has been demonstrated to be involved in sensing/modulating intracellular redox homeostasis *(198)*. Moreover, inactivation of Trx may cause the S-phase arrest by inhibiting the regeneration of ribonucleotide reductase *(173)*.

Even though some of the DNA damage recognition/repair proteins may not be vital for the repair of irofulven–DNA adducts, the possibility that the drug may compromise their function (either directly by formation of drug adducts or indirectly by global redox change) is relevant to the combinations of irofulven with other DNA-damaging agents, including the demonstrated potentiation of IR effects *(136)*. Studies on irofulven/IR combination in brain tumor cells implicated drug inhibitory effects on DNA-protein kinase (DNA-PK) *(199)*, one of the vital proteins in the recognition and repair of IR-induced DNA double-strand breaks. As irofulven is not a DNA strand scission agent, DNA-PK status has marginal effect on the cytotoxicity and apoptotic responses to the drug alone in glioma MO59K cells harboring wild-type DNA-PK versus its isogenic subline MO59J with a mutated DNA-PK. By contrast, the radiosensitization conferred by irofulven is markedly more pronounced in the inherently radioresistant MO59K cells than in MO59J cells *(199)*. Given that the drug inhibits DNA-PK activity in cell-free system, irofulven effects on DNA-PK may, at least partly, contribute to the greater radiosensitization in MO59K cells.

Another potential benefit of redox distortion by irofulven pertains to targeting hypoxic cells. In general, the activation of the Trx/Ref-1-dependent HIF-1 pathway is a frequent response of hypoxic cells that leads to chemoresistance and radioresistance.

Consistent with the ability to impede Trx function, however, irofulven inhibited HIF-1 transactivation and downstream HIF-1-mediated signaling in hypoxic cells *(191)*. Accordingly, irofulven retained fully its lethal potency against prostate cancer and glioma cells under hypoxia.

Given the pivotal significance of redox signaling to both DNA repair and apoptosis, it is conceivable that the levels, localization and the oxidation status of redox-regulating factors (such as Trx and/or Ref-1) might prove useful for predicting the overall death/survival outcomes in cells exposed to dual-action drugs. Further investigations with irofulven and other drugs are highly warranted to better understand therapeutic opportunities created by the environment with oxidatively distorted protein redox status.

6.2. Oxaliplatin and Other Pt Drugs—Apoptosis Promoted by Lethal DNA Lesions, but Enhanced by Protein Targeting?

6.2.1. Complicated Connections Between DNA Damage Signaling and Apoptosis

Apoptosis-inducing levels of Pt drugs result in fewer DNA adducts compared with irofulven ($\sim 4 \times 10^4$/cell for oxaliplatin), but the majority of these lesions are intra- and inter-strand DNA crosslinks as well as DNA-protein crosslinks *(29,200)*. In general, DNA damage produced by Pt drugs is believed to be a primary determinant of their lethal effects *(9,201)*. It is also clear that apoptosis is an important aspect of antiproliferative effects of cisplatin and other Pt drugs. In some models, the causative relationship between DNA damage and apoptosis induction is reasonably well established. For example, apoptotic responses to cisplatin are known to be particularly intense in testicular cancer cells that tend to have inherently low ability to repair DNA damage induced by Pt drugs *(202,203)*. In other models, however, the kinetics and magnitude of apoptotic effects relative to DNA damage may vary widely.

One particularly ambiguous and controversial aspect is the role of p53 that is expected to mediate responses to DNA damage by Pt drugs. Given a multitude of disparate outcomes, the still widespread notion that Pt drugs exert apoptosis in a manner dependent on wild-type p53 is an oversimplification. Even the distinct sensitivity of malignant testicular cells to cisplatin is not correlated with p53 status and reflects both p53-dependent and p53-independent pathways *(204,205)*. Mutated p53 may actually enhance cisplatin apoptosis *(206)*. In addition, cisplatin remains apoptotic in p53-negative hepatoma cells *(207)*.

Also oxaliplatin is capable of promoting similar levels of apoptotic responses in cancer cells differing in their p53 status *(200)*. Even though oxaliplatin is more growth inhibitory in some cells with wild-type p53 than in some cells with aberrant p53 *(200)*, the p53 status in a panel of 30 colorectal cancer cell lines fails to correlate with the sensitivity of these cell lines to oxaliplatin *(208)*. Although oxaliplatin was more apoptotic in p53+/+ colon carcinoma HCT116 than in its p53–/– subline *(208)*, another study concluded that p53 signaling only marginally contributes to oxaliplatin-induced apoptosis in HCT116 cells *(209)*. Furthermore, oxaliplatin was more lethal against colon carcinoma and breast cancer cells with mutated p53 than against their respective isogenic counterparts with wild-type p53 *(210)*. Finally, both cisplatin and oxaliplatin are able to promote an efficient response of p53 negative tumors *in vivo (211)*.

In addition to the controversy surrounding the role of p53 as a major mediator of apoptotic signaling from DNA damage, relationship between DNA damage induced by Pt drugs and apoptosis is not always unambiguous. For example, differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent cells was independent of the levels of drug accumulation and DNA adduct formation *(212)*. Lethality of DNA adducts formed by different Pt drugs is not uniform. Oxaliplatin needs to form significantly fewer DNA lesions than cisplatin for equitoxic effects. This difference is not attributable to altered repair of Pt–DNA lesions *(29,200,213)*. Moreover, direct comparison in a panel of several colon cell lines demonstrated that Pt–DNA adducts formation and repair were correlated with cisplatin but not with oxaliplatin cytotoxicity *(214)*.

More direct evidence suggesting that factors other than DNA damage at least modulate apoptotic responses to Pt agents is mounting. For example, experiments using enucleated cells (cytoplasts), which eliminate nuclear signaling, demonstrate that Pt drugs initiate apoptotic pathways that are independent of damage to nuclear DNA. In both nucleated cells and cytoplasts, cisplatin was shown to activate endoplasmic reticulum-specific caspase-12 and, subsequently, caspase-3 *(215)*. Likewise, enucleation did not prevent oxaliplatin-induced mitochondrial dysfunction *(209)*. Experiments with cytoplasts demonstrated that apoptotic resistance to oxaliplatin can be conferred not only by the nuclear compartment (which would be consistent with the prevailing significance of DNA damage) but also by cytoplasmic compartment *(209)*. In contrast to Pt drugs, topoisomerase poison etoposide fails to promote apoptotic signaling in cytoplasts, as expected for an agent that acts through damage to nuclear DNA *(215)*.

6.2.2. Interference of Pt Drugs with the Antiapoptotic TrxR

In addition to damage to nuclear DNA, protein reactivity of Pt drugs is one of the plausible factors that may contribute to apoptosis induction at several levels. Cisplatin macromolecule binding data *(23)* suggest that there are approximately 10^6 protein adducts/cell at apoptosis-inducing cisplatin concentrations. A higher number of protein adducts is expected for oxaliplatin, which is more reactive with cysteine and methionine residues than cisplatin *(72,81)*. Thus, apoptosis induction by Pt drugs is accompanied by protein damage, albeit most likely not as massive as for dual action agents such as irofulven.

It remains unclear how selective Pt drugs are in affecting cellular proteins. Moreover, the potential apoptotic consequences of protein damage by Pt drugs are by and large under-investigated, although there is one important exception. Namely, studies by several groups have implicated the interference with redox regulation and the antiapoptotic Trx system in the effects of Pt drugs *(83,84,216–220)*. Cisplatin and other Pt complexes have been demonstrated in cell-free and cellular models to inactivate redox-regulating proteins, in particular TrxR *(83,84,87,216)*. Recent data directly demonstrate that the inactivation of TrxR by oxaliplatin results from drug covalent adducts to this protein *(87)*. Oxaliplatin binding to TrxR was several-fold greater than binding to Trx under the same conditions. Consistent with TrxR being a preferred target, oxaliplatin is a more potent inhibitor of the enzymatic activity of purified and cellular TrxR than Trx *(87)*. The preference of Pt complexes (TrxR>Trx) is inverted relative to that of irofulven (Trx>TrxR), which illustrates the potential of chemically distinct drugs to differentiate among members of the same class of redox-regulating proteins.

Consistent with the role of TrxR targeting, attenuation of intracellular TrxR activity in cisplatin-treated cells correlates with the decrease in cell viability *(85)*. In addition, downregulation of TrxR expression sensitizes cells to cisplatin *(85)*, whereas forced overexpression of TrxR, but not the catalytically inactive TrxR mutant, confers resistance to oxaliplatin *(87)*. In addition to the effects of Pt drugs on cytoplasmic form of TrxR (TrxR1), a link was suggested between the inhibition of mitochondrial TrxR2 by cisplatin and drug-induced dissipation of the mitochondrial membrane potential *(221)*. Even though Pt drugs prefer TrxR, targeting of Trx may not be insignificant. In various types of cancer cells, elevated Trx (cellular or secreted) was associated with resistance to cisplatin *(219,220,222,223)*. A Trx inhibitor 1-methyl-propyl-2-imidazolozyl disulfide was shown to increase cisplatin-induced apoptosis and enhance growth inhibition *(224)*. In some models, however, Trx is not critical for cisplatin activity *(225,226)*.

6.2.3. Conclusion

Oxaliplatin typifies a dual-action drug for which DNA damage is most likely essential, whereas protein damage could be secondary. Still, the inhibition of TrxR is probably an important factor in apoptosis induced by oxaliplatin and various other Pt drugs.

6.3. Convergence of DNA Damage and Protein Damage Signaling in Mitochondrial Dysfunction

6.3.1. Caspase-Dependent and Caspase-Independent Cell Death Induced by Irofulven

Apoptotic signals resulting from both DNA damage and protein damage are predicted to converge at the level of mitochondrial dysfunction (Fig. 2). Recent investigations in our laboratory have confirmed that mitochondrial dysfunction indeed plays a central role in irofulven-induced apoptosis and have comprehensively characterized its nature and downstream consequences in prostate cancer cells *(116)*. In conjunction with data on irofulven responses obtained in other cancer models, these studies have allowed us to delineate irofulven effects leading to cell death and to suggest how the revealed overlapping pathways can be related to the initial drug-induced insult.

Irofulven promotes the "classical" Bax/Bcl-2-mediated mitochondrial dysfunction. The observed early (6–12 h) effects involve translocation of Bax to mitochondria, loss of mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome c release *(116)*. Downstream from mitochondrial dysfunction, irofulven activates the initiator caspase-9 and executioner caspases *(61,116,227)*. Activated caspases provide a positive feedback loop that enhances cytochrome c release and facilitates $\Delta\Psi_m$ collapse at later times. Noteworthy, caspase-3, which serves as a major executioner for various apoptotic stimuli, is dispensable in irofulven-induced apoptosis. Although irofulven activates caspase-3, other caspases are processed more extensively, and caspase-3 inhibition has marginal effect on the subsequent apoptotic DNA fragmentation *(116,227)*. Moreover, MCF-7 breast cancer cells, which are generally resistant to apoptosis because of caspase-3 deficiency, are as sensitive to irofulven as caspase-3-proficient breast cancer cells MDA-MB-231 *(227)*.

The initial manifestations of apoptosis by irofulven are accompanied by caspase-dependent DNA fragmentation, phospholipid remodeling and chromatin condensation *(48,49,61,116,227)*. General and specific caspase inhibitors abrogate irofulven-induced apoptotic DNA fragmentation with the following order of potency against their caspase targets: pan-caspase ≥ casp-9 > casp-8/casp-6 > casp-2 > casp-3/casp-7 > casp-1/casp-4 *(116)*. The potent inhibitory effect of the caspase-9 inhibitor corroborates the initiator role of this caspase.

A study with synchronized cells demonstrated that irofulven-induced apoptosis is pre-mitotic *(139)*. The drug arrested the S-phase progression and blocked cell entry from G1 into S. In either case, apoptotic cells were directly recruited from blocked cell population, indicating that neither cells traverse through mitosis nor active DNA synthesis is needed for irofulven-induced cell death *(139)*. These attributes could explain the ability of the drug to efficiently kill non-proliferating (contact inhibited) cancer cells *(191)*.

Progression of irofulven-induced apoptosis results in compromised cell membrane integrity, which is typically observed 24–36 h later than early manifestations of apoptosis *(61,116,227)*. A significant contribution of apoptosis to the antiproliferative effects of irofulven is further corroborated by (i) formation of sub-G1 particles, (ii) net cell loss in short-term cytotoxicity assays, and (iii) a long-term inhibition of clonogenic survival *(48,136,227)*. In addition to several prostate cancer cells, a similar pattern of responses to irofulven was observed in other types of cancer cells, including leukemic, breast, ovarian, colon and brain tumor cells.

It must be highlighted that the classical Bax/Bcl-2-mediated caspase-dependent pathway reflects only one facet of irofulven-induced apoptosis. As mentioned above (Section 5.), protein-reactive agents may cause mitochondrial dysfunction bypassing the antiapoptotic regulation by Bcl-2 or Bcl-X_L. In such cases, mitochondrial permeability transition is initiated by changes in redox-sensing mitochondrial membrane proteins and results in the release of AIF *(120,122,133,150,228)*. Several observations demonstrate that Bcl-2-independent effects play a role in responses to irofulven and contribute to the caspase-independent cell death *(116)*.

The timing of irofulven-induced loss of $\Delta\Psi_m$ relative to cytochrome c release suggests that the inner membrane depolarization is initially independent of Bax/Bcl-2-regulated outer mitochondrial membrane permeability changes that lead to cytochrome c release *(116)*. Accordingly, the early $\Delta\Psi_m$ collapse is not inhibitable by forced overexpression of Bcl-2 *(61)*. Moreover, forced Bcl-2 overexpression had marginal to weak inhibitory effect on irofulven-induced caspase-mediated DNA fragmentation *(61,139)*. Neither early phospholipids remodeling nor the inhibition of cell proliferation were affected by Bcl-2 overexpression. The relevance of Bcl-2-independent effects to cell death is further substantiated by the lack of correlation between Bcl-2 gene expression and irofulven cytotoxicity in the NIH panel of 60 cells lines and drug apoptosis in several of these cell lines *(61)*. Collectively, these data show that Bax/Bcl-2 imbalance mediates only a minor portion of irofulven-induced apoptotic effects.

Consistent with the observed caspase-independent death, irofulven binds to a subset of mitochondrial proteins and promotes the release of AIF (in leukemic CEM cells, Liang and Woynarowska, unpublished data). Moreover, preventing the reactivity of irofulven with the mitochondrial membrane proteins partially antagonizes the apoptotic and antiproliferative effects of irofulven in prostate cancer cells *(116)*. In contrast,

blocking the caspase cascade, which largely abrogates DNA fragmentation, only minimally protects against the loss of cell viability and does not affect cell growth inhibition *(116)*.

6.3.1.1. Conclusion. The fate of irofulven-treated cells is probably determined by a complex interplay of signaling routes originating from drug effects on its diverse cellular targets. These signals are integrated at the mitochondrial level and proceed along two coexisting routes: caspase dependent and caspase independent. This redundancy of death modes could be essential for the ability of irofulven to efficiently kill cancer cells, including those cell types that tend to be resistant to other apoptosis inducers.

6.3.2. Death Routes Promoted by Cisplatin and Oxaliplatin

Mechanistic attributes of apoptosis induced by Pt drugs show many similarities with irofulven. One potential difference is that in addition to the prominent role of mitochondrial dysfunction, apoptosis by Pt drugs may also involve, at least in some models, the death receptor-mediated apoptotic pathway. The activation of both pathways was implicated, in particular, in the rapid and efficient apoptosis of testicular cancer cells treated with cisplatin *(203)*. Likewise, oxaliplatin increased CD95 receptor and CD95 ligand levels in colon carcinoma cells *(229)*. A role for the death receptor pathway is consistent with the activation by oxaliplatin and cisplatin of caspase-8 (in addition to the caspase-9 and caspase-3 that are characteristic of the mitochondrial dysfunction pathway) *(229,230)*. However, the extent to which apoptosis by Pt drugs depends on the death receptor pathway remains controversial. For example, even in Fas-competent models, exogenously added anti-Fas/FasL reagents may fail to attenuate drug-induced apoptosis *(231,232)*. Moreover, caspase-8 activation by Pt drugs does not always reflect the death receptor pathway but can also be a by-product of activation of other caspases. Cisplatin activates caspase-8 in Hep3B cells that are Fas deficient *(207)*. The activation of caspase-8 by cisplatin in other models was also suggested to proceed in a mitochondria-dependent and Fas-independent manner *(233)*.

Mitochondrial effects of Pt drugs involve proapoptotic and antiapoptotic proteins of the Bcl-2 family. Bax upregulation is an early event in cisplatin-induced apoptosis in testicular tumor cells *(234)*. Oxaliplatin-induced apoptosis in colorectal carcinoma HCT116 cells involves Bax translocation to mitochondria, which precedes cytochrome c release *(208)*. Apoptotic effects of oxaliplatin in these cells are enhanced by down-regulation of Bcl-X_L *(235)*. Bax/Bak-dependent mitochondrial apoptosis was implicated in oxaliplatin resistance *(208,209)*. Bax-mediated mitochondrial dysfunction and the release of cytochrome c along with another caspase activator DIABLO characterized the responses of hepatoma cells to cisplatin *(207)*. Cytochrome c release was also observed in cisplatin-treated cervical carcinoma and squamous carcinoma cells *(236,237)*. Correspondingly, forced overexpression of Bcl-2 conferred partial protection from cisplatin and oxaliplatin in glioma cells and cervical carcinoma cells *(61,238)*. It needs to be noted, however, that a significant portion of caspase-dependent DNA fragmentation induced by both cisplatin and oxaliplatin in cervical carcinoma cells was resistant to Bcl-2 overexpression *(61)*. Interestingly, cisplatin-activated caspases were shown to promote a proapoptotic Bcl-2 cleavage to a Bax-like fragment in

melanoma cells, suggesting a positive feedback by which caspase activation may enhance mitochondrial dysfunction *(239)*.

In addition to caspase-8, the initiator caspase-9 and executioner caspase-3 are consistently found activated in responses of various models to cisplatin and oxaliplatin *(200,207,208,229,230,236,237,240)*. Reconstitution of caspase-3 sensitized caspase-3-deficient MCF-7 cells to cisplatin action *(241–243)*. Caspase activation by Pt drugs is typically accompanied by the cleavage of poly(ADP-ribose) polymerase (PARP).

Caspase-mediated apoptosis is clearly not the only death pathway activated by Pt drugs. In Fanconi anemia lymphoblasts, low concentration of cisplatin caused chromatin condensation and phosphatidylserine externalization in the absence of significant caspase activation (albeit caspase-3, caspase-8 and caspase-9 were activated at higher drug levels) *(230)*. The activation by cisplatin of caspase-9 and caspase-3 in ovarian cancer cells was blocked, as expected, by the overexpression of crosslinked inhibitor of apoptosis protein (XIAP) *(240)*. Yet, this inhibition had no effect on the magnitude of cisplatin-induced apoptotic cell morphology. Likewise, in cisplatin-treated melanoma cells, the inhibition of caspase-3 and caspase-7 abrogated neither the apoptotic morphology nor the proteolytic PARP degradation *(244)*. Pretreatment with the pan-caspase inhibitor Z-VAD-fmk inhibited cisplatin-induced phosphatidylserine externalization in some epithelial cancer cell lines but had no effect in L1210 cells *(245)*. Likewise, pretreatment with Z-VAD-fmk afforded only partial decrease in cisplatin-induced apoptotic morphology in hepatocellular carcinoma cells *(207)*. Finally, inhibition of caspases had no effect on the lethality of oxaliplatin in LNCaP-Pro5 prostate cancer cells *(87)*.

6.3.2.1. Conclusion. Mechanistic attributes underlying apoptosis by Pt drugs are consistent with a contributing role of protein damage. Mitochondrial dysfunction induced by Pt drugs appears to be partly Bcl-2 independent. In addition to caspase-dependent apoptosis, Pt drugs promote caspase-independent cell death. Caspase-independent component seems sufficient for cell inactivation caused by oxaliplatin, which is reminiscent of analogous effect promoted by irofulven.

6.4. Differential Apoptosis in Cancer Versus Normal Cells

As discussed in Section 5.2., redox-regulating systems may constitute the Achilles heel of cancer cells that need to cope with the challenges of a more oxidative state than normal cells have. If redox distortion plays a role in the effects of dual-action AA/Pt drugs, it is then reasonable to expect that apoptosis in cancer cells may be facilitated compared to responses in normal cells. Clinically effective AA/Pt drugs obviously have a degree of selectivity *in vivo*. Yet, a question remains whether inherent differences exist in basic responses to dual-action drugs in tumor versus normal cells. Somewhat surprisingly, until recent years, drug effects were rarely investigated in normal cell lines in a side-by-side comparison to tumor cell lines. Nonetheless, such comparative data for irofulven and, to a lesser extent, for oxaliplatin are available. These data suggest that both drugs can indeed preferentially induce apoptosis in cancer cells.

6.4.1. Non-Lethal Effects of Irofulven in Normal Cells—Buffering of Redox Changes?

Studies in our laboratories have extensively compared effects of irofulven in various types of cancer cells (including prostate, colon, breast, brain, cervical and leukemic cancer cell lines) against various normal cell lines (prostate, breast, renal and mammary epithelial cells, colon mucosa, fibroblasts and endothelial cells) *(48,136,141,191,227,246)*. Collectively, these studies demonstrate that the responses of normal cells to irofulven are qualitatively different from the responses of tumor cells.

These differences are manifested at several levels. An equivalent "load" of drug macromolecule adducts is 3-fold to 4-fold less growth inhibitory in all tested normal cell lines *(48)*. Most importantly, however, growth-inhibited normal cells remain viable and capable of resuming proliferation after the removal of the drug, whereas cancer cells are irreversibly inactivated *(48,136,227)*. In contrast to the profound apoptosis in tumor cells, marginal or very low levels of apoptosis are detected in normal cells. These qualitatively dissimilar responses are consistently observed for all tumor and normal cell lines examined, a range of treatment conditions and multiple apoptotic endpoints *(48,136,227)*. Normal cells remain non-apoptotic and fully viable after a prolonged (4 days) exposure to drug concentrations exceeding by 1–2 orders of magnitude levels that are apoptotic and lethal in cancer cells after 12–24 h *(136)*. Furthermore, contact-inhibited normal cells remain fully viable under treatment conditions that completely inactivate contact-inhibited prostate cancer cells *(191)*. Hence, irofulven is cytotoxic (lethal) in proliferating and non-proliferating tumor cells but merely reversibly cytostatic and essentially non-lethal in normal cells.

This resistance of normal cells to apoptosis reflects different responses to irofulven-induced protein damage and DNA damage, not altered drug accumulation or reduced binding to macromolecules *(48,107)*. Levels of irofulven macromolecular adducts that are similar or higher than those resulting in profound apoptosis in cancer cells are non-apoptotic in normal cells such as normal prostate epithelial cells (PrECs) and normal colon mucosa NCM-460 cells. Microarray analysis revealed that in response to irofulven, NCM-460 and PrEC cells rapidly upregulate several genes pertinent to apoptosis and cell-cycle regulation *(246)*. The significantly upregulated genes included transcriptional activator E2F, several damage recognition genes and cyclins and antiapoptotic survivin. In contrast to the normal cells, prostate cancer LNCaP-Pro5 cells extensively downregulated multiple antiapoptotic, pro-proliferative and DNA damage recognition genes. Proteome profiling by 2D gel analysis revealed corresponding patterns *(246)*. Normal cells displayed multiple upregulated proteins and no discernible downregulation, whereas the pattern for tumor cells indicated downregulation and degradation of multiple proteins. These data suggest that normal cells, but not cancer cells, are able to neutralize the irofulven-induced insult by a sustained corrective response, probably involving buffering of redox changes. In contrast to progressive inhibition of the activity of Trx, TrxR and GST, and the global distortion of protein redox state in sensitive cancer cells, in normal cells, even higher levels of irofulven caused only a transient and modest decrease in Trx activity with no inhibition of TrxR and GST *(107,191)*. After a few hours, normal cells were able to restore the pre-insult activity of Trx as well as the global protein redox balance. These results suggest that a more efficient protein redox regulation is a likely major factor in the ability of normal cells to avoid apoptosis in response to irofulven.

The buffering of oxidative changes in protein redox status is mainly governed by the cytoplasmic compartment, in which most of cellular Trx activity and the activities of other redox-regulating proteins are located. The role of the cytoplasmic compartment in apoptotic resistance of normal cells to irofulven is supported by the experiments with apoptosis mimicking cell-free systems consisting of isolated nuclei and cytosolic extracts. Incubation of nuclei from apoptosis-resistant normal cells with cytosols from irofulven-treated cancer cells resulted in characteristic apoptotic changes: partial chromatin condensation, nuclei breakage and the release of small chromatin fragments *(247)*. In striking contrast, cytosols from irofulven-treated normal cells had no effect on the morphology of nuclei from either tumor or normal cells. These findings demonstrate that nuclei in normal cells remain sensitive to irofulven-induced apoptosis. However, apoptotic signals in these cells are attenuated at the cytoplasmic level.

6.4.2. Oxaliplatin—a Greater Selectivity for Cancer Cells than Cisplatin?

Relevant data on oxaliplatin effects in normal cells are considerably less extensive than those for irofulven. Still, normal prostate cells (PrEC) and normal fibroblasts WI-38 were found to be remarkably resistant to oxaliplatin *(200)*. Both PrEC and WI-38 cells remained viable under conditions resulting in potent apoptosis induction in several tumor cell lines. Oxaliplatin is thus another dual-action drug that appears to have the ability to differentially kill cancer cells while sparing normal cells.

Not all the Pt drugs may be equally selective for cancer cells in their apoptotic effects. Cisplatin was reported to be apoptotic in normal WI-38 cells *(248)*. Likewise, consistent with the known renal toxicity and drug-related hearing losses, cisplatin induces significant apoptosis in normal renal cells and normal auditory cells *(245,249)*. It is plausible that the superior toxicological profile of oxaliplatin might in part reflect oxaliplatin's potential to better differentiate between tumor cells and normal cells in apoptosis induction.

6.4.2.1. Conclusion. Normal cells can neutralize the adverse consequences of high levels of irofulven macromolecular adducts that are lethal to cancer cells. The Trx system and a greater capacity of normal cells to maintain protein redox homeostasis are likely factors in these differential apoptotic responses. Also, oxaliplatin seems able to differentially kill cancer cells while sparing normal cells. Further exploration of redox regulation as the molecular basis underlying differential apoptosis should help to optimize the use of current AA/Pt drugs and facilitate the design of better agents.

7. CONCLUDING REMARKS

AA/Pt drugs remain among the most useful clinical anticancer agents. Yet, they are often perceived as "non-specific" agents that must be inherently inferior to newer strategies aimed at defined molecular targets. Reviewed literature suggests an alternative viewpoint—that the ability of AA/Pt drugs to affect multiple cellular targets underlies their apoptotic effects and that this poly-targeting could, in fact, be a crucial attribute of various clinically active AA/Pt drugs. Moreover, a potent lethal component provided by such drugs could be essential to complement narrowly targeted strategies *(16)*. It thus seems highly relevant to thoroughly explore and understand the connection

between the poly-targeted nature of these agents and their desirable proapoptotic properties.

Regrettably, the pleiotropic reactivity of AA/Pt drugs with many cellular biomolecules remains understudied and conceptually underdeveloped, being overshadowed by the assumption that DNA damage is by far the main, if not the only, biologically important effect. Unrepaired damage to nuclear DNA undoubtedly plays a critical role in cell inactivation by many AA/Pt drugs. However, DNA-specific alkylators (which do not react with other targets) tend to be less proapoptotic than protein-specific AA agents (which do not react with DNA) and dual-action drugs (that react with both DNA and proteins). Accordingly, there is a growing appreciation of targets other than DNA as factors that markedly contribute to cell death induced by AA/Pt drugs.

Promoting cell death at multiple levels accelerates the process and reduces dependence on known adverse factors that might limit apoptosis by narrowly targeted agents. The paradigm of dual-action drugs such as irofulven and oxaliplatin suggests that the combination of DNA damage and protein targeting elicits cumulative apoptotic effects that involve the co-activation of caspase-mediated and caspase-independent routes. Consequently, irofulven and oxaliplatin are consistently apoptotic not only in generally sensitive cell types but also in various apoptosis-resistant models. The idea that drug effects on multiple targets cumulate in enhanced apoptosis is pertinent to many other AA/Pt drugs that react with DNA and proteins. In essence, a poly-targeted agent can be viewed as a virtual drug combination (which may be pharmacologically advantageous over real combinations of multiple narrowly acting drugs).

The concept of poly-targeting affecting the cell homeostasis, rather than a unique biomolecular target, underscores the need to revise the still common perception of pharmacological apoptosis as an execution of a hardwired teleological "program," in which cell responses rigidly follow specific pathways. It is not surprising that in the conceptual frame of "programmed" death, networks of cell responses to poly-targeted AA/Pt drugs are "shockingly complex" *(15)* and difficult to generalize in predictive ways. However, pharmacological apoptosis can be viewed, more simply, just as cell demise mediated by a spontaneous, entropy-driven breakdown of cellular structures and organization.

The fundamental principle of life is struggle for entropy to maintain order and organization. According to the second law of thermodynamics, the sum of entropy of all the biochemical processes in the cell is always positive *(250)*. As information fluxes are formally equivalent to negative entropy *(250,251)*, drug-induced cellular responses ("signaling") distort the entropic steady state in the unfavorable direction. This "entropy penalty" must be compensated either by net entropy flowing into the system (through exchanges of energy and materials with the environment) or by cell de-organization, whichever is feasible and thermodynamically most advantageous for given states of the metabolome and environment *(250,251)*. Pharmacological apoptosis can thus be perceived as a "lost struggle for entropy," in which spontaneous cell degradation compensates for negative entropy change caused by cell responses to an overwhelming drug insult. De-compartmentalization, such as leakage of mitochondrial components, and proteolytic/nucleolytic cascades generate entropy. Likewise, hypercondensation of nuclear chromatin, one of the most characteristic morphological hallmarks of apoptosis, has been proposed to be driven by favorable changes in conformational entropy *(252)*.

The entropic perspective implies cell de-organization along the thermodynamic "path of the least resistance" rather than along fixed, predefined pathways. Accordingly, drug-induced apoptosis shows multiple and context-dependent manifestations. When one route (e.g., caspase cascade) is not available or becomes inhibited, cell de-organization may shift to alternative routes. This implies that some specific effectors/pathways that are important in some models may become marginalized in others. Another implication of practical significance is that the global state of the metabolome (reflecting the thermodynamic status of the cell) may represent the ultimate determinant of the "point of no return" and predictor of death/survival outcomes, prevailing over the status of any specific proapoptotic or antiapoptotic effector molecule.

Disruption of cell redox homeostasis by poly-targeted AA/Pt drugs exemplifies a scenario in which the global status of the cell affects a multitude of specific effectors and signaling pathways. Pro-oxidative changes in the protein redox status directly promote apoptosis and facilitate apoptotic signaling from other stimuli. AA/Pt drugs can distort protein redox status by a combination of (i) their total adducts in the entire proteome (serving as a collective target) and (ii) inactivation of the key proteins that regulate protein redox homeostasis. An increasing body of evidence demonstrates that various AA/Pt drugs interfere with the functions of the antiapoptotic Trx system that plays pivotal roles in the global regulation of cell redox status. Moreover, the state of redox imbalance profoundly affects various cellular functions, including DNA synthesis, DNA damage repair/signaling and transcriptional responses, as various proteins involved in these processes are redox sensitive. Protein redox status may be one of the decisive factors determining the death or life cellular switch and overriding some specific pathways and apoptotic effectors. An attractive possibility has emerged that, unlike cancer cells, normal cells may be able to avoid apoptosis in response to poly-targeted AA/Pt drugs because of their less strained redox regulation systems and, consequently, a greater capacity to buffer oxidative distortion.

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23 Contributions of Apoptosis and Senescence to Cytotoxicity Produced by Microtubule-Stabilizing Agents

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Summary

The antitumor drug Taxol is recognized for its efficacy in the treatment of cancer and has become the prototype for a class of drugs known as the microtubule-stabilizing agents (MSAs). Despite years of investigation, a clear and detailed mechanism by which these compounds bring about tumor cell death remains elusive. Apoptosis, with its well-defined biochemical and cell-structural characteristics, has often been presented as the most important direct result of treatment with Taxol. It is now clear that the major response to the MSAs is far more complex and depends on the cell type, the specific drug being studied, and the concentration of drug to which the cell is exposed. Although apoptosis may occur at a downstream step in the response of cells to these agents, it has not been demonstrated that this effect is the direct result of the binding of Taxol to its known target, the microtubules, except in hematopoietic cancer cells. Other cell death processes that do follow directly from the binding of Taxol to the microtubules include caspase-independent cell death, with or without an apoptotic morphology, and a characteristic aberrant mitosis that is often referred to as mitotic catastrophe. These processes appear to produce a significant amount of cell death well after that which would be produced by direct caspase activation. In addition to cell killing, cytotoxic agents are known to elicit the stress-induced, terminal cell proliferation referred to as premature or accelerated senescence. This response had been thought to play a minor role in the actions of the MSAs, but recently, one such agent, discodermolide, was shown to elicit a powerful induction of accelerated senescence. The senescence phenotype appeared after the characteristic aberrant mitosis seen with these agents, and it was invariably followed by a delayed form of cell death. The powerful effects of Taxol and much of the promise perceived in newer MSAs appear to result from their complex and widespread effects on cell function and from many options they have for inhibiting uncontrolled tumor cell proliferation.

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Key Words: Taxol; discodermolide; microtubules; accelerated senescence; microtubule-stabilizing agent; apoptosis; mitotic catastrophe.

1. INTRODUCTION

Taxol (paclitaxel) was the first microtubule-stabilizing agent (MSA) reported *(1,2)*, and today, many other compounds with a similar mechanism of action have been described *(3)*. Approved by the FDA for ovarian, breast, and small-cell lung cancer, it is used in the treatment of a wide variety of cancers. A second-generation taxane, Taxotere (docetaxol), is approved for metastatic- and anthracycline-resistant breast cancer *(4)*. Other naturally occurring compounds, not structurally related to Taxol, have been found to produce comparable microtubule-stabilizing effects in cancer cell lines and inhibition of tumor cell growth, while improving upon some of the problems encountered with Taxol, such as those involving solubility and resistance. The epothilones, discodermolide, and laulimalide have been extensively studied in an effort to identify important structural correlates with regard to microtubule effects, drug resistance, and pharmacological properties and have also been incorporated into clinical trials *(5–7)*. In addition, one of these agents, discodermolide, has demonstrated synergism when administered with Taxol to cell lines and to mice harboring tumor xenografts *(8,9)*.

Many have attempted to uncover the mechanism(s) by which MSAs suppress tumor growth. The primary effects of Taxol have long been believed to result from its interference with mitotic spindle assembly and function, leading to the failure of accurate chromosome segregation *(10)*. Yet, the path to tumor growth inhibition and cell death that follows MSA binding to the major target, microtubules, has not been adequately defined. Despite extensive experimentation that describes this cell death as being apoptotic, the story is more complex than what can be described as apoptotic cell death and varies with the specific drug, its concentration, and tumor type *(11–13)*.

Given the importance to an organism of a cell's decision to live or die, it is intuitively reasonable that redundant mechanisms may have evolved to assure that more than one cell death option is available. Many have criticized the use of the term apoptosis as a catchall for cell death accompanied by caspase activation following treatment with cytotoxic agents and have demonstrated other, caspase independent, mechanisms of cell death *(14–17)*. The actions of Taxol and the other MSAs also appear to support these observations in that many different cell signals are initiated after microtubule stabilization in different cell types, and these processes can initiate a delayed, downstream activation of caspases and/or other proteases, all of which may contribute to cell death *(18,19)*.

2. CELL DEATH AFTER MICROTUBULE STABILIZATION

Certain effects on microtubule structure and function are well established as being direct actions of Taxol and the other MSAs. Most of these agents bind to β-tubulin that is in the α/β-tubulin dimer and promote microtubule stabilization, inhibit microtubule disassembly, and suppress the normal dynamic behavior of microtubules because of the inhibition of lengthening and shrinking at their polymer ends *(20,21)*. This stabilization interferes with a host of diverse cell functions including the maintenance of cell structure, intracellular trafficking of proteins and vesicles by motor proteins and both the capture and the separation of sister chromatids during mitosis *(22–25)*. As mitotic spindle microtubules are more dynamic than those assembled during interphase, the

binding of an MSA is particularly disruptive of cell division and the checkpoint processes that insure its fidelity *(26)*. Depending on cell type and drug concentration, these initial effects can produce a number of secondary responses ranging from mitotic arrest leading to apoptosis to aberrant mitosis with multipolar spindles that yield aneuploid interphase cells. An impressive amount of delayed cell death may occur, sometimes involving caspase activity *(27–29)*.

2.1. Apoptosis

As Taxol-induced cell death has been reported to be accompanied by some caspase activation, and/or to produce an apoptotic morphology, it has frequently been said to result from apoptosis. The assumption had been, for many years, that MSAs induce programmed cell death through inhibitory actions on the mitotic spindle *(30–32)*. One of the weaknesses in this assumption is that in many of the studies cited, cancer cell lines were exposed to micromolar drug concentrations for prolonged periods of time. Under these conditions, cells can arrest in metaphase, prior to apoptosis, enter a G1 block, or be involved in endoreduplication (DNA proliferation uncoupled from cell division) *(28)*. Although Taxol infusions in humans may expose a small population of cells within the tumor to a brief spike of a micromolar drug concentration, drug metabolism and clearance quickly reduce this concentration to the nanomolar range *(33)*. Thus, long-term, high concentrations of Taxol cannot address the question of which cell growth-inhibitory mechanisms participate during drug treatment.

A second issue concerns the many studies that purport to assess cell killing of a specific cancer cell type by an MSA but consider only cell death that consists of the rapid appearance of classical apoptotic morphological characteristics. When Taxol or another MSA binds to microtubules at a more clinically relevant, nanomolar concentration, both non-transformed and cancer cells with wild-type p53 and p21 expression arrest in G1 and/or G2 of the cell cycle, protecting them from mitotic arrest. Cells that do not express either a functional p53 or a functional p21 are more sensitive to mitotic arrest and the associated caspase-dependent cell death with an apoptotic morphology *(28,34)*. In either case, the cells that manage to exit from mitotic arrest and enter into the G1 phase display aneuploidy and multiple micronuclei and are often referred to as having undergone slippage *(28,29)*. This leads to a poorly understood process that may play a major role in cell killing by these drugs and has been referred to as "slow death" *(13)*. In ovarian cancer cells treated with nanomolar concentrations of Taxol, it was reported that there was almost no decrease in cell viability after 24 h and only a 10% decrease in cell number at 48 and 72 h *(35)*. Although values for cell death were not obtained at later time points, these authors report that only a small percentage of the surviving cells were able to proliferate and form colonies. These findings support the idea that Taxol treatment acts through a mechanism leading to a delayed cell death. Such delayed cell killing was shown to occur despite the use of broad spectrum caspase inhibitors but was blocked by inhibitors of the lysosomal protease cathepsin B, suggesting that lysosomal permeabilization is affected by the binding of MSAs and may play a role in this delayed cell killing by Taxol *(36)*.

Taxol has also been reported to initiate apoptosis by various cell-signaling pathways. One of the most actively investigated areas is the phosphorylation of Bcl-2 following c-Raf-1 or Ras activation *(37–39)* or mTOR activation *(40)*. Although the inactivation of Bcl-2 undoubtedly contributes to Taxol-mediated cell killing, the subject

remains controversial given that the observed Taxol-mediated phosphorylation may reflect the increased percentage of cells blocked in mitosis *(41,42)*. Taxol also directly modulates the levels of Bcl-2 family members in certain cancer cell models *(43–46)*. The drug produces an aberrant activation of cyclin-dependent kinases with checkpoint protein activation that may play a role in the cell death resulting from microtubule stabilization *(47,48)*. Depending on cell type, these agents have been shown to produce activation/suppression of one or more of the mitogen-activated protein kinases (MAPKs), which may also play a role in the cell killing observed *(49–53)*.

Although many such studies can be cited as evidence of the ability of MSAs to produce cell death, this approach has demonstrated convincingly a prominent role for apoptosis only in the death of hematopoietic cancer cells *(54–57)*. In solid tumors, such studies have shown caspase activation to be delayed, often occurring downstream of the commitment to cell death. This suggests that apoptosis is only one of the many possible mechanisms involved in Taxol-mediated cell death *(12,13,18,58–61)*. Other studies have noted a lack of caspase activation prior to cell death resulting from Taxol administration *(11,62)* or unchanged cell killing after the inhibition of caspases produces a loss of apoptotic phenotype *(63)*. However, cell death with a classically apoptotic morphology may also be produced by other proteases *(19)*. Thus, although caspase activation has been shown to participate in cell killing by MSAs, and the treated cancer cells often present with a classical apoptotic morphology, apoptosis has not been demonstrated to be the major cell death pathway utilized by these agents.

2.2. Mitotic Catastrophe (Fig. 1)

Tumor cells grown in the presence of MSAs produce abnormal mitotic figures and yet often manage to exit this abnormal mitosis because of deficiencies in prophase checkpoint proteins. These cells undergo an apoptotic cell death or, more commonly, become enlarged, display multiple micronuclei, and undergo a delayed form of cell death. The term mitotic catastrophe has been used, by different investigators, to describe both the apoptotic cell death and the delayed death of the giant multinucleated cells *(64,65)*. Still others have suggested that mitotic catastrophe is not a mechanism of cell death, such as apoptosis or necrosis, but a condition that results from either a failure to complete cell division or a fusion of cells after mitosis *(66)*. In the scenario where mitotic catastrophe is defined and investigated as a special case of apoptosis, cells enter into and die in metaphase after DNA structure checkpoint inactivation, caspase activation, and mitochondrial damage. This pattern of cell killing has been demonstrated to require a functional spindle checkpoint in a series of experiments involving the disruption of kinetochore protein function and therefore the spindle checkpoint. When this occurred in p53-deficient cancer cells treated with DNA-damaging agents, they survived long enough to undergo an aberrant mitosis, form multinucleated cells, and undergo a delayed cell death *(67)*. Although some caspase activation may accompany the death of multinucleated cells following Taxol treatment, the absence of both DNA ladder formation and DNA breaks detectable with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining argues for this pathway being distinct from apoptosis *(68,69)*. Similarly, neither caspase inhibition nor Bcl-2 overexpression prevent aberrant mitosis or the formation of multinucleated cells, further supporting the contention that the resulting cell death is non-apoptotic *(70–72)*.

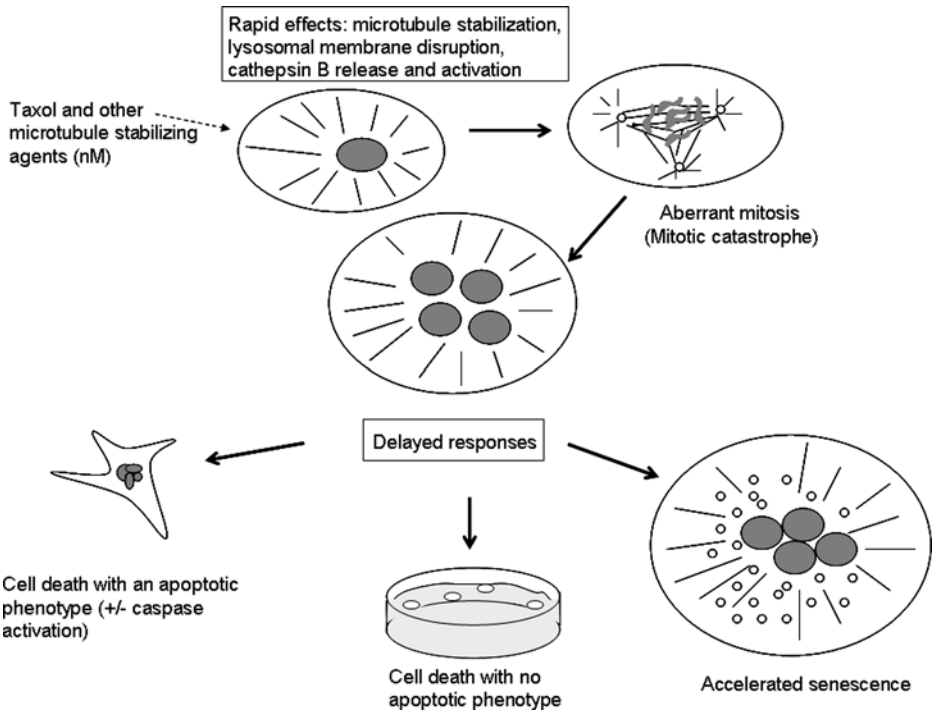

Fig. 1. The response of solid tumor cells to nanomolar concentrations of Taxol. Following Taxol exposure but prior to mitosis, microtubules are stabilized and lysosomal membranes disrupted. Owing to the latter effect, proteases, including cathepsin B, may be released and activated. After an aberrant mitotic division (mitotic catastrophe), cells enter G1 enlarged and multi/micronucleated. These cells later die with an apoptotic phenotype that may or may not be attributed to caspase activation, loose their ability to adhere to the plate, and die without any indication of apoptosis or present with an enlarged, flattened senescent phenotype (including an enhanced production of lysosomal vesicles) prior to cell death.

In their review on mitotic death, Erenpreisa and Cragg *(73)* describe and contrast rapid death occurring in sensitive cells within a few hours of genotoxic insult, with the delayed reproductive or "mitotic" death pathway seen in more resistant (p53-mutated) cancer cells. Some of the characteristics described for the latter include "a disruption of coupling between interphase apoptosis and the G1/S checkpoint, a delay during the G2 phase of the cell cycle, aberrant mitoses which can end in mitotic death, formation and disintegration of multinuclear giant cells" (which they say is often, but not always, called mitotic catastrophe), "delayed apoptosis and, sometimes despite all this, cell survival" *(73)*. They describe how this uncoupling of apoptosis from the G1/S checkpoint and the push toward a G2 arrest, or a subsequent endoreduplication, can yield increasingly greater repair capacity, favoring cell survival over apoptosis. More specifically, related to Taxol and the MSAs, they cite reports that down-regulation of the anaphase-promoting complex activity by spindle damage may retard exit from mitosis and allow entry into the endocycle *(74–76)*. From this perspective, the formation of multinucleated cells following treatment with MSAs may represent an attempt at repair and cell survival. Despite such attempts, the induction of aneuploidy following an aberrant mitosis creates chromosomal breakage and interchromosomal

concatenation, favoring increased genetic instability in tumor cells *(77)*. The observation that concentrations of MSAs required to inhibit cell proliferation correspond more closely to those concentrations that produce aneuploidy than to those required to induce mitotic arrest, suggesting that delayed, mitotic death plays an important role in the mechanism of MSA action *(29)*.

2.3. Caspase-Independent Programmed Cell Death

Although apoptosis is the best defined and most thoroughly investigated mechanism of programmed cell death, more recent work has begun to define an apoptosis-like programmed cell death that is an active, signaling-dependent process that is not necessarily driven by caspases but is distinct from both autophagic cell death and necrosis *(78)*. Such a process may or may not produce cells with an apoptotic morphology and may be initiated by processes other than the activation of caspases. One such mechanism was observed in human neuroblastoma cells where Taxol induced a rapid, caspase-independent mitochondrial production of reactive oxygen species and cell killing prior to any caspase activation *(79)*. Other proteases such as granzymes, lysosomal cathepsins, matrix metalloproteinases, and proteasomal proteases that have recently been presented as potential players in the cell death brought about by Taxol treatment have just begun to be investigated *(19)*.

One lysosomal protease is now known to participate in the production of aberrant mitosis and killing of cancer cells by MSAs. Taxol, epothilone, and discodermolide were shown to induce lysosomal disruption and cathepsin B activation in non-small-cell lung carcinoma cells *(36)*. The resulting aberrant mitosis and cell death were blocked by the inhibition of cathepsin B activation but not by inhibition of caspases or other lysosomal proteases. Although the underlying mechanism leading from the binding of an MSA to a microtubule to lysosomal membrane disruption is not certain, the authors suggested a possible role for signal transduction cascades. Oncogenic Ras in glioma and gastric cancer cell lines previously had been shown to produce lysosomal vacuoles and a genetically regulated, caspase-independent cell death mechanism *(80)*. Taxol produces just such a prolonged Ras activation in certain cell types, including human esophageal squamous cancer cells *(81)*. Discodermolide was seen to produce an even more powerful and sustained ERK activation than Taxol in A549 non-small-cell lung carcinoma cells *(82)*. These A549 cells subsequently presented as multinucleated senescent cells with extensive cytoplasmic vacuoles and finally underwent a delayed cell death. These observations suggest that the MAPK signaling pathway may play a role in connecting the binding of MSAs to microtubules, with the lysosomal disruption/cathepsin B activation observed in the findings of Broker et al. *(36)*.

3. ACCELERATED SENESCENCE

Premature or accelerated cell senescence occurs when radiation, DNA damage, oxidative damage, overexpression of mitogenic signals such as oncogene activation, or a host of other cell stressors produce a mitogen-resistant, terminal proliferation state in previously dividing cells *(83–86)*. The resulting cells present with a phenotype much like that observed when repeated passaging of normal primary cells produces telomere shortening leading to replicative senescence *(87,88)*. They display the characteristic enlarged, flattened, granular morphology, an interphase (either G1 or G2) cell-cycle

block, changes in protein expression levels and activities, including a β-galactosidase activity at pH 6 *(89)*, but may or may not have the shortened telomeres seen in replicative senescence. The developing consensus is that this "stress-induced" senescence is an important fail-safe mechanism that must be bypassed for either tumorigenesis or resistance to chemotherapy to allow unchecked cell proliferation *(90,91)*. The involvement of many signaling pathways in the production of this proliferation barrier and what was believed until recently to be the irreversible nature of senescence have caused it to be likened to apoptosis and have encouraged a search for a similarly well-defined mechanism *(92)*.

The signaling mechanisms controlling the onset of accelerated senescence are complex and redundant, and this, in itself, suggests the physiological importance of this tumor suppressor mechanism. Stressors have been thought to set off various cell signals that can activate one or both the pathways considered critical for the induction and maintenance of this state *(93)*. Activation of the p19ARF/p53/p21 and the p16INK4a/retinoblastoma (Rb) pathways leads to the dephosphorylation and activation of the Rb protein. The Rb protein associates with and silences the transactivation functions of several transcription factors, significantly the E2F proteins, and thereby down-regulates critical cell-cycle proteins (such as cyclins E and A), inhibits proliferation, and induces the many characteristic features of senescence *(94)*. In many cell types, as in the case with replicative senescence, up-regulation of p21 initiates this process, and the p16/INK4a gene expression is activated at a later time point and maintains the senescence phenotype *(95–97)*. Although inactivation of p53 can reverse the proliferation arrest established by shortened telomeres, inactivation of pRB is unable to overcome the proliferation barrier established by p16 and permit proliferation to resume *(98)*. However, a small percentage of p53 null, p16-deficient H1299 carcinoma cells were observed to escape the senescence produced by any number of chemotherapeutic agents, including the small induction by Taxol. Depending on the drug used, between 5 and 90% of the cells displayed a senescence phenotype after 3 days and a small percentage of these were able to resume proliferation between day 18 and day 24 *(99)*. These "senescence escape cells" overexpressed the cyclin-dependent kinase Cdc2/Cdk1, and these authors proposed that this up-regulation may constitute a mechanism responsible for tumor recurrence.

Although the idea that disruption of cytoskeletal function may initiate a stress response leading to accelerated senescence is intuitively satisfying, the actual mechanisms involved have only recently been explored. An overproduction of vimentin intermediate filaments was observed to accompany senescence in cultured human fibroblasts and promoted a senescent cell morphology in young fibroblasts *(100)*. The stress produced by representative microtubule-binding agents (Taxol and vincristine) was also shown to make a contribution, albeit a weak one when compared with the effect produced by DNA-damaging agents, to the induction of accelerated senescence *(69)*. Discodermolide was recently shown to produce a powerful senescence induction in various carcinoma cell lines *(82)*. Given the similar microtubule-stabilizing actions of Taxol and discodermolide, the synergy they produced, as noted in the introduction, was surprising and difficult to explain *(8)*. Although a relative difference in senescence induction provides one explanation for this synergy, the cause of this differential senescence induction remains unexplained. One of the differences observed between Taxol and discodermolide signaling was that the latter produced a far more powerful

and sustained ERK activation in the wild-type p53, p16 null A549 cells studied. Previous work had demonstrated that oncogenic Ras and Raf could induce senescence in IMR-90 lung fibroblasts through induction of p16 *(85,86)*. More recently, Deng et al. *(101)* demonstrated that high-intensity signaling from the Ras pathway was required to induce senescence. Along with previous studies, this research implicated a strong and sustained MEK–ERK activation, leading to activation of p38, as central to the production of the senescence phenotype produced by oncogenic Ras and as playing a critical role in the common senescence signaling pathway produced by telomere shortening and other cell stressors *(102–104)*. That this same mechanism might be triggered downstream of microtubule stabilization and mediate the accelerated senescence produced by discodermolide is of interest.

4. CONCLUSION

A quick perusal of the mechanisms by which MSAs might inhibit the proliferation of tumor cells suggests a reason for the proven effectiveness of Taxol and its value in the treatment of cancer. By interfering with the normal dynamic behavior of microtubules, which are critical to a host of physiological cell activities, these agents can potentially initiate multiple cell death mechanisms or interfere with cell proliferation by interfering in many processes. Clearly, classical apoptosis is important in Taxol-mediated killing of leukemia and lymphoma cells and may participate, to varying extents, in the cell death produced in solid tumors. Yet, cell death from MSAs is certainly not synonymous with apoptosis. The aberrant mitosis and delayed cell death produced by these agents, primarily through caspase-independent mechanisms, are now recognized as being critical measures of their efficacy. Accelerated senescence, as a naturally occurring blockade to uncontrolled cell proliferation, is another potentially useful program to be targeted in the development of ever more effective treatment. Given the powerful senescence induced by discodermolide, it may serve as a prototype for the development of microtubule-stabilizing drugs that can arrest the proliferation of cells in aggressive tumors. The especially attractive feature of accelerated senescence induced by discodermolide is that it appears to be preceded by aberrant mitosis and followed, ultimately, by a delayed cell death. The net effect is that rapid cell proliferation is arrested prior to cell death. The effective pairing of this course of action with a second type of agent, such as Taxol, that would begin the cell killing at an earlier time point could help to undermine any escape from senescence that might occur because of the inactivation of p53 or silencing of p16 that are so common in cancer cells.

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24 Tyrosine Kinase Inhibitors

Michael Deininger, MD, PhD

Summary

The extraordinary success of imatinib for the treatment of chronic myeloid leukemia (CML), gastrointestinal stromal tumors and subgroups of patients with hypereosinophilic syndrome and chronic myelomonocytic leukemia has greatly stimulated the development of small molecule inhibitors for targeted therapy of malignant diseases. Nothing short of a major breakthrough, imatinib has undoubtedly set a precedent and provided proof of principle for a completely new concept in cancer therapy. Unfortunately, diseases other than CML may prove more resilient to targeted tyrosine kinase inhibition, and even in CML, acquired resistance is a significant clinical problem. This chapter will review the current status of tyrosine kinase inhibitors for therapy of malignant disease. Much space will be given to imatinib, as the experience gained from the development of this agent is applicable to other conditions. The emerging concept is that for the patient's maximum therapeutic benefit, disease classifications will have to integrate therapeutic targets, and this will have implications for clinical trial design.

Key Words: targeted therapy; BCR-ABL; FLT3; EGFR; tyrosine kinase inibitors; imatinib; gefitinib; erlotinib; cancer; leukemia

1. TYROSINE KINASES IN MALIGNANT DISEASE

That protein phosphorylation plays a role in the regulation of protein function, that is, reversible phosphorylation regulates the activity of glycogen phosphorylase, was first discovered by Fischer and Krebs, almost 50 years ago. With the advent of more sophisticated molecular biology technology, it has become clear that protein phosphorylation is central to the regulation of metabolism in eukaryotic organisms. Protein kinases catalyze the transfer of phosphate from ATP to serine, threonine or tyrosine residues of proteins according to the basic reaction $MgATP^{1-} + \text{protein-OH} \rightarrow \text{protein-}OPO_3^{2-} + MgADP + H^+$. Large-scale sequencing revealed the existence of 518 human protein kinases, encompassing 1.7% of all human genes and representing the fifth largest protein family in the human genome *(1)*. Protein kinases are grouped in eight families, according to structural features and their ability to phosphorylate serine/threonine or tyrosine residues *(1)*. There are 90 recognized tyrosine kinases, 58

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of which are receptor tyrosine kinases and 32 non-receptor or cytoplasmic tyrosine kinases. In addition, 43 tyrosine kinase-like kinases have been identified.

Tyrosine kinases are key mediators of growth and survival signals, which is why their involvement in the pathogenesis of malignant conditions may be disproportionately high compared to serine/threonine kinases. Cytokine receptors, such as KIT or the platelet growth factor receptors (PDGFRs), are themselves tyrosine kinases. Signaling through other receptors such as the erythropoietin, interleukin-3 or granulocyte monocyte-colonystimulating factor (GM-CSF) receptors involves the recruitment of tyrosine kinases, such as JAK family kinases as early critical events after ligand binding. The activity of tyrosine kinases is counterbalanced by tyrosine phosphatases that remove the phosphate groups transferred by the kinases. There are 87 members in this family, including 53 classical tyrosine phosphates, 33 dual specificity phosphatases and one low molecular weight phosphatase *(2)*. Under physiological conditions, this equilibrium is very much in favor of non-phosphorylation, and it has been estimated that less then 1% of tyrosine residues are phosphorylated in a cell at any given time. This ascertains a constantly high level of responsiveness to signals such as growth factors, while at the same time limiting the duration of "dangerous" growth stimuli. Constitutive activation of a tyrosine kinase uproots the delicate balance and, in simplistic terms, leads to a state of permanent activation of growth and survival signals (Fig. 1). This endows the malignant cells with a growth advantage over their normal counterparts, whose proliferation is dependent on external signals. The majority of the known tyrosine kinases have been implicated in human malignancies, either directly or indirectly (Table 1) *(3)*.

1.1. Kinase Activation by Mutation

Tyrosine kinase activation may occur as a result of several types of mutations. In receptor tyrosine kinases, dimerization is physiologically inhibited by intrinsic mechanisms that usually involve amino acid sequences in the juxtamembrane domain, the region of the receptor immediately adjacent to the inner leaflet of the cell membrane (Fig. 2). Binding of ligand overcomes the intrinsic inhibition of dimerization. Conversely, mutations that disrupt the mechanism that prevents spontaneous dimerization also activate the kinase. An example of this mechanism of action is the internal tandem duplications (ITDs) in the juxtamembrane domain of FLT3 *(4)*. Point mutations can have the same effect, for example, in some gastrointestinal stromal tumors (GISTs) *(5)*. Similar to receptor tyrosine kinases, cytoplasmic tyrosine kinases may also be activated by dimerization. However, dimerization is the result of chromosomal translocations that fuse unrelated sequences with the capacity to form dimers N-terminally to the kinase domain. Examples include BCR fused to ABL in the Philadelphia translocation *(6)*, the hallmark of chronic myeloid leukemia (CML) or TEL-PDGFR in the (5;12)(q33;p13) reciprocal translocation that characterizes a subset of patients with chronic myelomonocytic leukemia (CMML) *(7)*. It is thought that the unphysiological dimerization enables phosphorylation in trans. Both cytoplasmic and receptor tyrosine kinases may also be activated by point mutations, insertions or deletions in the kinase domain that disrupt autoinhibitory mechanisms. The same tyrosine kinase may be activated by both types of mutation, not infrequently in a tumor type-specific manner. For example, mutations of the juxtamembrane domain of KIT are common in GISTs, while point mutations affecting D816 in the activation loop of the kinase domain are typical of systemic mastocytosis (SM) *(5,8)*. From the

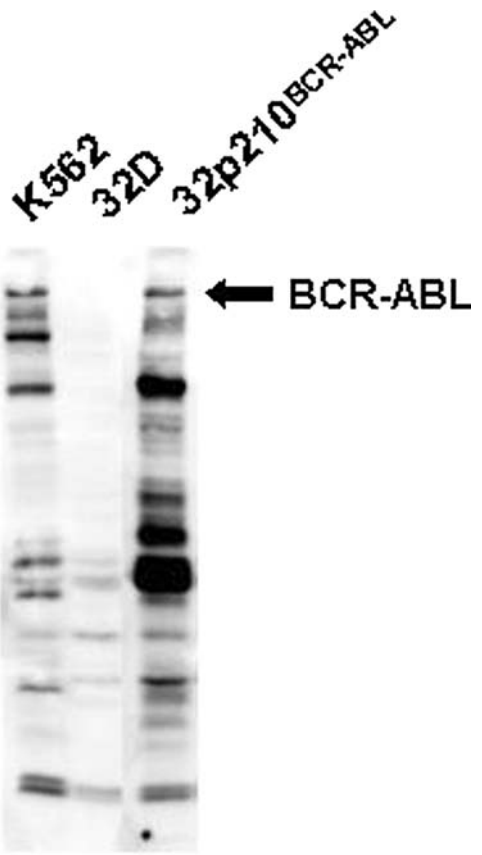

Fig. 1. Increased tyrosine phosphorylation of proteins in cells expressing an activated tyrosine kinase. Lysates from K562 cells, a chronic myeloid leukemia cell line, 32D cells, a murine myeloid cell line that grows in interleukin-3, and 32Dp210$^{BCR-ABL}$ cells (32D cells transduced with BCR-ABL) were analysed for tyrosine phosphorylated proteins by Western blot analysis.

viewpoint of targeted therapy this is relevant, as mutations of the enzyme active site of the kinase may alter substrate binding, while this structure is not altered by abnormal dimerization. Thus, an inhibitor that binds the catalytic site of the unmutated kinase with high affinity may have much lower affinity if a mutation is present. For example, imatinib inhibits unmutated KIT in the nanomolar dose range but is practically inactive against the D816V activation loop mutant *(9,10)*. In contrast, AP23464, an ABL/SRC inhibitor, is more active against the D816V mutant than wild-type KIT *(11)*. Yet, another mechanism of kinase activation is mutations in the extracellular domain that alter ligand affinity. This has recently been shown for KIT exon 4 mutants, which show increased responsiveness to low concentrations of stem cell factor compared to wild-type KIT *(12)*.

1.2. Activation by Increased Expression

In contrast to mutations, the significance of overexpression of the tyrosine kinases compared to phenotypically matched non-neoplastic cells is less well defined. For example, KIT is highly expressed on the blasts of many patients with acute myeloid

Table 1
Tyrosine Kinases Involved in Human Cancer

	Oncogenic alteration and target tissue/disease phenotype
Receptor tyrosine kinases	
EGFR/ErbB1	GOF point mutations, small deletions, insertions: NSCLC Overexpression (amplification): NSCLC, breast and ovarian cancer Extracellular domain deletions: glioblastoma multiforme
ERBB2/HER2/Neu	Overexpression (amplification): breast, ovarian, gastric, colon, lung cancer GOF point mutations: NSCLC
ERBB3/HER3	Overexpression: breast cancer
ERBB4/HER4	Overexpression: breast cancer, granulose cell tumors
IGF-1R	Overexpression: cervical and other carcinomas, sarcomas
PDGFR-α	Overexpression (amplification): glioma, glioblastoma, ovarian carcinoma Gene fusions: FLP1L1-PDGFR-α fusion [del(4q12)]: hypereosinophilic syndrome BCR-PDGFR-α [t(8;22)]: atypical CML GOF mutations: GISTs
PDGFR-β	Gene fusions: ETV6–PDGFR-β fusion [t(5;12)] and other fusions: CMML Overexpression: glioma
CSF-1R (FMS)	GOF point mutations, overexpression: acute and chronic myelomonocytic leukemia, monocytic tumors, malignant histiocytosis, endometrial cancer, glioma
KIT/SCFR	Overexpression: AML, SCLC, other carcinomas GOF point mutations and small deletions: GISTs, AML, SM, seminomas/dysgerminomas
FLK2/FLT3	GOF point mutations, internal tandem duplications: AML, myelodysplastic syndrome
FLT1/VEGFR1	Expression: tumor angiogenesis
FLT1/VEGFR2	Expression: tumor angiogenesis
FLT4/VEGFR3	Overexpression: tumor angiogenesis; vascular tumors (Kaposi's sarcoma, Hemangiosarcoma, lymphangiosarcoma)
FGFR1	Overexpression: various tumors Gene fusions: ZNF 198-FGFR1 [t(8;13)] and other fusions: myeloproliferative syndrome, T-cell lymphoma, AML Point mutations: autosomal skeletal disorders/dysplasias
FGFR2/K-SAM	Overexpression (amplification) and C-terminal truncation: gastric, breast, prostate cancer
FGFR3	Overexpression: IgH locus/MMSET translocation [t(4;14)] placing FGFR3 downstream of IgH/MMSET: multiple myeloma GOF point mutations: achondroplasia, thantophoric dysplasia, hypochondroplasia
FGFR4	Overexpression (amplification): breast, ovarian cancer
TRKA	Gene fusions: TPR-TRKA [t(1;1)], TFG-TRKA [t(1;3)]: Papillary thyroid carcinomas, neuroblastomas
TRKC	Gene fusion: ETV6-TRKC [t(12;15)]: congenital fibrosarcoma, AML

HGFR	Gene fusion: TPR-MET [t(1;7)]: Papillary thyroid carcinomas Overexpression: rhabdomyosarcoma, hepatocellular carcinoma GOF point mutations: renal carcinoma, SCLC
RON	Overexpression/increased kinase activity of splice variants: colon cancer, hepatocellular cancer
EPHA2	Overexpression: melanoma
EPHB2	Overexpression: gastric, esophageal and colon cancer
EPHB4	Overexpression: infiltrating ductal mammary carcinomas
AXL	Overexpression: AML
TEK/TIE2	Expression: tumor angiogenesis (endothelium)
RET	Gene fusions and inversions: H4-Ret (PTC1), Rlα-Ret (PTC2); ELE1-Ret (PTC3 & PTC4); RFG5-Ret (PTC5); HTIF1-Ret (PTC6); RFG7-Ret (PTC7); KTN1-Ret (PTC8); ELKS-Ret: papillary thyroid carcinomas GOF point mutations: multiple endocrine neoplasia type 2A+B: medullary thyroid carcinoma, parathyroid hyperplasia, pheochromocytoma, enteric mucosal ganglioneuromas; familial medullary thyroid carcinoma
ROS	Overexpression: glioblastomas, astrocytomas
Non-receptor tyrosine kinases	
ALK	Gene fusions: NPM-ALK [t(2;5)]; Igλ-ALK PTK [t(2; 22)]; other fusions: anaplastic large cell lymphoma
SRC	C-terminal truncation (increased kinase activity): colon cancer Overexpression: breast and pancreatic cancers, neuroblastomas, others
FGR	C-terminal truncation and GOF point mutations: AML, chronic lymphocytic leukemia, EBV-associated lymphomas
YES	C-terminal truncation, GOF point mutations, overexpression: colon cancer, melanoma, other cancers
LCK	Overexpression due to gene fusion: TCRβ -LCK [t(1; 7)]: T-cell ALL
ABL1	Gene fusions: BCR-ABL1 [t(9;22)]: CML, ALL, AML ETV6-ABL1 [t(9;12)]: myeloproliferative syndrome, ALL NUP214-ABL1: T-cell ALL
ABL2 (ARG)	Gene fusion: ETV6-ABL2 [(t(1;12)]: AML
JAK1	Overexpression: various leukemias
JAK2	Point mutation: V617F in myeloproliferative diseases Gene fusion: TEL-JAK2 [(t(9;12)]: T-cell childhood ALL, AML, atypical CML
JAK3	Point mutation: Megakaryocytic leukemia Overexpression: various leukemias and B-cell malignancies
FAK	Overexpression and/or altered tyrosine kinase activity: modulation of adhesion, invasion and metastasis of diverse malignancies
PYK2	Overexpression and/or altered tyrosine kinase activity: modulation of adhesion, invasion and metastasis of diverse malignancies
BRK	Overexpression: breast cancer
SYK	Downregulation (acts as a tumor suppressor): breast cancer

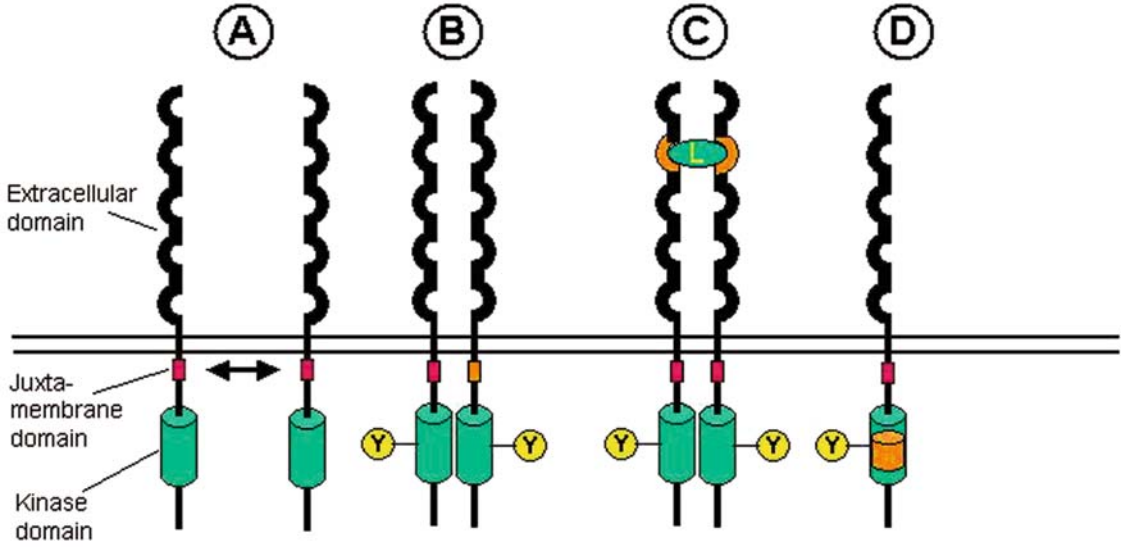

Fig. 2. Mutational activation of receptor tyrosine kinases. (A) Under physiological conditions, dimerization in the absence of ligand is prevented by a motif in the juxtamembrane domain (magenta). (B) Disruption of this mechanism by mutation leads to constitutive dimerization and kinase activation. (C) Mutations in the extracellular domain may increase affinity for ligand (L), leading to dimerization in the presence of low concentrations of ligand. (D) Mutations in the kinase may activate the kinase independently of dimerization.

leukemia (AML), but most of these patients do not respond to imatinib *(13)*. However, there may be exceptions to this rule. For example, FLT3 mutations are found in approximately 30% of patients with AML *(14)*. Although responses to specific FLT3 inhibitors tend to correlate with the presence of a mutation, activity was also seen in some patients with high surface expression of FLT3 *(15,16)*. It is thought that a very high concentration of FLT3 molecules on the cell surface may lead to kinase activation by spontaneous dimerization in the absence of ligand. Enhanced signaling via the wild-type receptor may also occur as a result of increased expression of the ligand. For example, autocrine loops involving the PDGF/PDFGR system are operational in some glioblastoma cell lines, which provided the rational for therapeutic trials of imatinib in patients with glioblastoma *(17)*. It is not possible to predict with certainty how dependent a particular tumor is on signaling from a specific tyrosine kinase that is overexpressed. Generally, increased expression of a wild-type kinase that is caused by gene amplification is more likely to be critical to pathogenesis than simply high levels of expression in the absence of an underlying mutation. For example, responses to the epidermal growth factor receptor (EGFR) kinase inhibitor gefitinib in patients with non-small cell lung cancer (NSCLC) are not correlated with high expression of EGFR as measured by immunohistochemistry but with the presence of activating EGFR mutations *(18,19)* and gene amplification *(20)*. Myeloma cells that overexpress FGFR3 as a result of t(4;14)(p16;q32) are responsive to treatment with FGFR3 inhibitors *(21)*. This suggests that the mutant cell clones have been selected on the basis of the newly acquired genetic feature and are thus "addicted" to the respective growth and survival signals.

2. DEVELOPMENT OF IMATINIB

The development of imatinib (STI571, CGP57148), a specific inhibitor of the ABL tyrosine kinase, as a therapeutic agent for CML has become a paradigm for successful translational research and stimulated intense interest in the exploitation of tyrosine kinase inhibition for the treatment of malignant disorders. Although a rare disease, CML has been a pacemaker for important steps in oncology. The clinical presentation of a CML patient led Virchow *(22)* to coin the term leukemia, the Philadelphia chromosome was the first chromosomal abnormality consistently associated with a human malignancy *(23)* and t(9;22) the first reciprocal translocation *(24)*, whose translocation partners, the BCR and ABL genes, were identified *(25,26)*. From the perspective of targeted therapy, the most important discovery was that the chimeric BCR-ABL protein had constitutive tyrosine kinase activity that correlated with its ability to transform cells *(27)*. With this in mind, attempts were initiated to develop inhibitors of ABL kinase activity for potential therapeutic use. In 1992, Anafi and colleagues *(28,29)* reported on a "tyrphostin," a small molecule that inhibited the tyrosine kinase activity of BCR-ABL and suggested that it might be possible to design specific compounds for the treatment of ABL-associated human leukemias. In subsequent studies, the derivatives AG568, AG957 and AG1112 were identified as the most specific compounds. Despite their promising *in vitro* activity, the tyrphostins were never developed for clinical use.

Starting in the late 1980s, scientists at Ciba Geigy (now Novartis), under the direction of N. Lydon and A. Matter, initiated medicinal chemistry studies to identify compounds

with inhibitory activity against protein kinases. In one such project focusing on protein kinase C (PKC) as a target, a 2-phenylaminopyrimidine derivative was identified as a lead compound *(30,31)*. Modifications of this compound greatly increased activity against tyrosine kinases, while all but eliminating activity against serine/threonine kinases and improved solubility, eventually leading to CGP57148, which was subsequently re-named STI571 and eventually imatinib (Gleevec, Glivec) (Fig. 3).

Imatinib has activity against ABL, the ABL homolog ARG (also referred to as ABL2), KIT and the PDGFRs in the micromolar dose range, whereas most other kinases, including SRC and serine/threonine kinases, are not inhibited at concentrations of up to 100 μM *(32,33)*. Given the high level of sequence homology between the kinase domains of ABL and SRC, this selectivity was initially surprising but its structural basis became clear when the crystal structure of the ABL kinase domain in complex with an imatinib analog was solved *(34)*. The catalytic domains of protein kinases have a rather uniform bi-lobar structure, with a smaller N-terminal lobe that consists predominantly of antiparallel beta-sheets and a larger C-terminal lobe that is predominantly α-helical. The amino acid residues critical for catalysis line the groove between the two lobes, and kinase activity is dependent on their mutual position. Equally important, access of the catalytic cleft to substrate and ATP is controlled by the activation loop of the kinase, a highly flexible structure whose conformation is regulated by tyrosine phosphorylation. While the activation loop conformations of active kinases are similar, they are quite distinct in the inactive states. Contrary to expectations, the crystal structure revealed that imatinib bound the kinase inactive form of ABL, with the activation loop in a closed position (Fig. 4). Given the much greater structural differences between inactive ABL and inactive SRC, this explained imatinib's high level of specificity. Thus, imatinib is not a straightforward ATP-competitive inhibitor but rather acts by stabilizing the kinase in a unique inactive conformation. The interaction with the catalytic site is tight, engaging no less than 19 amino acid residues in hydrogen bonds or hydrophobic interactions *(35)*.

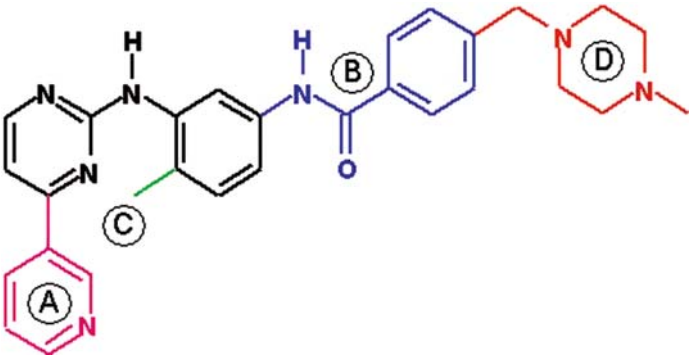

Fig. 3. Development of imatinib from a 2-phenylaminopyrimidine backbone (shown in black). (A) Activity in cellular assays was improved by introduction of a 3′ pyridyl group (magenta) at the 3′ position of the pyrimidine. (B) Activity against tyrosine kinases was further enhanced by addition of a benzamide group (blue) to the phenyl ring. (C) Attachment of a "flag-methyl" group (green) ortho to the diaminophenyl ring strongly reduced activity against protein kinase C. (D) Addition of an *N*-methylpiperazine (red) increased water solubility and oral bioavailability. Adapted from Deininger et al. *(33)* with permission.

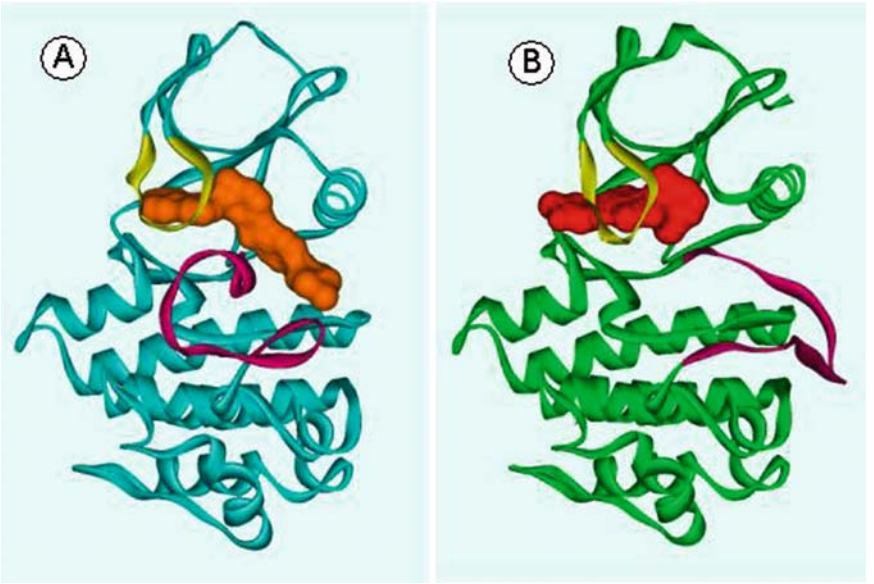

Fig. 4. (A) Conformation of ABL (blue) in complex with imatinib (orange), with the A-loop (magenta) in a "closed" conformation. (B) Conformation of ABL (green) in the PD180970 (red) complex with the a-loop (magenta) in an "open" conformation. The p-loop (yellow) folds down over the inhibitor in both cases. Adapted from Deininger et al. *(33)* with permission.

2.1. Imatinib for Therapy of CML

From the perspective of targeted therapy, CML is an almost ideal disease. There is a more than 90% consistency between typical morphology and the presence of BCR-ABL, a much more favorable situation than in most other types of malignancy *(36)*. Only polycythemia vera has a similarly tight association with a specific mutation ($JAK2^{V617F}$) *(37)*. Equally important, BCR-ABL is both necessary and sufficient for induction of CML. Transplantation of murine bone marrow cells expressing BCR-ABL into lethally irradiated recipient mice induces a myeloproliferative syndrome that resembles CML *(38)*. Even more convincing, the reversibility of BCR-ABL-positive myeloproliferative disease was recently demonstrated in a transgenic mouse model that used an inducible expression system *(39)*. Thus, the acquisition of BCR-ABL by a hematopoietic progenitor cell appears to be an early, probably the initiating event of CML, which may not be the case with tyrosine kinase mutations found in AML *(40,41)*. Last, most CML patients are diagnosed in the chronic phase, a situation that may be compared with an adenoma and represents an early stage of leukemia evolution.

2.1.1. Phase I Studies

Under the direction of Brian Druker, a phase I trial with imatinib was initiated in spring 1998. This study initially included patients with CML in late chronic phase but was later extended to patients in blast crisis, based on extremely promising efficacy data *(42,43)*. In chronic phase, patients treated with at least 300 mg of imatinib daily, there was an almost 100% rate of complete hematological response (CHR, defined as normal blood counts and absence of splenomegaly or other signs of disease) and major

cytogenetic response rate (defined as the presence of the Philadelphia chromosome in less than 35% of metaphases). Based on the efficacy of doses >300 mg daily, a decision was made to use 400 mg daily as the standard dose for the phase II studies. At this dose, median trough plasma levels were 1.43 μM, approximately 5-fold higher than the IC_{50} for *in vitro* inhibition of cell proliferation *(32,44)*. Imatinib was generally well tolerated, with most non-hematological toxicities being grade 1 or grade 2. Compared to late chronic phase, results in blast crisis were less impressive and remissions were usually not maintained.

2.1.2. Imatinib for Newly Diagnosed CML in Chronic Phase

Imatinib has become the first-line drug therapy for CML in all phases of disease. The rates of CHR and complete cytogenetic response (CCR) are highest in patients with early disease, that is, those with newly diagnosed CML in chronic phase. A randomized phase III comparison with cytarabine/interferon-alpha, the previous standard drug therapy, showed imatinib vastly superior with respect to rates of CHR and CCR and freedom from progression to accelerated phase and blast crisis (Table 2). Due to crossover design of the study and the much higher efficacy and tolerability of imatinib, the interferon/cytarabine arm collapsed, when imatinib was approved and many patients in the interferon/cytarabine arm withdrew consent. This meant that the original randomized trial essentially became an one-armed study. A recent update of the patients who received imatinib up-front showed a 98% cumulative rate of CHR and 87% cumulative rate of CCR *(45)*. Freedom from progression to accelerated phase or blast crisis was 93% and overall survival 89%. Importantly, the annual rates of progression to accelerated phase or blast crisis have decreased with longer follow-up (Table 3), suggesting that a plateau may be reached eventually. Another crucial observation is that the clinical response to imatinib seems to override pre-therapeutic risk factors. Thus, patients who attain CCR or major molecular response [MMR, defined as a more than 3-log reduction of BCR-ABL mRNA compared to a standardized baseline *(46)*] have a very low risk of relapse or disease progression, irrespective of their individual pre-therapeutic risk. There is some evidence that higher doses of imatinib (800 mg) may be superior to the standard dose of 400 mg daily. As of now, this notion is not derived from randomized trials but rather from sequential studies performed at

Table 2
Responses to Imatinib vs. Interferon (IFN) Plus Cytarabine in Newly Diagnosed Chronic Myeloid Leukemia Patients in Chronic Phase

	CHR	*MCR*	*CCR*	*Progression-free survival (14 months)*
Imatinib (%) ($n = 553$)	95.3	85.2	73.8	92.1
IFN+Cytarabine (%) ($n = 553$)	55.5	22.1	8.5	73.5
P value	0.001	0.001	0.001	0.001

CCR, complete cytogenetic response; CHR, complete hematologic response; MCR, major cytogenetic response.

Median duration of follow-up is 19 months.

Table 3
Progression Events in Newly Diagnosed Patients Treated with 400 mg Imatinib Daily Up-Front

Year	*All progression events*[a]*(%)*	*AP/BC (%)*
1	3.4	1.5
2	7.5	2.8
3	4.8	1.6
4	1.5	0.9
5	0.9	0.6

[a]Includes loss of complete hematologic response, loss of major or complete cytogenetic response and progression to accelerated phase (AP) or blast crisis (BC).

the MD Anderson Cancer Center. These trials showed higher rates of CCR, MMR complete molecular response (CMR, undetectability of BCR-ABL transcripts by RT-PCR) in the patients treated with 800 mg *(47,48)*. It remains to be seen whether this will ultimately translate into a survival advantage, as the low-dose cohort appears to catch up with time in terms of rates of MMR and CMR *(49)*. Nonetheless, a more recent update of the studies showed a trend toward reduced rates of early progression events with high doses of imatinib. Thus, the advantage of this approach may be to prevent early progression in some patients, presumably individuals with high Sokal risk. Unfortunately, being non-randomized, these studies do not meet vigorous quality standards, but the question will ultimately be resolved by several large multicenter trials that are under way in Europe and the United States. Additional evidence that higher doses may be more effective comes from the phase II studies in advanced CML, where 600 mg were superior to 400 mg, although again this was not a randomized comparison *(50,51)*. Lastly, in some patients, dose escalation from 400 to 800 mg is able to overcome hematologic or cytogenetic refractoriness *(52)*. Given that the choice of 400 mg as the standard dose was largely arbitrary [based on an almost 100% rate of CHR in patients with late chronic phase treated with 300 mg and the observation of cytogenetic responses at 400 mg *(43)*], this is not surprising. Overall, the issue of optimal dosing of targeted therapy has wider ramifications. There is currently no established test to monitor effective target inhibition in patients on imatinib, although the phosphorylation of CRKL, a rather specific substrate of BCR-ABL, is used in a research setting *(43)*. This test is not very sensitive and it is likely that a substantial fraction of BCR-ABL kinase remains active in patients treated with standard doses of imatinib. Better alternatives may emerge in the form of FACS analysis of patient cells either on therapy or *in vitro (53,54)*. It could be argued that instead of being based on a reliable and sensitive test, dosing should be based on the maximum tolerated dose (MTD), just like with conventional chemotherapy. There is some suspicion that in the setting of NSCLC, suboptimal dosing may have contributed to the failure of gefitinib, a EGFR kinase inhibitor *(55,56)*. This is discussed in detail in Section 4.

2.1.3. Imatinib for Advanced CML

Imatinib is effective in patients with CML in accelerated phase and blast crisis. However, the rates of CHR and CCR are much lower in these patients, and relapse after an initial response is common (Table 4). Thus, long-term imatinib monotherapy

Table 4
Responses to Imatinib in Advanced Phases of Chronic Myeloid Leukemia

	Overall hematologic response/CHR	*Sustained hematologic responses (> 4 weeks)*	*MCR*	*CCR*	*Median survival*
Myeloid blast crisis (%) ($n = 229$)	52/15	31	16	7	6.8 months
Ph-positive ALL[a] (%) ($n = 56$)	59/22	27	Not available	Not available	4.9 months
Accelerated phase (%) ($n = 181$)	82/53	69	24	17	Not reached
Chronic phase after failure of IFN	6	95	60	41	Not reached

ALL, acute lymphoblastic leukemia CCR, complete cytogenetic response; CHR, complete hematologic response; IFN, interferon; MCR, major cytogenetic response.

[a] Includes lymphoid blast crisis of chronic myeloid leukemia only CHR reported.

in this group of patients is problematic, and more aggressive strategies are justified if long-term remission is the therapeutic goal. Most experts agree that allogeneic stem cell transplantation should be offered to patients with advanced CML, if this is an option *(49,33)*. Fortunately, there is no evidence that imatinib therapy preceding an allogeneic transplant has a negative impact on transplant-related mortality *(57)*.

2.1.4. Side Effects of Imatinib

Perhaps as surprising as the excellent therapeutic efficacy of imatinib is the fact that side effects are usually minor. ABL knockout mice have a high rate of neonatal lethality, and the viable offspring suffers from impaired immune function, bone defects and an ill-defined wasting syndrome *(58,59)*. Even more significantly, disruption of both ABL and ARG (ABL2) generates an embryonic lethal phonotype, due to a complete defect of neurulation *(60)*. Mice null for PDGFRβ have vascular defects and edema *(61)*, and KIT is important for early hematopoietic development *(62)*. While imatinib does have some typical side effects, such as fluid retention, joint and musculoskeletal pain and skin rashes, one would expect more significant side effects from prolonged inhibition of these kinases. One explanation for the relative lack of side effects is that the relevance of the target kinases during embryonal development (reflected by the knockout mice) may be much greater than during adult life. Another reason may be that the inhibition of kinase activity is incomplete, which though (probably) undesirable from the pharmacodynamic point of view may help to reduce undesired drug effects. An exception to the rule that side effects are mild is hematologic toxicity, which is frequently grade 3 or grade 4, particularly in patients with advanced disease. Evidently, one could argue that hematologic toxicity reflects effective suppression of leukemic hematopoiesis by imatinib and is thus not a side effect *sensu strictu (63)*. Despite the generally benign profile of side effects, it remains possible that additional adverse effects may be detected as experience with imatinib grows or with longer follow-up. For example, severe congestive heart failure has recently been reported in a small series of patients *(64)*. Although the overall incidence of this may be low, it is a reminder that imatinib is a potent agent that should be used by experienced physicians.

2.1.5. Resistance

Primary resistance or refractoriness is frequent in blast crisis *(50,51,65)*. The underlying mechanisms are largely unknown but may be related to drug transport mechanisms *(66)*. Acquired resistance after an initial response is common in advanced disease, less frequent in late chronic phase and quite rare in newly diagnosed chronic phase *(67,68)*. One key observation from the study of patients with acquired resistance to imatinib was that relapse is almost invariably accompanied by reactivation of BCR-ABL signaling. Thus, the transforming principle responsible for pathogenesis in the first place retains its central role at the time of relapse. Reactivation of BCR-ABL signaling may occur as a result of point mutations within the kinase domain of ABL (Fig. 5) or, less frequently, as a result of increased expression. Point mutations have been described in more than 40 different positions *(46)* and may impair drug binding by one of three mechanisms *(69–76)*. Bulky substitutions in spatially confined areas may sterically clash with imatinib. The pivotal example is the exchange of isoleucine for threonine at position 315 (T315I), which in addition to causing sterical

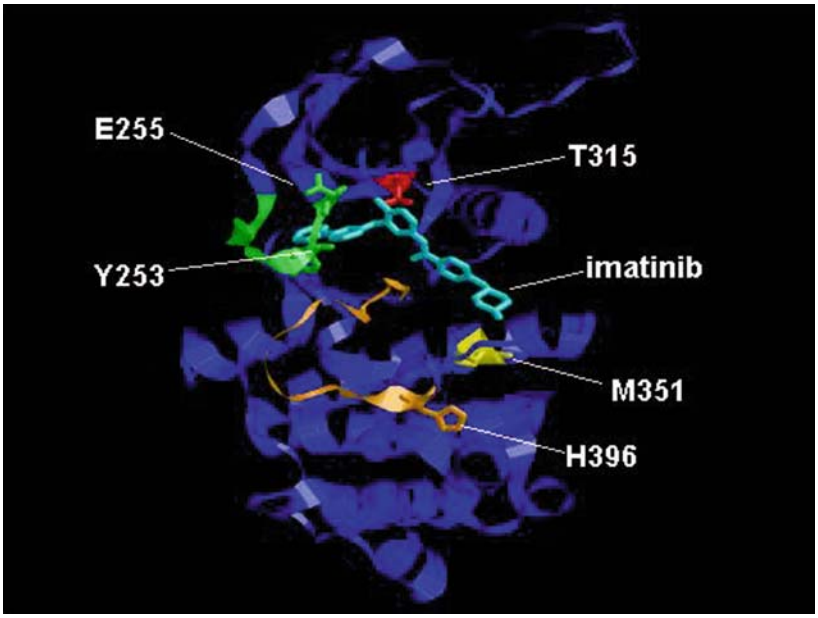

Fig. 5. Amino acids frequently mutated in patients with imatinib resistance. E255 and Y253 are localized in the ATP binding loop (p-loop, green), T315 (red) is the site of a hydrogen bond to imatinib, M351 (yellow) contacts the SH2 domain of the ABL in the autoinhibited conformation of the kinase, and H396 is localized in the activation loop (brown). Courtesy of Dr. Ian Griswold, Orgeon Health & Science University.

hindrance abolishes a hydrogen bond between the pyrimidine nitrogen of imatinib and threonine 315. A second type of mutation prevents the conformational changes that are required for optimally accommodating imatinib. Examples include mutations in the ATP-binding loop (p-loop) of BCR-ABL. This structure undergoes extensive conformational changes on imatinib binding *(34)*. Thus, mutations in the p-loop may prevent the structural changes required for drug binding. Lastly, activation loop mutations probably favor the active conformation of the kinase to which imatinib is unable to bind *(77)*. The frequent M351T mutation may also belong to this category, as M351 is the site of an intramolecular kinase inhibitory interaction in ABL that may be abolished as a result of the mutation, favoring the kinase active state *(33)*. The degree of resistance depends on the specific type of mutation. Some mutations retain almost wild-type drug sensitivity *(78)*, while others like T315I are completely resistant. For the clinical management of patients with mutations, this has major implications, as dose escalation of imatinib is unlikely to benefit patients with highly resistant mutations but may recapture responses in the case of mutations that confer low or intermediate levels of resistance *(79)*.

2.1.6. Strategies to Overcome Imatinib Resistance

A common strategy to overcome imatinib resistance is combining imatinib with conventional chemotherapeutic agents. The consensus reading from a large number of *in vitro* studies is that most combinations with conventional agents are additive or synergistic, as long as the resistant cells retain some sensitivity to imatinib *(80)*.

Exceptions include the combination of imatinib with methotrexate or topotecan *(81)*. In addition, imatinib has been combined with many other signal transduction inhibitors that target pathways known to be activated in BCR-ABL-positive cells. Several imatinib combinations are currently in phase I/II testing (reviewed in ref. *(33)*). However, the most promising development is alternative inhibitors of ABL that retain activity against the mutant ABL kinase. These alternative ABL inhibitors belong to different chemical classes. With the exception of AMN107 (nilotinib) and NS-187, they were originally developed as SRC inhibitors (Table 5) *(82–88)*. Crystal structure analysis revealed that PD173955, a pyridopyrimidine initially developed as a Src inhibitor, binds the active conformation of the ABL kinase domain, with less stringent conformational requirements compared to imatinib (Fig. 4) *(35)*. Similar binding modes have subsequently been demonstrated for other Src/Abl inhibitors, including dasatinib (BMS354825) *(89)*, which has recently been approved by the FDA for the treatment of patients with imatinib failure. In contrast, nilotinib (AMN107) and NS-187, both derivatives of imatinib, bind the inactive conformation of ABL *(87,88)*. Their increased potency is the result of improvements to the imatinib molecule. Thus, in AMN107, the piperazinyl group was replaced by a trifluorinated imidazole, resulting in the elimination of two energetically unfavorable hydrogen bonds, with more free binding energy available for the remaining molecule *(90)*. The activity profiles of these alternative ABL inhibitors against the various kinase domain mutants are quite similar. Most mutants are covered, with the exception of T315I which is completely resistant. At a closer look however, differences become apparent that reflect the different binding modes. Thus, while in the case of nilotinib and NS-187 the relative differences between wild-type and mutant ABL are preserved, this is not the case for dasatinib. This suggests that while nilotinib and NS-187 "overpower" the mutants by their overall increased binding affinity, dasatinib exhibits an entirely different mode of binding.

There are very significant differences in bioavailability that prevented the clinical development of some of the compounds. All in all, four compounds are in clinical development. Dasatinib (Sprycel™) has recently been approved by the FDA, nilotinib (AMN107) is in advanced phase II testing, and NS-187 as well as SKI-606 are in phase studies. Both dasatinib and nilotinib have shown considerable clinical activity (Table 6) *(91,92)*. Similar to imatinib, response rates are highest in patients with chronic phase and lowest in blast crisis. A direct comparison at this time is impossible, due to slightly different inclusion criteria and the different length of follow-up.

The Achilles heel of all the novel inhibitors in clinical development is that they are inactive against the T315I mutant. Thus, it is possible that this particular mutant will emerge as the "default mutant" in patients treated with compounds like dasatinib or nilotinib *(93,94)*. This is suggested by *in vitro* data showing selection of T315I in the presence of nilotinib, dasatinib and both combined in BaF3 cells engineered to express BCR-ABL, while culture in the presence of imatinib produced a range of different mutations that rather faithfully represented the spectrum of mutations observed in patients with clinical resistance *(95,96)*. Efforts are underway to develop inhibitors of T315I. One compound with anti-T315I activity termed ON012380 appears to act as a non-ATP-competitive inhibitor of BCR-ABL kinase activity. The profile of off-target effects of this compound is not yet clear, so it remains to be seen whether it will be a therapeutically viable compound *(97)*. VX-680, an aurora kinase inhibitor, has moderate activity in the low micromolar dose range *(77)*. This agent is in phase 1 trials for

Table 5
Alternative ABL Kinase Inhibitors with Activity Against Kinase Domain Mutants

Compound	*Class*	*IC_{50} (cell proliferation) (nM)*					*References*
		Wild-type BCR-ABL	*E255K*	*M351T*	*H396P*	*T315I*	
PD180970	Pyridopyrimidine	25	140	45	15	840	*82*
SKI606	Pyridopyrimidine	~ 11	~30	ND	~10	>500	*83*
AP23848	Trisubstituted purine	14	94	24	8	9050	*88*
BMS-354825	2-amino-thiazole-5-carboxamide	0.087–1	Low nanomolar range			>1000	*158,159*
AMN107	2-phenylaminopyrimidine	20	150	31	ND	>10,000	*88*
NS-187	2-phenylaminopyrimidine	22	98	150	95	>10,000	*87*

ND, not detected.

Table 6
Results of Phase I trials of Nilotinib and Dasatinib

Disease phase and response	*Dasatinib*	*Nilotinib*
Chronic phase	$n = 40$[a]	$n = 12$[b]
Major hematologic response	37 (92%)	11 (92%)
Complete cytogenetic response	14 (35%)	6 (55%)
Accelerated phase	$n = 11$	$n = 24$
Major hematologic response	9 (82%)	24 (52%)
Complete cytogenetic response	2 (18%)	6 (13%)
Myeloid blast crisis	$n = 23$	$n = 24$
Major hematologic response	14 (61)	4 (17)
Complete cytogenetic response	6 (26)	1 (4)
Lymphoid blast crisis and Ph-positive ALL	$n = 10$	$n = 9$
Major hematologic response	8 (80%)	1 (11%)
Complete cytogenetic response	3 (30%)	1 (11%)

[a]Eight patients in chronic phase, two in accelerated phase and one each in myeloid and lymphoid blast crisis were intolerant of imatinib.
[b]Additional 10 patients had clonal cytogenetic evolution as the only feature defining accelerated phase. All five patients with active hematologic disease attained complete hematologic response and two patients attained complete cytogenetic response.

refractory leukemia. Arguably the most promising candidate is a series of compounds that have been generated with a crystallography-based approach. Compounds of this class including SGX393 are active against T315I in the nanomolar dose range *(98)*. The structures have not been published yet, and no clinical trials have been initiated.

Will it be possible to cure CML with BCR-ABL kinase targeted therapy? The jury is out. Most with CCR remain positive by RT-PCR, in contrast to recipients of stem cell transplants *(99,100)*, and recurrence of disease is the rule if imatinib is discontinued *(49)*. The causes underlying the persistence of minimal residual disease are not well understood but may include mechanisms that are non-dependent on BCR-ABL kinase activity or not even on BCR-ABL *(33,101)*. For example, it is conceivable that leukemic stem cells are able to utilize physiological signals, such as growth factors, to maintain viability in the face of BCR-ABL inhibition. This would obviously limit the potential of BCR-ABL kinase targeted therapy.

2.2. Imatinib for the Treatment of Other Hematologic Malignancies

Some 50% of patients with hypereosinophilic syndrome (HES) harbor a FIP1L1-PDGFRα fusion gene that is generated as a result on an interstitial deletion on chromosome 4 *(102)*. These individuals respond extremely well to imatinib, even at doses that are ineffective in CML, consistent with the higher imatinib sensitivity of PDGFRα compared to ABL *(88)*. Interestingly, it was the imatinib responsiveness of HES that directed the path to the identification of the underlying genetic lesion *(103)*. CMML with translocations that activate PDGFRβ is also extremely responsive to imatinib *(104)*. Lastly, some patients with SM express juxtamembrane mutants of KIT that are imatinib sensitive, in contrast to the activation loop mutants that characterize the majority of SM patients *(105,106)*.

Imatinib was tried in many other types of hematologic cancer, sometimes without clear biological rationale. Given the favorable profile of side effects, this could be done safely, but not unexpectedly the results were generally disappointing. For example, there were no significant responses in 23 patients with idiopathic myelofibrosis (IMF) *(107)*. Similarly, only isolated patients with AML responded *(13,108)*. An exception is polycythemia vera, where responses have been observed rather consistently in the form of reduced phlebotomy requirements, although usually only at high doses of imatinib *(109)*. In some patients, this leads to a significant reduction but not elimination of the polycythemia vera cell clone as detected by quantitative PCR for the V617F mutation of JAK2 *(110)*.

2.3. Imatinib for the Treatment of Gastrointestinal Stromal Tumors

GISTs are rare GI tract tumors that were previously classified as leiomyomas or leiomyosarcomas. As a result of inconsistent histopathological diagnosis, the incidence of GISTs is not exactly known but may be in the range of 2 per 10^5 population and thus slightly higher than the incidence of CML *(111)*. GISTs are derived from the interstitial cells of Cajal, the pacemaker cells of the intestine, and are most frequently located in the stomach and small intestine. Activating mutations of KIT are found in 80–85% of patients, and activating mutations of PDGFRα in 7%. Studies in very small tumors revealed a high incidence of KIT mutations, which is a direct evidence that the acquisition of KIT mutations is an early event in the pathogenesis of GISTs *(112)*. Several families with germline KIT mutations have been identified. Such individuals have diffuse interstitial cell hyperplasia and frequently develop multiple GISTs at an early age *(5)*.

Mutations in the form of deletions, insertions, ITDs as well as point mutations occur most frequently (66.7%) in the juxtamembrane domain, which is thought to inhibit dimerization of the receptor in the absence of ligand. The mutations disrupt the autoinhibitory function of the juxtamembrane domain, leading to constitutive dimerization and activation of the kinase. Mutations in the extracellular domain, kinase domain I and kinase activation loop are less frequent *(5)*. Expression of mutant KIT renders cytokine-dependent cell lines factor-independent, consistent with gain-of-function status.

In vitro studies showed that imatinib was active against a cell line juxtamembrane mutant KIT *(113)* and led the manufacturer to allow compassionate use of the drug in a patient with metastatic GIST *(114)*. The impressive response seen in this individual quickly led to clinical trials, a multicenter study that compared 400 vs. 600 mg imatinib daily *(115)* and a phase I EORTC trial that used 400–1000 mg daily *(116)*. Objective partial responses were seen in approximately half the patients and stable disease in another 25%. Responses were generally stable during the 6–9 month observation time of these trials. One crucial observation from these studies was that response rates depended on KIT mutation status. The partial response rate in patients with juxtamebrane mutations was 83.5%, but only 48.7% in patients with mutations in the extracellular domain and 0% in patients without mutation *(5)*. This validated KIT as the target of imatinib in GISTs. Moreover, as the *in vitro* sensitivity to imatinib of extracellular domain and juxtramembrane domain mutants of KIT is not different, these data show that the specific type of mutation must influence the biology of the disease irrespective of its sensitivity to imatimib *(5)*. Differential sensitivity to imatinib was also observed in the rare GIST patients with mutations of PDGFRα. Patients with

mutations in the juxtamembrane domain have imatinib responsive disease, while the disease in patients with activation loop mutations is imatinib resistant *(117)*.

Unfortunately, as with imatinib in advanced phases of CML, GIST patients tend to relapse after an initial response. The mechanisms underlying resistance appear to be similar to those observed in CML patients, involving overexpression and mutations of KIT that impair drug binding. In addition, some patients with resistant disease exhibit downregulation of KIT expression, consistent with the activation of alternative signaling pathways, and there is "functional resistance" in some cases, with sensitive disease *in vitro* but resistance *in vivo (5)*. Similar to imatinib-resistant CML, the most promising approach to overcome resistance in GIST is alternative inhibitors of KIT that retain activity against the kinase domain mutant protein *(118)*.

2.4. Imatinib for the Treatment of Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans is an infiltrative skin tumor that presently is treated with surgery. Although these tumors rarely metastasize, there is a high risk of local recurrence after surgery. The causal genetic lesion is a chromosomal rearrangement fusing the collagen type Iα1 (*COLIA1*) gene to the PDGF B-chain (*PDGF-B*) gene. The resulting COLIA1/PDGF-B fusion protein is processed to mature PDGF-BB, which in an autocrine loop stimulates tumor growth. Studies in cell lines showed that interruption of this autocrine loop by imatinib leads to apoptosis, and anti-tumor activity was also documented in a murine xenograft model *(119)*. Clinical activity was demonstrated in several patients with metastatic disease *(120)*.

3. FLT3 INHIBITORS FOR ACUTE MYELOID LEUKEMIA

3.1. Biological Basis

Approximately 30% of patients with AML have mutations of FLT3, a type III receptor tyrosine kinase expressed on early hematopoietic progenitor cells. FLT3 mutations are associated with monocytic or promyelocytic differentiation and high white cell counts and in most studies with an adverse prognosis. The most frequent type of mutation, found in approximately 25% of patients with AML, is ITDs within the juxtamembrane domain. These duplications encompass between 5 and 40 amino acids, undermine the ability of the juxtamembrane domain to prevent dimerization and result in constitutive activation of receptor signalling *(4,14)*. Another 7% of AML patients exhibit mutations in the activation loop, typically in position D835 that is homologous to D816 in KIT *(121)*.

FLT3 ITDs or activation loop mutants transform BaF3 cells independent of growth factor but do not induce AML in the standard transduction/transplantation model of murine leukemia. Rather, they lead to a myeloproliferative syndrome *(122)*. However, in combination with PML-RARA, the fusion gene typical for acute promyelocytic leukemia (APL) induces AML with a promyelocytic phenotype, consistent with the high prevalence of FLT3 ITDs in patients with APL *(123)*. These experiments show that the roles of BCR-ABL in CML and FLT3 mutations in AML are different. BCR-ABL alone is capable of inducing a CML-like myeloproliferative syndrome while the induction of AML by constitutively active FLT3 requires cooperating genetic lesions. As discussed in Section 3.2., this has consequences for the success of FLT3 targeted therapy of AML.

3.2. Clinical Trials

Several FLT3 inhibitors have entered clinical development (Table 7). The various compounds are structurally diverse and have a variety of other kinase targets, mainly other type III receptor tyrosine kinases, such as KIT or PDGFR. The IC_{50} for inhibition of FLT3 autophosphorylation *in vitro* ranges between 2 nM for CEP-701 and 528 nM for PKC412, a compound previously developed as a PKC inhibitor *(124)*. Overall, the results of early clinical trials were quite variable. For example, MLN518 was tested in a phase I trial of 40 patients with refractory or relapsed AML, or myelodysplastic syndrome (MDS) who were not pre-screened for FLT3 mutations. Stable disease was

Table 7
FLT3 Inhibitors in Clinical Development

Compound	*Class*	*Target*	*FLT3 IC_{50} (nM)*	*Clinical trials*	*References*
PKC412	Benzoylstaurosporine	PKC PDGFR KDR KIT FLT3 ABL	528	Phase 2: AML with/without FLT3-ITD	*128*
CEP-701	Indolocarbazole	FLT3 TRKA KDR PKC PDGFR EGFR	2–3	Phase 2: AML with FLT3-ITD	*160*
CT35318	Piperazinyl quinazoline	KIT PDGFR FLT3 FMS	170–220	Phase 1: AML/MDS with/without FLT3-ITD Phase 2: AML with FLT3-ITD	*125,161*
SU5416	Indolinone	FLT3 KDR KIT	250	Phase 2: refractory AML/ MDS/MPD Phase 2: refractory AML (c-KIT$^+$)	*126,127*
SU11248	Indolinone	FLT3 KDR PDGFR	10	Phase 1: AML	*162*

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; PDGFR, platelet growth factor receptor; PKC, protein kinase C.

reported in two patients without evidence of FLT3 mutations and a partial response in one patient with a FLT3 ITD *(125)*. Similarly, only one complete and seven partial responses were seen in 43 patients with advanced AML treated with SU5416 *(126)*. This study had been conceived with VEGFR rather than FLT3 as the therapeutic target. Nonetheless, FLT3 ITDs were demonstrated in seven patients, but none of these patients responded to treatment. Another study tested SU5416 in patients with relapsed or refractory AML or MDS and reported only three partial responses in 55 patients *(127)*. More promising results were obtained with PKC412 in a cohort of 20 relapsed or refractory AML patients with FLT mutations *(128)*. In this trial, a reduction of peripheral blood blast counts by 50% or more was seen in 70% of patients. Half of these patients completely cleared blasts from the peripheral blood. Two patients achieved a complete response in the marrow (<5% blasts). Collateral studies demonstrated inhibition of FLT3 autophosphorylation in blasts from patients on treatment, an indication of efficient target inhibition. Another trial with an orally bioavailable FLT3 inhibitor, SU11248, reported responses in 4/4 patients with FLT3 mutations, including three partial responses and complete marrow response without platelet recovery. However, responses were generally short-lived. Altogether these data are reminiscent of the results of imatinib for CML in blast crisis, not surprising, given that mutant FLT3 is unable to induce an AML phenotype in the absence of cooperating mutations *(122)*. In fact, it is likely that the acquisition of the tyrosine kinase mutation is the second rather than the first hit on the path to AML, and reversal of a late event would naturally not be capable of eradicating the disease. Consistent with this, it has been demonstrated that FLT3 mutations may be acquired at the time of relapse but may also "disappear" with disease recurrence after chemotherapy, when another clone with wild-type FLT3 becomes dominant *(14,41)*. Interestingly, this sequence has also been shown for AML with mutations of the core binding factors such as AML1-ETO and CBFbeta-MYH11 in association with mutations of KIT. In patients in long-term remission to chemotherapy, AML1-ETO remains demonstrable, but not the activating mutation of KIT, suggesting that the latter is the secondary event *(40,129)*.

Bearing all this in mind, it is clear that monotherapy with FLT3 inhibitors will not be the future. Rather, it will be crucial to integrate these compounds into conventional therapy regimens and/or allograft approaches. One concern is that drugs with a broad spectrum of activity against several kinases (FLT3, KIT, in some cases KDR) with important roles in early hematopoietic development may significantly increase the stem cell toxicity of conventional agents. Fortunately, studies in mice suggest that CT53518 does not adversely affect recovery from chemotherapy-induced myelosuppression or engraftment after bone marrow transplantation *(15)*. MLN518 is currently being tested in combination with standard chemotherapy (cytarabine+daunorubicin) in a phase I study of newly diagnosed patients.

4. DEVELOPMENT OF GEFITINIB AND ERLOTINIB FOR NON-SMALL CELL LUNG CANCER

The EGFR is highly expressed on various types of epithelial tumors, including NSCLC *(130)*. Based on this observation, EGFR was proposed as a drug target in NSCLC, and several murine and humanized murine antibodies were developed, the best

known of which is cetuximab *(131)*. These antibodies target the extracellular domain of EGFR where they block the binding of ligand. In the early 1990s, a drug discovery program at AstraZeneca led to the identification of anilinoquinazoline derivatives as specific ATP-competitive inhibitors of the EGFR tyrosine kinase *(132,133)*. Gefinitib (Iressa) was chosen as the compound for clinical development. Activity was demonstrated against a number of cell lines derived from NSCLC and ovarian, breast, colon, and head and neck tumors and in various xenograft models. Phase I trials in patients with many different types of refractory tumors showed mild toxicity *(134–138)*, the MTD being 1000 mg/day. Importantly, target modulation, that is, inhibition of EGFR phosphorylation, was demonstrated in skin biopsies obtained during treatment *(139)*. Antitumor activity was observed at doses greater than 150 mg/day, with no clear dose response above this level. As a consequence, similar to imatinib, the phase II trials did not use the MTD but rather settled at 250 and 500 mg gefitinib daily. Based on the promising phase I data, two large phase II trials (IDEAL-1 and IDEAL-2) were initiated in patients with NSCLC who had failed cis-platinum-based chemotherapy. Each of these trials compared 250 and 500 mg gefitinib daily in more than 200 patients *(140,141)*. Disease control was seen in 42–54% of patients treated with 250 mg and 36–51% of patients treated with 500 mg gefitinib daily. One-year overall survival was approximately 30%, which compared very favorably with historical controls. Objective response rates were around 20% in the IDEAL-1 trial and 10% in the IDEAL-2 trial, a difference that may reflect the inclusion of patients with slightly more advanced disease in the IDEAL-2 study. Severe (grades 3 and 4) toxicity was more frequent with 500 mg compared to 250 mg (18 vs. 7% in the IDEAL-2 trial), which led to the choice of 250 mg daily for the phase III trials, which tested gefitinib plus gemcitabine/cis-platinum (INTACT-1 study) or gefitinib plus paclitacel/carboplatinum (INTACT-2 study) *(142,143)* vs. chemotherapy alone or gefinitib vs. placebo and best supportive care in patients previously treated with chemotherapy (ISEL study, $n = 1692$) *(55)*. Unexpectedly, the chemotherapy combination studies did not show an advantage over chemotherapy alone, although synergistic effects of the drugs under investigation had been shown *in vitro*. The precise reasons why the *in vitro* data did not translate into a clinical benefit are not known, but may include dose administration (continuous in the trial, but pulsed in animal studies), antagonistic effects and the fact that no three-drug chemotherapy regimen had shown superiority over the two-drug combinations *(56)*. The ISEL trial showed a significantly higher objective response rate in the gefitinib group (8 vs. 1%, $p < 0.0001$), but failed to show an overall survival benefit (median survival 5.6 vs. 5.1 months, $p =$ not significant). However, significantly superior results were observed for patients who never smoked (8.9 vs. 6.2 months, $p = 0.012$) and in patients of Asian origin (9.5 vs. 5.5 months, $p = 0.01$) in pre-planned subgroup analysis.

The results of the ISEL trial became available one month after erlotinib (Tarceva™) had been approved by the FDA based on the results of a similar but smaller study (BR21; $n = 732$) *(144)*. In contrast to the ISEL trial, the BR21 study showed a significant improvement of overall survival in the erlotinib group compared to placebo (6.7 vs. 4.7 months, $p < 0.001$) and a more substantial improvement for patients of Asian origin (12.3 vs. 5.5 months, $p < 0.001$). On the basis of the BR21 results, the FDA relabeled gefitinib for use in patients already receiving it and obtaining

a clinical benefit from it. It should be noted that erlotinib in combination with carboplatin/placlitaxel or cisplatin/gemcitabine was not superior to placebo in two studies (TALENT and TRIBUTE trials) with a design analogous to the IMPACT studies *(145)*.

The reasons for the discrepancy between the ISEL and the BR21 trials have been the subject of much discussion. The profile of responders was similar in both studies and included female gender and adenocarcinoma histology (and Japanese ethnicity in the IDEAL-2 trial). Subsequent studies showed that the striking responses seen in a minority of patients were associated with the presence of mutations in the kinase domain of EGFR *(18,19)* or multiple gene copies of EGFR or high levels of protein *(20)*. Various types of mutations were detected, including deletions, point mutations and in frame insertions. Compared to the wild-type EGFR, the mutant proteins exhibit more prolonged and stronger tyrosine phosphorylation in response to EGF. At the same time, there is increased sensitivity to gefitinib. This suggests that the mutations may increase the affinity to ATP but also to gefitinib. There are also differences in the signaling pathways activated by mutant compared to wild-type receptor, with the mutant receptor preferentially activating AKT *(146)*. As the phase II trials had not shown an association between response and EGFR expression as analyzed by immunohistochemistry, EGFR status was not used as a selection criterion for either the ISEL or the BR21 trials. Retrospective EGFR copy number analysis in both trials showed a significant association between gene amplification and response *(56)*. Mutation analysis for the ISEL trials has not yet been completed. As information on less than half of the patients is available, it is currently impossible to determine whether the inclusion of more patients with a favorable "molecular make-up" in the BR21 trial explains the superior results. A recent comparison of baseline characteristics showed equal distributions of major risk factors, including smoking history and previous chemotherapy. Fewer patients of Asian origin enrolled in the BR21 study (13 vs. 21% in the treatment arms), but more patients with a complete or partial response to previous chemotherapy (38 vs. 18%) *(56)*. The latter could explain the failure of the ISEL study, as in an exploratory analysis of the BR21 trial a significant survival benefit for erlotinib was demonstrated in patients with a prior response to chemotherapy but not for those with stable or progressive disease as their best response *(144)*. Another factor not related to patient selection may be the dose. Erlotinib was used at the MTD of 150 mg daily, while gefinitib was used at 250 mg daily, significantly below the MTD. These data are concordant with the lower rate of skin and gastrointestinal side effects in the ISEL trial, which suggest lower levels of systemic exposure. If a suboptimal dose was indeed the reason for the failure of the ISEL study, it would evidently be an "easy fix" for the drug.

Interestingly, in a recent study, mutations in the kinase domain of ERBB2 (also known as HER2 or Neu) were identified in 4% of NSCLC samples (10% in the adenocarcinoma subtype). These mutations (mainly one specific type of insertion) activate the kinase, consistent with their position within the kinase domain that is similar to the localization of activating EGFR mutations *(147)*. Inhibition of this type of mutations has recently been shown for HKI-272, an orally available arylaminoquinoline *(148)*.

Unfortunately, as with advanced CML and GISTs, acquired gefitinib resistance due to a point mutation in the kinase domain has recently been demonstrated in a

patient who relapsed after two years of gefitinib therapy *(149)*. Notably, the mutation (T790M) is structurally homologous to T315I in ABL, the notorious imatinib-resistant mutant first described by Gorre et al. *(150)*. It is currently unclear, however, whether point mutations are as important in the scenario of NSCLC as in BCR-ABL-positive leukemia, as at least in some reported cases, mutant alleles constitute only a small minority of all alleles *(151)*. Regardless, irreversible inhibitors of EGFR and ERBB2 have shown promise in preclinical studies of T790M mutant EGFR and are currently in phase I trials *(151,152)*.

5. PERSPECTIVE

The success of imatinib for the treatment of CML, HES and GISTs has sparked enormous interest in targeting tyrosine kinases for therapy of malignant disease. Large-scale sequencing projects suggest that tyrosine kinase mutations may be a widespread principle in carcinogenesis. For example, sequencing of the "tyrosine kinome" in colorectal cancer cell lines and tumor specimens revealed somatic mutations in 14 kinase genes. Although the localization of the mutations in critical regions of the kinases suggest that they may be activating, these data still require confirmation in biochemical assays *(153)*. Additionally, even with this approach, tyrosine kinase mutations were detected in only one-third of the samples. This suggests that alternative mechanisms of kinase activation may be operational, such as amplification or activation by translocations that would be missed by the sequencing approach. The other possibility is that mutations may be present in serine/threonine kinases, lipid kinases or phosphatases. There are precedents for all of these. Mutations that lead to constitutive activation of BRAF are frequent in melanoma and pancreatic carcinoma and occur at lower rates in several other types of solid tumors *(154)*. Mutations of the p110 subunit of the phosphatidyl inositol 3′ kinase have recently been detected in a variety of solid tumors *(155)*, and sequencing of the human tyrosine phosphatome revealed mutations in several phosphatases *(2)*.

Will it be possible to repeat the success of imatinib? It appears that this will largely depend on the genetics of the disease in question. One extreme of the spectrum may be diseases like polycythemia vera, where a rather uniform clinical entity exhibits a slowly progressive phenotype and is associated with an activating tyrosine kinase mutation ($JAK2^{V617F}$) in almost all cases *(37,155,156)*. The prediction is that a JAK2 inhibitor would be at least as effective as imatinib in CML. The same should hold true for patients with $JAK2^{V617F}$-positive essential thrombocythemia and perhaps even for IMF, although the frequent chromosomal abnormalities present in IMF suggest that the pathogenesis may be more complex, involving mechanisms in addition to JAK2 activation *(157)*. In contrast, it is quite clear that the pathogenesis of solid tumors is much more complex, leading to more potential targets but also to more redundancy. Nonetheless, the example of gefitinib shows that tyrosine kinase inhibitors can be effective even in very advanced cancers. To fully exploit the potential of tyrosine kinase and other specific signal transduction inhibitors, it will be crucial to get the right drug to the right patient. For this to be accomplished, future classifications of malignant disorders will have to use biochemical rather than morphological criteria as the basis for a personalized approach to therapy. The first steps have been taken but much work still remains to be done.

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VI Recent and Developing Cancer Therapies

25 Monoclonal Antibodies in Lymphomas

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Summary

Immunotherapy has markedly altered the treatment options available for patients with non-Hodgkin's lymphoma (NHL). Monoclonal antibodies have revolutionized the treatment of cancers in two respects. First, they represent a therapy with a different mechanism of action than chemotherapy. By providing an alternative type of attack, they may improve outcomes through better efficacy. Second, they represent a major step forward in improving the tolerability of cancer therapies. As a more targeted therapy, monoclonal antibodies enable patients to receive treatment that might not have otherwise been tolerated, potentially extending their lives and decreasing symptomatology. Although each monoclonal antibody was initially developed for one indication, their approvals have enabled exploration of other possible indications. We are only beginning to understand the breadth of diseases, malignant and non-malignant, that might benefit from treatment with these monoclonal antibodies, and how best to use them. Attempts to further improve outcomes in NHL are being explored, including the role of modifications of dose and schedule, chemotherapy combinations, and through the use of other biologics. Monoclonal antibodies represent the epitome of targeted therapy and have the potential to move forward the treatment of lymphoma more than any other development since the first use of multiagent chemotherapy.

Key Words: Monoclonal antibodies; immunotherapy; targeted therapy.

1. INTRODUCTION

The goal of cancer therapy is to eliminate the malignant cells without harming the patient. Current cancer treatment is dependent primarily upon three different modalities: surgery, chemotherapy, and radiation. Surgery and radiation therapy are effective at removing local disease while only damaging non-malignant tissue in the immediate

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vicinity. Thus, although surgery and radiation have no damaging effects on distant cells, they also do not treat malignant cells that have spread outside of the immediate area. Chemotherapy, on the contrary, penetrates throughout the body and is able to eliminate malignant cells that have spread beyond the local area. Unfortunately, chemotherapy will also damage normal tissues.

Although chemotherapy might demonstrate specificity by inhibiting a particular enzyme or damaging a particular protein, the targets of chemotherapeutic drugs are also typically found in normal tissue. This results in chemotherapy harming normal cells, with the primary impact generally expressed in rapidly dividing tissues, such as the bone marrow and gastrointestinal epithelium. The "selectivity" of chemotherapy is generally ascribed to the increased sensitivity of cancer cells to these agents. This increased sensitivity results, in part, from the malignant cells being under greater biologic stress because of their greater cell proliferation rate and energy demands, and because of less time and opportunity for repair prior to the next cell division. Although this strategy is somewhat effective, there is a great deal of damage to normal cells. This damage is seen as toxicities of therapy. These toxicities often result in painful and undesirable outcomes and limit the use of these conventional treatments.

If cancer treatments could be delivered specifically to the cancer cells, there would be the expectation of less associated toxicity. The therapy could also be delivered more safely and possibly in higher doses, potentially with greater effectiveness. Antibodies offer a means to target specific cells in the body, thereby limiting the toxicity to non-targeted tissues.

Antibodies are composed of two identical heavy chains and two identical light chains held together by disulfide bonds. Each antibody can bind two copies of its target antigen. The antigen binding domain is composed of the variable region of one light chain and one heavy chain. Each light chain possess one variable and one constant region. Each heavy chain possess one variable region and either 3 or 4 constant regions depending upon the immunoglobulin class. The effector functions of an antibody are mediated by the constant domains of the two heavy chains. (See Figure 1).

The specificity of antibody therapy results from features of both the antibody and the antigen. Through the processes of VDJ recombination, somatic hypermutation, and nucleotide base addition, the immune system is able to generate antibodies that possess extraordinary specificity. The second level of specificity is derived from the selection of an appropriate antigen whose expression is restricted to the targeted cells. The ideal target for a therapeutic antibody would be an antigen expressed only on malignant cells. Unfortunately, the identification of such malignancy-associated antigens, or tumor markers, has been difficult. The epitome of such selective expression would be the idiotype demonstrated by a particular lymphocyte clone. This target, created by the hypervariable region of the surface immunoglobulin of the lymphocyte, is unique to that one clone of lymphocytes. Currently, many investigators are working on developing anti-idiotype vaccines as treatments for lymphomas. This approach is difficult as it requires that a novel treatment be developed for each individual.

Lymphocyte differentiation is well characterized by the expression of a series of surface proteins termed "cluster of differentiation" (CD) antigens. Given the ontogenic relationship between lymphomas and normal lymphocytes, the antigens expressed on lymphomas are almost always expressed by their non-malignant counterparts. The damage that might result from targeting of an antigen on both normal and malignant

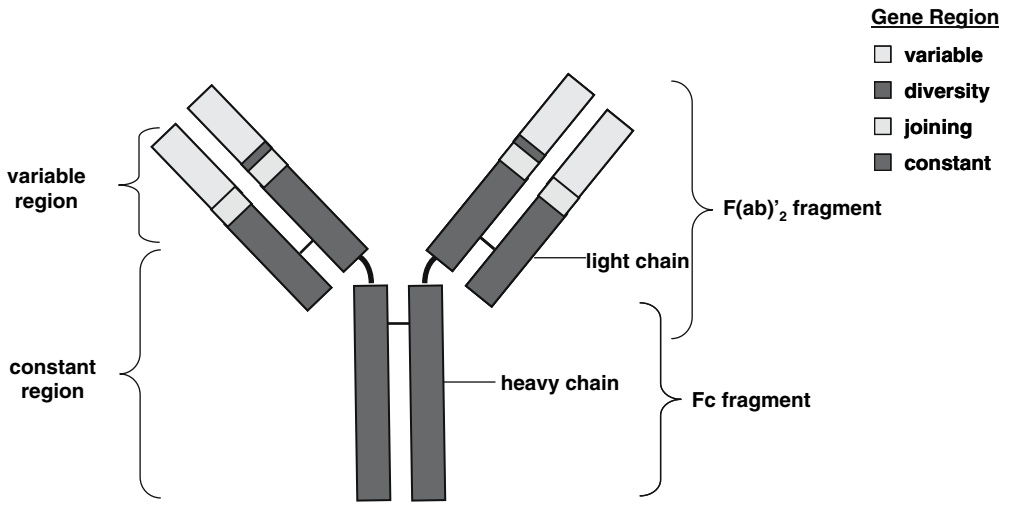

Fig. 1. Antibody structure. Each heavy chain is composed of variable region derived from the V, D, and J gene regions and a constant region derived from the C gene region. Each light chain possess a variable region derived from V and J gene regions and a constant region derived from the C gene region. The variable regions from both the light and heavy chains make up the antigen binding pocket. The F(ab)'2 fragment, derived from pepsin digestion, contains the antigen binding regions. The Fc fragment, derived from papain digestion, contains the immune effector functions.

lymphocytes is minimized by the ability to regenerate normal lymphocytes after therapy, limiting the duration and severity of immunosuppression.

The antigens that are targeted by monoclonal antibodies are cell surface-bound or soluble molecules. The features that make each of these types of antigens contributes to target differ. For cell surface-based targets, an ideal antigen would fulfill several criteria, including (i) expression of the antigen should be restricted so as to spare normal tissues; (ii) expression of the antigen on the tumor cell should be sufficiently intense in order to bind adequate numbers of antibodies; (iii) the antigen's cell surface expression should not be modulated in order that the antibody will remain on the surface once bound to the antigen (with the exception for toxin conjugates that require intracellular delivery); (iv) the antigen should be present in almost all cases of the malignancy; and (v) the antigen should not be present in a soluble or secreted form that would prevent the antibody from reaching the targeted cell or lead to soluble immune complexes.

For soluble targets, the ideal target would be a molecule that will have its activity neutralized once bound by antibody; also interference with its function will not impact upon other cells. The binding of an antibody to a soluble molecule would stereotactically inhibit its ability to bind to its receptor. Alternatively, the binding of the antibody might lead to increased clearance of the antigen, reducing its availability to activate its receptor. This latter mechanism of action requires antibodies of very high affinities and concentrations, as soluble molecules are typically meant to act over very short distances and at very low concentrations.

Antibodies eliminate cancer cells through several potential mechanisms: (i) complement-dependent cytotoxicity (CDC); (ii) antibody-dependent cellular cytotoxicity (ADCC); (iii) delivery of a toxin or radiation to the target cell; (iv) signaling

through the target antigen; and (v) deprivation of a necessary survival factor. Mechanisms (i–iii) are mediated by the Fc portion of the antibody. Mechanisms (iv and v) of action are mediated by the antigen-binding domain of the antibody. Currently, there are nine monoclonal antibodies approved for therapeutic oncology indications (Table 1). There are also many antibodies under clinical development whose targets include CD3, CD20, CD22, CD23, CD25, CD30, CD40, CD74, and CD80, vascular endothelial-derived growth factor, interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α.

Several different processes are used to generate monoclonal antibodies, resulting in antibodies of varying degrees of similarity to human immunoglobulins. Monoclonal

Table 1
Nomenclature System for Monoclonal Antibodies

guidelines:

1. the suffix –mab is used for monoclonal antibodies and fragments.
2. infixes identify the product source and the disease or target class.
3. distinct syllable selected to create unique name selected as prefix
4. stems combined in the following sequence: prefix + target disease infix + product source infix + stem

target:

viral	-vir-
bacterial	-bac-
immune	-lim-
infectious	-les-
cardiovascular	-cir-
colon	-col-
melanoma	-mel-
mammary	-mar-
testis	-got-
ovary	-gov-
prostate	-pr(o)-
miscellaneous	-tum-

product source identifiers:

o	= mouse
a	= rat
e	= hamster
xi	= chimera
zu	= humanized
u	= human
i	= primate

stem (for all monoclonal antibodies):
-mab

examples:
murine: tositumomab: “-tum-o-mab”
chimeric: rituximab: “ -tu(m)-xi-mab”
humanized: alemtuzumab: “-tu(m)-zu-mab”
human: ofatumumab: “-tum-u-mab”

antibodies are named based upon a system that has been adopted by the World Health Organization's Program on International Nonproprietary Names and the United States Adopted Names Council. Each antibody is named using a prefix, two infixes each representing: 1) the antibody target and 2) the species from which the antibody is derived, and a suffix. (See Table 2). The first monoclonal antibodies were generated in 1975 utilizing hybridoma technology and were murine in nature *(1)*. To generate these monoclonal antibodies, mice were vaccinated with the target antigen and then splenectomized. The B lymphocytes from the spleen were fused with a murine myeloma cell line. The myeloma cell line provided a means for immortalizing the B lymphocytes from the mouse. Fused cell lines were then screened for production of the desired antibody, and the monoclonal antibodies were collected from the supernatant of the cultured hybridomas. Antibodies generated in this manner were fully murine in character.

One of the initial and more significant obstacles to producing monoclonal antibodies was the need for the vaccinated antigen to maintain its native conformation. Processing of antigens that destroyed relevant epitopes would not produce relevant antibodies. Later, when murine antibodies were investigated as therapeutic tools, the murine nature of the antibodies presented three additional problems. First, murine antibodies (or almost any non-human species) have shortened half-lives in human serum, as an antibody's half-life in serum is determined by its Fc sequence and the protection it affords from proteolysis. Second, murine antibodies are immunogenic and might lead to the development of antibodies by the human recipient against the murine antibodies, human anti-murine antibody (HAMA). These HAMAs would have the potential to neutralize the therapeutic antibodies by either interfering with the antibody binding its antigen or increasing its clearance from the serum. They could also lead to serious infusion reactions because of the formation of antibody–antigen complexes rapidly in the serum upon infusing. Third, and perhaps most importantly, murine antibodies are unable to trigger the immunologic functions performed by the Fc portion of antibodies, including CDC and ADCC. These are both potent activities of the immune system that would lead to elimination of the targeted tumor cells.

In an attempt to rectify some of these issues, chimeric monoclonal antibodies were generated. Chimeric monoclonal antibodies utilize the murine variable domains and human constant domains. These antibodies were generated by cloning the cDNA for the heavy and light variable chains from the murine antibody to the cDNA for the heavy and light chain constant regions of human immunoglobulin *(2,3)*. These antibodies typically contain sequences that are 33% murine and 67% human. These chimeric antibodies are able to retain the specificity of the murine antibody, possess the effector functions of human immunoglobulins leading to CDC and ADCC, and lessen the likelihood for antibody production against the chimeric antibodies [human anti-chimeric antibodies (HACAs)].

Subsequent work generated what are termed "humanized" monoclonal antibodies. Humanized antibodies involve combining the hypervariable, complementarity-determining regions (CDRs) of a murine antibody, which fold to form the antigen-binding pocket, with human variable chain framework regions *(4)*. The DNA coding the murine CDRs are sequenced and cloned into a human immunoglobulin gene of an antibody-producing cell line. These humanized antibodies contain 90–95% human and only 5–10% murine sequences. The only murine sequences are the amino acids that

Table 2
Approved mAbs for Oncology Indications (as of 1/1/07)

Name (generic, trade)	*Target*	*approval*	*source*	*conjugate*	*Indication*	*Mechanism of action*
rituximab (Rituxan)	CD20	11/26/97	chimeric	naked	CD20+ follicular lymphoma	CDC, ADCC, induction of apoptosis by binding CD20
trastuzumab (Herceptin)	EGFR2/HER2	9/25/98	humanized	naked	breast cancer	toxin conjugate (calicheamicin)
gemtuzumab (Mylotarg)	CD33	5/17/00	humanized	calicheamicin	AML	inhibition of receptor activation by blocking ligand
alemtuzumab (Campath)	CD52	5/7/01	humanized	naked	CLL	CDC, ADCC
ibritumomab tiuxetan (Zevalin)	CD20	2/19/02	murine	RIT: yttrium-90	lymphoma	radioconjugate (yittrium-90)
tositumomab-I 131 (Bexxar)	CD20	6/27/03	murine	RIT: iodine 131	lymphoma	radioconjugate (iodine-131
cetuximab (Erbitux)	EGFR1/HER1	1/12/04	chimeric	naked	colorectal cancer	receptor blocking
bevacizumab (Avastin)	VEGF	2/26/04	humanized	naked	colorectal cancer	inhibition of receptor activation by blocking ligand
panitumumab (Vectibix)	EGFR	9/27/06	human	naked	colorectal cancer	inhibition of receptor activation by blocking ligand

comprise the CDR. The humanized antibodies afford all the Fc functions of human antibodies with minimal immunogenicity.

The newest technique produces "human" monoclonal antibodies. These antibodies are generated by two methods. The first method utilizes a transgenic mouse whose murine immunoglobulin genes have been replaced by human immunoglobulin genes *(5,6)*. These mice will generate a series of human antibodies in response to an antigen challenge. Antibody-producing hybridomas are then generated. The second process, known as phage display, utilizes bacteriophage containing almost every possible nucleotide sequence to produce antibodies with different specificities *(7)*. Colonies of bacteriophage are screened and then the sequences cloned.

The initial therapeutic use for monoclonal antibodies involved heavily treated patients with non-Hodgkin's lymphomas (NHLs). These patients' immune systems were quite impaired because of the underlying lymphoma or the immunosuppressant treatments delivered as lymphoma therapy. As a result, there was little concern for these patients developing a HAMA. This is best exemplified by the early studies of Bexxar in patients with follicular lymphoma. In the initial pivotal study, HAMAs developed in 8% of the patients from this heavily pretreated population. When Bexxar was studied as therapy for untreated patients with follicular lymphoma, the HAMA rate reached 63% *(8,9)*. Both of these studies utilize Bexxar as a single agent. When Leonard et al. *(10)* administered three cycles of fludarabine prior to Bexxar in patients with previously untreated follicular lymphoma, HAMAs developed in only 6% of the patients. Although the fludarabine was used in this study to debulk the patients with the hope of making the Bexxar more effective and safer, it had the additional benefit of immunosuppressing the patients sufficiently to prevent the generation of HAMA responses. Since, there was little chance that these patients would have sufficient effector cell function to take advantage of the presence of an Fc receptor, the use of a murine antibody is not problematic. Additionally, owing to the lack of effector cells, the antibodies would have to possess alternate means for inducing apoptosis, either radioactivity or a toxin conjugate.

Subsequently, chimeric, humanized, and human antibodies were developed that allowed the range of antibody use to increase.

In the remaining parts of this chapter, data will be presented regarding specific antibodies utilized in the treatment of patients with lymphomas.

2. RITUXIMAB

Rituximab (IDEC-C2B8), the first monoclonal antibody to be approved for a cancer indication in the USA, received approval from the Food and Drug Administration (FDA) in November 1997. Rituximab is approved as treatment for patients with relapsed or refractory, low-grade or follicular, CD20-positive, B-cell NHL. Rituximab is an IgG1 kappa chimeric monoclonal antibody directed against the CD20 molecule, whose expression is restricted to B lymphocytes *(11)*. The variable regions of rituximab are derived from the murine anti-CD20 antibody, 2B8 *(12)*. Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids, with an approximate molecular weight of 145 kDa. Rituximab is produced by Chinese Hamster Ovary (CHO) cells that contain the human/murine cDNA sequences for rituximab and secrete the antibody in large quantities into culture, where it is purified by affinity

and ion exchange chromatography. Rituximab binds to the CD20 antigen with a high affinity, $5 \times 10–9$ mol/L *(12)*.

CD20 is expressed only on normal and malignant B cells *(13,14)*. Its expression is first detectable at the pre-B-cell stage of lymphocyte development, and its expression remains present at high levels until the lymphoplasmacytoid stage, when it begins to become down-regulated. CD20 is not expressed on plasma cells, the most terminally differentiated B cell. This is of significance as rituximab will not target the earliest B-lymphocyte progenitors or plasma cells, allowing continuation of some antibody production and replenishment of normal B lymphocytes. The CD20 molecule is expressed in over 90% of B-cell lymphomas *(11,13)*.

CD20 is believed to play a role in regulating the activation, proliferation, and differentiation of B cells through its regulation of transmembrane calcium conductance that is responsive to signaling through the B-cell receptor *(15–17)*. CD20 possesses four transmembrane domains with its C-termini and N-termini both located within the cytoplasm. Initial experiments supported the idea that CD20 does not internalize *(18)*, is tightly bound in the membrane, and not present in the plasma *(13)*. Subsequent work demonstrated the presence of low amounts of circulating CD20 in the plasma in healthy controls and significantly elevated levels in patients with CLL *(19,20)*. In the CLL patients, the level of circulating CD20 was identified as an independent prognostic indicator *(20)*. In addition to its spectrum of expression profile, CD20 serves as a good therapeutic target, because it is a transmembrane protein whose presence is most likely required for B-lymphocyte survival. Thus, it is well anchored to the cell, and there is very low potential for the development of CD20-negative B cells through selection.

The exact mechanism of action for rituximab remains debated, but most likely depends upon a combination of (i) complement activation (Complement-dependent cell cytotoxicity, CDC) *(12,21–23)*; (ii) ADCC *(21,24,25)*; and (iii) antibody-mediated apoptosis involving signaling through the CD20 protein *(26–31)*. In support of CDC are several observations. The Fc portion of rituximab binds human C1q effectively, initiating the complement cascade *(12)*. Blocking CD55 and CD59, cellular-based inhibitors of complement, increased the sensitivity of lymphoma cells to rituximab, and levels of CD55 correlated with resistance to rituximab *in vitro (21,23)*. Finally, cells cultured in complement-depleted media appeared to be less sensitive to the effects of rituximab *(22)*. However, other investigators have not found a correlation between inhibitors of complement and sensitivity to rituximab *(32)*. It is interesting to note that rituximab has anecdotally demonstrated some activity when used for intrathecal treatment, given that cerebrospinal fluid is not expected to have complement or cells to mediate ADCC *(33,34)*.

The importance of ADCC has been demonstrated in a series of experiments by Clynes et al *(24)*. They demonstrated that mice deficient in the activating FcγRIIIa receptor were unable to control transplanted tumors *in vivo* with rituximab treatment. In contrast, mice deficient in the inhibitory FcγRIIb receptor demonstrate more ADCC and better control with rituximat. In humans, the best evidence in support of a role for ADDC comes from studies of gene polymorphisms for two activating Fc receptors, FcγRIIIa and FcγRIIa *(25,35)*. For FcγRIIIa, the polymorphism results in either a phenylalanine (F) or a valine (V) being present at amino acid position 158. Human IgG1 binds more strongly to V/V phenotype compared with the F/F or F/V phenotypes. As FcγRIIIa mediates ADCC involving natural killer (NK) cells and macrophages, it is

hypothesized that the Fc receptors with higher binding avidity would result in a better response to rituximab. The overall response rates at 2 and 12 months after rituximab therapy were 100 and 90% for the patients with V/V phenotype compared with 67 and 51% for patients with either F/V or F/F phenotypes. An additional study by Weng and Levy supported the finding by Cartron, et al. and also identified a polymorphism of amino acid 131 of the FcγRIIa receptor that correlated with outcome.

Although rituximab is not the physiologic ligand for CD20, its binding initiates a series of signaling cascades that often leads to apoptosis. Alas et al. *(31)* demonstrated that rituximab leads to a decrease in STAT3 activity, resulting in decreased Bcl-2 expression and apoptosis. Others have shown similar effects on signaling pathways involving anti-CD20 and anti-B-cell receptor-mediated apoptosis *(27)*. Jazirehi et al. *(28,29)* demonstrated a decrease in Bcl-XL levels in response to rituximab and that this was mediated through inhibition of nuclear factor-κB (NF-KB).

The initial phase II study of rituximab as treatment for low-grade lymphomas demonstrated response rates of 46%, with 8% complete responses (CRs) and 38% partial responses (PRs) *(36)*. The pivotal study utilized this same dosing scheme of 375 mg/m^2 four times weekly in a multicenter, open-label, single-arm study involving 166 patients with relapsed or refractory low-grade or follicular B-cell lymphomas *(37)*. The overall response rate was 48% with 6% CR and 42% PR rates. The median duration of response was 11.2 months, with a range of 1.9–42.1+ months. Multivariate analysis of the data demonstrated a higher response rate in patients with International Working Formulation (IWF) histologic subtypes B, C, and D compared with IWF subtype A (58 vs. 12%), higher in patients whose largest lesion was <5 vs. >5 cm in greatest diameter (53 vs. 38%), and higher in patients with chemosensitive relapse as compared with chemoresistant relapse (53 vs. 36%).

Most patients demonstrated a rapid decrease in the number of peripheral blood B cells by day 4, which remained nearly undetectable until approximately 6 months post-treatment, followed by a gradual recovery. Mean serum immunoglobulin levels remained stable over the course of the study, with IgG and IgA levels remaining within the normal range but IgM levels falling slightly below the lower limits of normal. One out of 166 patients developed a HACA response at day 50, which was not associated with any clinical or laboratory abnormalities. Tumor response correlated with serum rituximab concentrations, with responding patients having a higher median serum concentration at all time points during treatment, but with the difference being statistically significant predose for infusions 2 and 4 and post-treatment infusion 4.

Given the tolerability and effectiveness of rituximab in the relapsed setting, Witsig et al. from the North Central Cancer Treatment Group studied rituximab in patients with untreated grade I follicular lymphomas. As might be expected with untreated patients, they demonstrated a higher overall response rate of 72%, with a 36% CR rate. The median time to progression was 2.2 years with only 56% of the patients progressing with a median follow-up of only 2.6 years *(38)*. Rituximab thus may serve as a means to delay the exposure of patients to chemotherapy and its resulting toxicities. It will be even more important to determine the numbers of these patients who respond to retreatment with rituximab. When rituximab retreatment was investigated in patients with relapsed, low-grade NHL who had previously demonstrated a response of at least

6 months, Davis et al. *(39)* reported an overall response rate (ORR) of 40%, with 11% CRs and 30% PRs.

Although rituximab does demonstrate substantial activity with acceptable toxicity, the benefits of the standard dosing schedule are transient, with relapse inevitable. A number of investigators have attempted to improve outcomes through modifications of the dose and schedule. Although these studies have demonstrated some improvement in response rates and time to treatment progression, it is unclear whether they improve overall survival *(37,39–44)*.

2.1. Chemoimmunotherapy Combinations for Indolent NHL

Another means for utilizing rituximab is in combination with chemotherapy. These chemoimmunotherapy regimens attempt to take advantage of an additive or even synergistic effect of combining chemotherapy with rituximab through concurrent or sequential administration. Two hypotheses are put forward to explain the rationale for combination chemoimmunotherapy. In the first theory, rituximab increases the sensitivity of the lymphoma cells to chemotherapy. This occurs by rituximab altering intracellular signaling pathways or by causing a "double hit", resulting in the cells being more sensitive to chemotherapy. The second hypothesis is that the rituximab will eliminate those cancer cells that are not sensitive to the chemotherapy.

The first clinical trial to evaluate the safety and efficacy of combination chemoimmunotherapy was performed by Czuczman et al. *(45)* between April 1994 and March 1996. Forty patients, 38 of whom had received prior therapy, with low grade and follicular lymphoma received six cycles of CHOP chemotherapy at standard doses with two infusions of rituximab administered before the first cycle, single infusions before the third and fifth cycles, and two infusions after the sixth cycle. They demonstrated an ORR of 100% (87% CR) with a median time to progression of 82.3 months. Ten years after the study's inception, 42% of the patients remained in remission.

Czuczman and colleagues also studied concurrent fludarabine and rituximab in patients with low-grade and follicular NHL *(46)*. They treated 40 patients with six cycles of fludarabine (25 mg/m^2/day for 5 days every 28 days) plus two infusions of rituximab prior to the first cycle, single infusions with the second, fourth, and sixth cycles, and two final infusions 4 weeks after the final fludarabine treatment. The ORR was 90%, with 80% CRs and median duration of response that had not been reached at 40+ months. Molecular remissions were achieved in 88% of the patients who were positive for the 14;18 translocation at enrollment.

The first randomized study comparing concurrent chemoimmunotherapy to chemotherapy alone in follicular lymphoma was reported by Marcus and colleagues *(47)*. About 321 patients with previously untreated, stages III and IV follicular lymphoma were randomized to treatment with eight cycles of CVP chemotherapy every 21 days (notably at a perhaps attenuated cyclophosphamide dose of 750 mg/m^2/cycle) with or without concurrent rituximab administered on day 1 of each cycle. Overall and CR rates were 81 and 41% in the R-CVP arm and were 57 and 10% in the CVP arm, respectively ($p < 0.0001$). Median time to progression was 32 vs. 15 months ($p < 0.0001$) for the two arms respectively. One important feature was that there was no evidence of increased toxicity when rituximab was added to CVP with the exception of infusion-related toxicities. Although these data are compelling, the reported follow-up was short and not reported for overall survival.

The German Low-grade Lymphoma Study Group compared treatment with fludarabine, cyclophosphamide, and mitoxantrone with and without rituximab as treatment for relapsed and refractory follicular and mantle cell lymphoma *(48)*. The addition of rituximab increased the ORR to 79% (33% CRs and 45% PRs) from 58% (13% CRs and 45% PRs) and was associated with a significantly longer progression-free survival (16 vs. 10 months) and overall survival (not reached vs. 24 months).

Another interesting approach investigated has been the use of rituximab followed by short-duration chemotherapy (CVP or CHOP for 3 cycles) *(49)*. The intention of this combination is to minimize chemotherapy-related toxicities by limiting the amount of chemotherapy necessary to achieve the necessary response. This type of approach is of particular importance when treating elderly patients or patients who might be expected to receive many repetitive cycles of chemotherapy, as seen in patients with follicular lymphomas. A trial of 86 patients showed an overall response rate of 93% (55% CR) with 4-year progression-free survival of 62%. The promising results in these initial studies are limited by the small sample size (and potential patient selection issues) and lack of a chemotherapy-only control group, though they have served as the basis for larger follow-up studies in broader settings.

Zinzani and coinvestigators investigated the use of chemotherapy followed by rituximab. They compared a regimen of fludarabine plus mitoxantrone (+/− rituximab) with CHOP (+/− rituximab) in 140 patients with untreated follicular lymphoma *(50)*. This study added rituximab treatment after chemotherapy only in those patients with persistent clinical or molecular evidence of lymphoma. The FM arm demonstrated higher CR rates than the CHOP arm (68 vs. 42%), with 39% of the FM-treated and 19% of the CHOP-treated patients achieving molecular remissions after chemotherapy alone. Overall, rituximab improved the rate of molecular CR from 29 to 61% (71% in FM-treated and 51% in CHOP-treated patients). However, with a median follow-up of 19 months, there was no difference observed in progression-free or overall survival amongst any of the different treatment groups. When these data mature, an important analysis will be to determine whether there is a benefit to be gained by using rituximab to convert a clinical CR to a molecular CR in patients with follicular lymphoma.

Patients treated in the study by Zanzani et al. received four doses of rituximab after completing their chemotherapy. The next step in the use of rituximab was to investigate utilizing "maintenance" rituximab after chemotherapy. Two randomized, controlled studies investigating the use of maintenance rituximab after chemotherapy were undertaken by McLaughlin et al. and the Eastern Cooperative Oncology Group (ECOG). McLaughlin compared treatment with fludarabine, mitoxantrone, and dexamethasone (FND) plus concurrent rituximab followed by interferon-α (IFN-α) with FND followed by IFN-α plus rituximab in patients with follicular NHL. After a relatively short 30 months of follow-up, no significant differences in response rates or survival have been identified between the treatment groups *(51)*.

In the study conducted by ECOG (E1496), untreated patients with indolent NHL were initially treated with CVP chemotherapy followed by randomization to either observation or maintenance rituximab administered as four weekly doses every 6 months for 2 years *(52)*. Patients who received rituximab after CVP had significantly higher progression free survival (PFS) at 2 and 4 years after randomization (74 and 58%, respectively) compared with those who only received CVP (42 and 34%, respectively). This improvement in PFS with rituximab maintenance was seen in all tumor histologies,

in patients with bulky disease, and in patients with evidence of minimal residual disease following induction therapy. Although a difference in survival has not been observed after a median follow-up of 1.2 years, this trial provides the first randomized evidence that adding rituximab maintenance to chemotherapy in untreated patients who achieve a response or stable disease can prolong response duration.

The European Organization for Research and Treatment of Cancer (EORTC) conducted a randomized study of CHOP vs. concurrent CHOP-R followed by a secondary randomization to either observation or maintenance rituximab (375 mg/m^2 for one dose every 3 months) in patients with recurrent follicular lymphoma *(53)*. The trial was stopped at the second planned interim analysis because of a significant difference in CR rate for CHOP-R vs. CHOP (30.4 vs. 18.1%). Additionally, rituximab maintenance has also demonstrated a longer PFS at 1 and 3 years compared with observation (80.2 and 67.7% vs. 54.9 and 31.2%, respectively).

2.2. Chemoimmunotherapy in Aggressive Lymphoma

Coiffier et al. *(54)* demonstrated in a phase II study of single agent rituximab in aggressive lymphomas an ORR of 31% (9% CRs and 22% PRs). Even with single agent rituximab fairing much worse in aggressive lymphomas than in indolent lymphomas, there still remains the potential benefit of combination chemotherapy and immunotherapy. A phase II study of rituximab given on day 1 combined with standard dose CHOP given on day 3 demonstrated an ORR of 94% (61% CRs and 33% PRs). With a minimum follow-up of 24 months, 88% of the patients remained alive and free of disease.

The Groupe d'Etude des Lymphomes de l'Adulte (GELA) conducted the first phase III, randomized study comparing eight cycles of CHOP with rituximab plus CHOP (R-CHOP) as treatment for diffuse large B-cell lymphoma (DLBCL) in previously untreated patients over the age of 60 years *(55,56)*. In this study, patients randomized to receive R-CHOP received both the rituximab and CHOP chemotherapy on day 1 of the cycle. With a median follow-up of 5 years, R-CHOP produced statistically significant improvements compared with CHOP in terms of CR rate (76 vs. 63%), 5-year PFS (54 vs. 30%), and 5-year overall survival (OS) (58 vs. 45%).

More recently, the Mabthera International Trial (MinT) evaluated the addition of concurrent rituximab to a CHOP-like regimen in good-prognosis patients younger than 60 years of age *(57)*. With median follow-up of 2 years, the addition of rituximab to chemotherapy resulted in higher CR rates (81 vs. 67%), longer 2-year time-to-treatment failure (TTF) rates (76 vs. 60%), and better 2-year OS (94 vs. 87%). Follow-up continues, and data on potential benefits of rituximab in patient subgroups (chemotherapy type, bulk of disease, use of radiation treatment, and prognosis) are anticipated.

The ECOG 4494 Trial investigated the use of CHOP plus rituximab in elderly patients with diffuse large B-cell lymphoma utilizing a 2×2 factorial design *(58,59)*. Patients were first randomized to treatment with either CHOP chemotherapy with rituximab administered prior to cycle 1 and concurrently with cycles 3, 5, and 7 or CHOP alone. Responding patients were then randomized to either observation or maintenance rituximab administered as a 4-week course every 6 months for 2 years. The ORR was not different between the induction regimens, 79% for R-CHOP and 76% for CHOP alone. Weighted analysis was performed to enable each randomization

to be examined individually. The data demonstrated an improvement in TTF for R-CHOP compared with CHOP induction at 3 years (52 vs. 39%) and in overall survival at 3 years (67% vs. 58%). Likewise, maintenance rituximab prolonged TTF compared to observation but with no difference in overall survival. Overall, in the patients reaching the second randomization, the 2-year TTF was 77, 79, 74, and 45% for R-CHOP, R-CHOP+MR, CHOP+MR, and CHOP, respectively. These findings indicate that maintenance rituximab improved TTF in patients treated with CHOP alone [hazard ratio (HR) = .45, $p = .004$] but did not provide any additional benefit to patients treated with R-CHOP (HR = .93, $p = .81$). The primary findings of the study confirm the benefits of CHOP-R induction therapy over CHOP alone and also suggest that with R-CHOP induction regimen, maintenance rituximab therapy is unnecessary in DLBCL. It could thus be said that it does not matter when rituximab is administered as part of a CHOP induction regimen for DLBCL, so long as it is administered.

Current studies are evaluating the effects of rituximab in combination with other regimens (such as dose-adjusted EPOCH) in DLBCL and other lymphoma histologies with varying results *(48,60,61)*. It is not clear that all aggressive lymphomas benefit from the addition of rituximab. In some studies involving HIV-associated aggressive lymphomas, an improvement in response rate is counter balanced by an increase risk of toxic death, leading to no improvement in overall survival *(62)*. Several hypotheses have been generated to explain the increased toxicity, including hypogammaglobulinemia *(63)*, prolongation of the neutropenia through cytokines *(64)*, and autoantibody production *(65)*. This prolonged neutropenia and infection-related deaths were not seen in the studies investigating the combination of rituximab and chemotherapy in non-HIV-associated lymphomas, suggesting that the deaths may have been the result of the combination of hypogammaglobulinemia and the CD4 cell lymphopenia seen in HIV patients. But other studies have refuted these findings *(66–69)*.

As can be seen from these studies, response rates are increased, but at the risk of serious infections. These studies were not standardized with regard to infectious prophylaxis or the use of granulocyte colony-stimulating factors. Therefore, in the right patients with the correct precautions, there may be much to gain from the use of rituximab in combination with chemotherapy for aggressive lymphomas in HIV-positive patients.

3. ALEMTUZUMAB

Alemtuzumab (Campath-1H) is a genetically engineered humanized IgG1 monoclonal antibody directed against CD52. Alemtuzumab, named Campath after *Cam*bridge *Path*ology, was first developed in 1983 as a rat monoclonal antibody that was very effective in reducing graft-versus-host disease following allogeneic bone marrow transplantation. The antigen-binding determinants of the alemtuzumab antibody were eventually cloned into human immunoglobulin-producing cells generating a humanized monoclonal antibody. As a humanized antibody, it was believed that alemtuzumab would be more effective at mediating Fc-mediated activities with decreased immunogenicity. Alemtuzumab was first used clinically in 1988 in a patient with refractory lymphoma *(70)*. But it was not until 1991 that the CD52 antigen was cloned and recognized as the target for alemtuzumab.

CD52 is a non-modulating glycosylphosphatidylinositol-anchored glycoprotein that is expressed at high density on all lymphocytes and monocytes but not on granulocytes,

erythrocytes, platelets, and hematopoietic stem cells *(71,72)*. CD52 is one of the most abundantly expressed membrane glycoproteins, with lymphocytes possessing almost 450,000 molecules per cell. CD52 is almost universally expressed on all lymphomas, with more intense expression seen on normal and malignant T cells compared with normal and malignant B cells, as well as low-grade lymphomas compared with aggressive lymphomas *(73)*. Alemtuzumab was demonstrated to be more effective against blood and bone marrow disease compared with lymph node disease, as well as against indolent disease compared with aggressive disease *(74)*. These data led to the initial development of alemtuzumab for use as treatment of patients with CLL. Alemtuzumab is able to fill a niche that rituximab could not because of the low intensity of CD20 expression present on CLL cells. Based on data generated in the pivotal study, Campath was approved by the FDA in 2000 for treatment of patients with CLL who have relapsed after fludarabine therapy *(75)*.

As T-cell lymphomas do not express CD20, they represent another area where rituximab is not of value. Peripheral T-cell lymphomas represent 5–10% of NHLs and typically have a poorer prognosis than B-cell lymphomas *(76)*. Anthracycline-containing chemotherapy regimens are expected to result in long-term survival in only approximately 30–40% of the patients *(77,78)*. Early studies suggested that responses to alemtuzumab correlated with levels of CD52 expression, further supporting a role for alemtuzumab in T-cell lymphomas *(79)*. Investigators began to utilize Campath in T-cell lymphomas in a manner analogous to that of rituximabs in B-cell lymphomas. Several phase II studies have been performed utilizing alemtuzumab as single-agent treatment delivered either by intravenous or by subcutaneous injection for either B-cell or T-cell lymphomas.

Three studies investigating alemtuzumab in patients with NHL demonstrated response rates of 16, 20, and 44% *(80–82)*. When Lundin et al. analyzed their eight patients with mycosis fungoides separately, they identified an ORR of 50%, with two CRs. Enblad et al. *(83)* conducted a trial involving patients with chemotherapy refractory peripheral T-cell lymphomas and demonstrated an ORR of 36% (5 of 14), including three CRs. In their study, hematologic and infectious complications were considerable and resulted in the study closing prematurely with five deaths attributed in part to alemtuzumab. Given the refractory nature of these patients' disease, the authors concluded that alemtuzumab has considerable activity and may be sufficiently safe in less heavily treated patients. The same group tested alemtuzumab in 22 patients with advanced mycosis fungoides *(84)* and achieved an ORR of 55%, with 32% of the patients achieving a CR. In this study, 10 patients developed infections, resulting in one death because of pulmonary aspergillosis. Four patients developed CMV reactivation, with all four responding to IV ganciclovir. The infectious complications in this study appear more manageable, supporting a role for the further development of alemtuzumab as a treatment for lymphomas. Another approach to reduce the infectious complications was studied by Zinzani et al. *(85)* utilizing a dosing regimen of 10 mg (instead of 30 mg) administered intravenously three times a week for 4 weeks. In the 10 patients treated, there were six responses, with four PRs and two CRs. Only one infection was noted, which was CMV reactivation adequately treated with ganciclovir. As supportive therapies improve, such as, valganciclovir, and more clinical experience is obtained with alemtuzumab, higher response rates with safer outcomes could be expected.

3.1. Other Immunotherapies in Use in NHL

The use of other biologic agents either alone or in combination with rituximab is an appealing strategy to avoid the toxicity of chemotherapy or to enhance its efficacy. Immunostimulatory agents are under active investigation for this purpose. IL-2 administration expands and activates circulating NK cell populations, which potentially improves ADCC. As ADCC is an important mechanism of rituximab activity, IL-2 in combination with rituximab might have synergistic effects. Additionally, as previously discussed, less benefit is derived from rituximab use in patients with certain Fc receptor polymorphisms. In an attempt to investigate this combination, IL-2 was administered at escalating doses from 4.5 to 14 million units subcutaneously three times per week in conjunction with four weekly standard rituximab doses *(86)*. In this phase I study, most patients responded. Higher circulating NK cell numbers and increased ADCC activity were seen with IL-2 therapy and correlated with clinical response. Eisenbeis and colleagues *(87)* have evaluated the combination of IL-2 plus rituximab in patients with rituximab-refractory indolent lymphoma. As this patient population was resistant to rituximab, any responses would be significant. Clinical responses were identified in five of 23 patients. When the Fc receptor polymorphisms of these patients were examined, it was noted that all of the responding patients analyzed were homozygous for phenylalanine polymorphism at position 158. This polymorphism is generally associated with less effective ADCC and less favorable outcome with rituximab. These findings are consistent with the hypothesis that IL-2 may be able to overcome rituximab resistance when it is related to relatively less effective ADCC because of Fc receptor polymorphism.

Immunostimulatory sequences of DNA in the form of CpG oligonucleotides have a wide range of immune effects including enhancement of antigen presentation and cytokine induction (supporting ADCC). Friedberg and colleagues *(88)* conducted a phase I trial with four doses of a CpG oligodeoxynucleotide (1018 ISS) with rituximab and noted clinical responses with minimal toxicity. Interestingly, correlative studies demonstrated that CpG administration was associated with a dose-related increase in expression of several IFN-inducible genes, a reassuring sign of biological effect. CpG administration is under active exploration both as a single agent and in combination with other therapies in lymphoma as well as other malignancies.

Other investigators have attempted to improve on rituximab therapy by creating new variations of CD20-directed antibodies. Preclinical studies have been conducted with a number of these agents that have been designed to either have human or humanized structure (other than murine or chimeric) to improve pharmacokinetics and reduce immunogenicity or to have enhanced complement-mediated cytotoxicity or ADCC *(89,90)*. Such agents have moved into phase I clinical trials in some cases, and clinical responses have been observed. Whether the theoretical advantages to these antibody modifications translate into a clinical benefit for patients remains to be determined.

Alternative targets for monoclonal antibodies (particularly in B-cell malignancies) are also under evaluation. The CD22 antigen is widely expressed in normal and malignant B cells and plays a role in B-cell activation and trafficking *(91)*. We conducted phase I trials of epratuzumab, a humanized anti-CD22 antibody, and demonstrated a favorable toxicity profile at doses of up to 1000 mg/m^2/week administered for 4 weeks *(92,93)*. Clinical responses were noted in follicular NHL and in diffuse

large B-cell lymphoma. In some cases, these have lasted for >1 year, and second responses (upon retreatment at progression) have been noted. Epratuzumab is currently under active investigation in combination with rituximab and CHOP-R chemotherapy, as well as in other B-cell malignancies and in autoimmune disease.

3.2. Radioimmunotherapy

Given the exquisite radiosensitivity of lymphomas, the attachment of a radioisotope to a monoclonal antibody can enhance the activity of the antibody through the addition of targeted radiation *(94)*. The monoclonal antibody serves as the means to deliver the radiation directly to the intended target. When conventional radiation is delivered by external beam radiation, normal and malignant tissues are irradiated. For tumors that are not localized, avoiding the normal tissues becomes impossible. The antibody delivers the radioisotope into proximity of the intended target. When the radioisotope decays, the radiation is released to damage the target cell, typically by inducing DNA strand breaks, leading to apoptosis of the cell. The radiation may be delivered not only to the antibody-bound targeted cell but also to neighboring cells that are located within the path length of the radiation. This "cross-fire" effect results in therapeutic benefit if the neighboring cells are tumor cells not bound by antibody or toxicity if the neighboring cells are non-malignant cells. This is of particular concern with low-grade lymphomas as they frequently involve the bone marrow and hematopoietic stem cells that are very radiosensitive. Thus, myelosuppression is one of the dose-limiting toxicities for radioimmunotherapy (RIT).

Two such agents have been FDA approved, yttrium-90 ibritumomab tiuxetan (Zevalin, Biogen Idec) and iodine-131 tositumomab (Bexxar, Corixa, and Glaxo-SmithKline), for use in patients with CD20+ transformed or non-transformed follicular lymphoma whose disease is refractory to rituximab and has relapsed following chemotherapy. Both radioimmunoconjugates chelate their radioisotope to a murine antibody targeting the CD20 antigen. As the therapeutic modality is radiation, there is no need for a human Fc portion of the immunoglobulin to be present to elicit ADCC or CDC. Utilization of the murine antibody allowed for quicker and simpler therapeutic development of RIT, as well as afforded a means to limit infusional-related toxicities associated with humanized or chimeric antibodies.

One problem with the use of Zevalin and Bexxar antibodies is that they are murine antibodies, leading to the potential for developing HAMAs. These HAMAs may impair the effectiveness of the RIT or subsequent immunotherapy-utilizing murine antibodies or result in the development of infusion-related toxicities. Patients treated with RIT as part of the initial studies were at low risk for developing HAMAs due to their immune dysfunction resulting from the high number of prior chemotherapy regimens they received. The pivotal trial of I-131 tositumomab in low-grade NHL demonstrated a HAMA rate of only 8% *(9)*. Patients enrolled on this study had a median of four prior chemotherapy regimens. In the phase I/II study of Y-90 ibritumomab tiuxetan, 2% of the patients developed HAMAs or HACAs *(95,96)*. These data compare to a rate of 63% for the development of HAMAs when Kaminski studied I-131 tositumomab as initial therapy in patients with follicular lymphoma *(8)*. Once again, it is unclear what the clinical implications of a HAMA are, but Kaminski reported that among the 23 patients in whom HAMA levels were more than five times the lowest level of detection, the 5-year rate of PFS was 35% compared with 70% for the remaining 53

patients. Additionally, 75% of the patients in the subgroup with high HAMA titers experienced grade 2 or higher fever, myalgias, arthralgias, or rash as compared with 12% of the other patients *(8)*.

I-131 tositumomab and Y-90 ibritumomab tiuxetan utilize different radioisotopes. The I-131 beta emissions travel on average 0.4 mm or several cell diameters. The beta emissions from Y-90 are of higher energy, resulting in a longer path length compared with I-131 (5 mm). This translates into an optimal target diameter for Y-90 of 3.4 cm compared with 0.34 cm for I-131 *(97)*. This higher beta energy may be an advantage where deeper penetration is desired but a disadvantage where it will result in greater toxicity. Additionally, I-131 emits gamma radiation that allows for imaging to be performed using external gamma cameras to determine dosimetry. As Y-90 does not emit gamma radiation, dosimetry needs to be performed utilizing an indium-111-labeled ibritumomab tiuxetan.

I-131 tositumomab and Y-90 ibritumomab tiuxetan are both individually dosed for each patient based on dosimetry calculations. For I-131 tositumomab, the dose is calculated to deliver an exposure of total-body radiation of 75 cGy; whereas for Y-90 ibritumomab tiuxetan, a dose of 0.4 mCi/kg (to a maximum of 32 mCi) is delivered *(95,98)*. Both RITs were found to require dose reductions in patients who had platelet counts between 100 and 149,000/μL, as well as recommendations for not treating patients with >25% bone marrow involvement with lymphoma.

In its pivotal study, responses to I-131 tositumomab treatment were compared with the efficacy seen with the patient's last qualifying chemotherapy (LQC) regimen *(9)*. This enabled refractory patients to be studied without needing to utilize a comparator arm, as these patients had already demonstrated refractoriness to chemotherapy. All patients treated on protocol had either not responded or a response of <6 months to their LQC. An ORR of 65%, with 20% CRs, was seen for I-131 tositumomab compared with 28% (with 3% CRs) after the LQC. The median response duration for those patients achieving a CR after I-131 tositumomab had not been reached after 47 months of follow-up compared with 6.1 months after LQC. Given the refractory nature of these patients' disease, these responses are of particular significance.

In the pivotal study for Y-90 ibritumomab tiuxetan, patients with low-grade, follicular, or transformed B-cell NHL were randomized to treatment with Y-90 ibritumomab tiuxetan or rituximab *(99)*. The ORR and CR rate for the two groups were 80 and 30% for Y-90 ibritumomab tiuxetan and 56 and 16% for rituximab, respectively. Additionally, more durable responses (>6 months in length) were seen with Y-90 ibritumomab tiuxetan than rituximab.

In the study by Witzig et al. just discussed, the labeled and unlabeled antibodies differed not just in regard to the presence of the radioisotope but also in that Y-90 ibritumomab tiuxetan is a murine antibody, whereas rituximab is a chimeric antibody. Two studies were conducted utilizing the same antibody with and without an attached radioisotope that confirmed the importance of the radioisotope to the anti-CD20 therapy. In a study that compared I-131-labeled tositumomab to unlabeled tositumomab, Davis et al. demonstrated ORRs of 55 vs. 19%, with 33 vs. 8% CRs, for I-131-labeled tositumomab vs. unlabeled tositumomab *(94)*. In a second study, Horning et al. studied I-131 tositumomab in patients who had previously received rituximab *(100)*. The prior responses to rituximab demonstrated by the 40 patients in this study included 24 patients without any response, 11 patients with responses of < 6 months,

and five patients with responses >6 months. The ORR was 65%, with a CR rate of 38%, for all the patients in the study, and a progression-free survival for responders of 24.5 months. There was no correlation between the response to I-131 tositumomab and that of the rituximab, indicating that refractoriness to rituximab does not predict for a poor response to I-131 tositumomab.

In an attempt to maximize the benefits of RIT for patients, I-131 tositumomab was evaluated in indolent or transformed B-cell NHL at the first or second relapse. Garlier use of the agent demonstrated a response rate of 76%, with a CR rate of 49% *(101)*. Median overall duration of remission was 1.3 years, and approximately one-fourth of the patients remained in a continuous remission ranging from 2.6+ to 5.2+ years after treatment. Kaminski and colleagues *(8)* assessed the utility of radioimmunotherapy as initial therapy for follicular lymphoma. Seventy-six patients with untreated, advanced stage follicular lymphoma were treated with a single course of I-131 tositumomab. The overall response rate was 95%, including 75% CRs. Eighty percent of the assessable patients achieved a molecular remission based on polymerase chain reaction assessment for BCL2 gene rearrangements. After a median follow-up of >5 years, estimated progression-free survival was 6.1 years. Treatment was well tolerated. As previously discussed, HAMA formation occurred in 63% of these patients, far greater than the 8% reported in the earlier studies of heavily pretreated patients.

These findings have led to further studies that will ultimately help to determine the utility of RIT as a component for initial therapy for indolent lymphoma. A phase II study conducted by the Southwest Oncology Group (SWOG) investigated CHOP followed by I-131 tositumomab as initial therapy in patients with follicular lymphoma *(102)*. Preliminary results appear quite favorable, with an ORR 90%, a CR rate of 67%, and a 2-year progression-free survival of 81%. As a follow-up, the SWOG and Cancer and Leukemia Group B (CALGB) are currently conducting a randomized trial of CHOP plus concurrent rituximab vs. CHOP followed by I-131 tositumomab as first therapy for follicular NHL.

Another potential use studied for I-131 tositumomab has been in conjunction with autologous stem cell transplantation. These studies investigated I-131 tositumomab as a single agent at myeloablative doses of approximately 25 Gy in place of chemotherapy as part of an autologous stem cell transplant for patients with relapsed lymphoma *(103,104)*. The dose-limiting toxicity for RIT is myelosuppression. By utilizing stem cell rescue to overcome this toxicity, the dose of radiation can be increased to the tolerance of the next most sensitive organ. This allows the radiation to be increased from 75 cGy to 25 Gy, an increase in radiation dose of more than 30-fold. This study consisted primarily of patients with low-grade lymphoma. The strategy was to utilize radiation to overcome the presence of any resistance to chemotherapy in previously treated patients. Eighteen of 21 patients (86%) responded, with a 76% CR rate and a 62% 2-year progression-free survival.

A second approach for utilizing RIT as part of stem cell transplants is in combination with chemotherapy. A phase I study of RIT in combination with BEAM (carmustine, etoposide, cytarabine, and melphalan) chemotherapy, was investigated in lymphoma patients refractory to chemotherapy. In this regimen, the RIT is being used to provide an additional, non-cross tolerant mode of therapy. Investigators were able to reach a dose of I-131 tositumomab of 75cGy *(105)*. The 3-year overall survival and event-free survival for this study after a median follow-up of 38 months were 55 and 39%,

respectively. These patients would not have been considered eligible for standard autologous stem cell transplants given the chemorefractory nature of their disease, making any responses noteworthy.

One concern related to RIT is the potential exposure of hematopoietic stem cells in the bone marrow to radiation. As most lymphomas invade the bone marrow, there is significant opportunity for toxicity because of "cross-fire." In the short term, this results in pancytopenia which in most cases, will resolve without complications. Long term, there is concern that the use of RIT will lead to myelodysplastic syndromes (MDS) and secondary acute myeloid leukemia (AML), both of which carry very poor prognoses. Patients with lymphomas who have been heavily pretreated with chemotherapy are at a significant risk for developing MDS or AML. When the data for I-131 tositumomab are analyzed in aggregate, the annualized incidence for the development of either AML or MDS is 1.6% per year *(106)*. This rate is consistent with what would be expected for this group of patients treated only with chemotherapy, suggesting no increase risk of AML or MDS from the addition of RIT. To further support this, when the patients who underwent RIT as initial therapy, and thus had no chemotherapy exposure, were examined, no cases of AML or MDS were identified.

4. CONCLUSIONS

The addition of monoclonal antibodies to the armamentarium available for treatment of lymphomas has dramatically altered the care of lymphoma patients. Monoclonal antibodies represent effective therapies that are typically not cross-resistant with standard cytotoxic chemotherapies and often without the associated toxicities of chemotherapy. This has enabled treatment to be available for many patients who would otherwise be unable to receive treatment because of comorbidities or cytopenias. Additionally, delaying the exposure of patients to chemotherapy through the use of monoclonal antibodies helps patients avoid the accumulation of toxicities that can impair longevity and quality of life.

Monoclonal antibodies have been successfully placed into all stages of treatment algorithms for lymphomas. The first antibodies were limited by the choice of targets determined by the immune response generated by the vaccinated mouse. With molecular genetics and computer modeling of antigens, future antibodies will be able to target more effective epitopes and with higher affinities. These antibodies will also have new radioisotopes and novel toxin conjugates of which to take advantage.

It should also be hoped that as our knowledge regarding cell biology expands, additional cell surface molecules can be identified which can be manipulated by the binding of monoclonal antibodies. These antibodies could activate or inhibit, leading to turning on or off cellular processes to generate desired responses. These responses could include apoptosis, enhanced antigen presentation, cytokine production or inhibition of cytokine production, or any of a number of other possibilities. This would enable physicians to manipulate a patient's own cells as a treatment of cancer.

With the improved tolerability and more targeted approach, monoclonal antibodies offer the potential to make indolent lymphomas, among others, truly chronic diseases. Repetitive dosing without the accumulation of toxicities would allow continued control of a disease until resistance develops. Future research needs to focus on means to circumvent the ability of lymphomas to resist treatment.

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26 Role of Apoptosis in Anti-Angiogenic Cancer Therapies

Jennifer A. Doll PhD, Jennifer E. Hobbs PhD, and Gerald A. Soff MD

Summary

Angiogenesis, the growth of new blood vessels from the existing vasculature, is necessary for normal growth and development and in the adult during wound healing and the reproductive cycles. In most adult tissues, however, the vasculature is maintained in a quiescent state by the balanced presence of both angiogenic inducers and inhibitors in the tissue milieu. For progressive growth and metastasis, cancer cells must shift this balance to favor angiogenic induction. When inducers predominate, vascular endothelial cells (VECs) become activated, proliferating and migrating toward the source of the angiogenic inducer. In the activated VECs, distinct cell signaling pathways are initiated compared with that of quiescent VECs, providing the specificity of anti-angiogenic therapies to the tumor vasculature. VEC apoptosis has been well documented in regressing vessels, and it has been shown that, in addition to activating the VECs, some inducers such as vascular endothelial growth factor also up-regulate Fas expression, thus sensitizing the cell to apoptotic stimuli. Endogenous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1) and pigment epithelium-derived factor (PEDF), stimulate signaling cascades within the VECs and also induce the expression of Fas ligand in activated VECs. Therefore, when inhibitors predominate, the apoptotic cascade is initiated. Depleting the supply of angiogenic inducers can also induce apoptosis, and thus, anti-angiogenic therapies can target the inducer supply or directly target the VECs. Although clinical studies are promising, most to date suggest that anti-angiogenic therapies may prove to be most effective when used in combination with traditional therapies.

Key Words: Angiogenesis; apoptosis; thrombospondin-1; endothelial cell; anti-angiogenic.

1. INTRODUCTION: ANGIOGENESIS IN TUMOR GROWTH

Angiogenesis, the growth of new blood vessels from the existing vasculature, is a normal and necessary process during growth and development. It also occurs in the adult during wound healing and the reproductive cycles. However, in most normal adult

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tissues, the vasculature is maintained in a quiescent state by the balanced presence of both pro-angiogenic and anti-angiogenic molecules in the tissue milieu *(1)*. Vascular endothelial cells (VECs) have a very low turn over time, estimated any where from 1000 days *(2,3)* to 7 years *(4)*. Judah Folkman first proposed in 1971 that tumor growth is dependent on the induction of angiogenesis *(5)*. Prior to angiogenic induction, tumors remain in a dormant stage, not growing beyond a few millimeters in diameter. Folkman and colleagues *(6,7)* demonstrated that this is due to a balance between tumor cell proliferation and apoptotic rates. In this non-angiogenic environment, tumor cell apoptosis is triggered by hypoxia, cell starvation, and a build-up of cellular waste products. The same paradigm applies to micrometastases, which remain dormant until a pro-angiogenic environment arises *(7,8)*. Tumors shift the balance to favor angiogenic induction by increasing expression of angiogenic inducers, decreasing expression of angiogenic inhibitors, or a combination of both *(9)*. These changes in expression result from the same genetic and epigenetic changes that drive tumor progression, such as oncogene expression, loss of tumor suppressor gene expression, and tissue hypoxia *(9,10)*. For example, Bouck and colleagues demonstrated that in fibroblasts progressing toward malignancy, loss of the p53 tumor suppressor gene resulted in decreased expression of thrombospondin-1 (TSP-1), a potent angiogenesis inhibitor, and increased expression of vascular endothelial growth factor (VEGF), a potent angiogenesis inducer, resulting in a pro-angiogenic phenotype *(11,12)*. Figure 1 illustrates this change in angiogenic mediator balance.

The angiogenic balance can also be altered indirectly through tumor cell stimulation of the stromal cells to produce pro-angiogenic factors and/or to decrease inhibitor production *(13)*. A large number of angiogenic inducers and inhibitors have now been identified, and they span a variety of molecule types, including growth factors, cytokines, hormones, and lipids *(9,14)*. Table 1 lists some of the most well-studied endogenous angiogenic regulators.

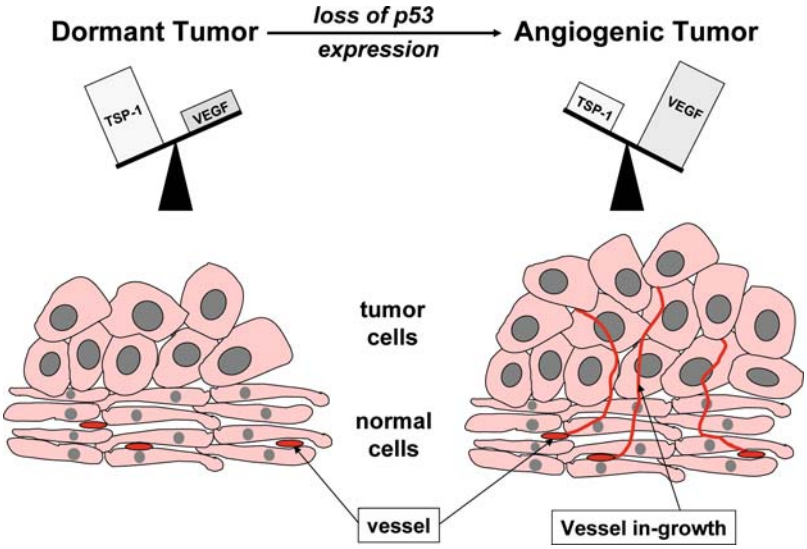

Fig. 1. Shift in balance of angiogenic mediators to favor angiogenic induction in tumors. Model based on data presented in refs. *11,12*.

Table 1
Examples of Endogenous Angiogenic Mediators

Pro-angiogenic factors	Anti-angiogenic factors
Angiogenin	Angiostatin isoforms (fragments of plasminogen)
Epidermal growth factor	Endostatin (fragment of collagen XVIII)
Interleukin-8	Interferon-α
Hepatocyte growth factor	Interleukin-1, interleukin-6, and interleukin-12
Fibroblast growth factor-1 (acidic FGF)	Pigment epithelium-derived factor
Fibroblast growth factor-2 (basic FGF)	Platelet factor-4
Platelet-derived growth factor	Thrombospondin-1
Proliferin	Thrombospondin-2
Prostaglandins E_1 and E_2	Tissue inhibitor of metalloproteinase-2 and metalloproteinase-3
Vascular endothelial growth factor	1,25-Dihydroxy-vitamin D_3

Reviewed in refs. *9,14*.

When a sufficient number of cells develop a pro-angiogenic phenotype, vessels grow into the tumor, supplying nutrients and oxygen and removing wastes. Subsequent to vascularization, the tumor cell apoptotic rate decreases significantly, whereas the proliferative rate persists, resulting in net tumor growth *(7)*. In addition, this neovascularization can also further stimulate tumor growth by providing the tumor with paracrine growth factors secreted by the VECs themselves, such as basic fibroblast growth factor (bFGF), interleukin-6, insulin-like growth factor, and platelet-derived growth factor, and also factors released by macrophages or other blood cells delivered to the tumor through the vasculature *(13,15,16)*. Furthermore, these vessels provide an avenue for tumor cells to metastasize to distant locations. Thus, the transition to a pro-angiogenic phenotype provides significant advantages for tumor growth and metastases.

2. ANGIOGENIC INDUCTION AND THE TUMOR VASCULATURE

The formation of new vessels requires several steps, including extracellular matrix degradation, migration, proliferation, and tube formation. When the balance of angiogenic mediators shifts to favor angiogenic induction, VECs become activated, resulting in changes in gene and protein expression patterns. The specific signal transduction pathways activated will be discussed in detail in Sections 5 and 6 below. Proteases released by the activated VECs degrade the basement membrane, allowing migration toward the angiogenic inducer source. VEC proliferation subsequently ensues, followed by lumen formation *(17)*. In addition, coculture experiments of tumor cells (U87 human glioma cells) with one type of VEC, human umbilical vein endothelial cells, have demonstrated coordinated increases in both expression of specific ligands and their receptors in the endothelial cells in response to the tumor cell secretions, creating autocrine stimulation of the VECs *(18)*, thus further promoting angiogenesis.

The tumor vessels formed are markedly different than the vessels in normal tissues and organs. They are associated with vascular leakage and the presence of inflammatory cells *(17,19)*. In addition, they often do not have associated pericytes and have reduced basement membrane formation, likely contributing to their leakiness *(17)*. Pericytes and

the basement membrane structures are thought to provide maturation signals as well as survival factors and anti-apoptotic signals to the VECs, thus maintaining the quiescent vasculature *(20)*. For example, anchorage of VECs to the basement membrane results in increased expression of focal adhesion kinase (FAK), anti-apoptotic bcl-2, as well as repression of p53 and pro-apoptotic bax *(20,21)*. Furthermore, the tumor vessels are constantly undergoing remodeling and expansion in response to the angiogenic signals produced by the tumor. Thus, the tumor vasculature and the associated activated VECs differ significantly from the normal vasculature and quiescent VECs, histologically and in gene and protein expression profiles. This provides the specificity of anti-angiogenic therapies for the activated VECs associated with the tumor vessels. Achieving a better understanding of these differences, at the histological and cellular and molecular levels, is one of the major hurdles in developing specific and effective therapies targeting the tumor vasculature.

3. TARGETING THE VASCULATURE IN CANCER THERAPY

The advent of anti-angiogenic therapies has added a new option for cancer treatment, supplementing the traditional approaches of surgery, chemotherapy, and radiation therapy. It is important to note that angiogenic therapies (both pro- and anti-angiogenic agents) have potential therapeutic benefits in other diseases as well. The focus here, however, is solely on their role as anti-cancer agents. In principal, anti-angiogenic cancer therapies offer several advantages over the traditional cytotoxic chemotherapies and radiation therapies. First, they are not mutagenic, so secondary tumors are unlikely *(9,22)*. Second, they target the tumor vasculature specifically; therefore, they do not cause the side effects associated with traditional chemotherapies, such as nausea, hair loss, and bone marrow suppression *(9,22)*. Although, this does not preclude the occurrence of other types of side effects, expected or unexpected. Third, they can act synergistically with current chemotherapies, radiation and gene therapies *(23–27)*. Fourth, they can be easily delivered through the circulation to their target cells *(9,22)*. Last, as they do not target the tumor cell, but instead the genetically stable VECs, the tumor cells are unlikely to develop resistance to therapy *(28,29)*. However, in this last advantage also lies a disadvantage. As the VECs, and not the tumor cells, are the target, these therapies do not kill the tumor cells directly and are therefore not curative. With these therapies, theoretically, the tumor would be reduced to avascular size limits. Thus, to overcome this issue, two approaches have been taken. One approach is to view anti-angiogenic therapies as chronic, long-term therapies. Another approach is to combine anti-angiogenic therapies with cytotoxic therapies that do directly target the tumor cell.

Traditionally, chemotherapies are given on a maximum tolerated dose (MTD) schedule over a short period of time with extended breaks between treatments to allow recovery of normal tissues. Most data to date suggest that anti-angiogenic therapies are more effective when administered on a more frequent, or metronomic, low dose schedule *(30)*. Folkman prefers the term "anti-angiogenic schedule" to describe this type of dosing to emphasize that the VECs are the target of the treatment rather than the tumor cells, as metronomic implies only a regular interval of dose delivery; however, the term metronomic is most often used. The MTD schedule appears to allow for recovery of the tumor vessels between treatments and thus does not effectively suppress vessel growth. The continuous presence of the anti-angiogenic agent

prevents vascular re-growth, therefore, suppressing tumor growth more efficiently. For example, continuous delivery of endostatin, an endogenous angiogenesis inhibitor, through osmotic pump implantation had significantly greater anti-tumor affect than the same dose given once a day as a bolus injection, and only the osmotic pump delivery achieved tumor regression *(31)*. As anti-angiogenic agents have fewer side effects in general, especially when used on lower, metronomic dosing schedules, it is feasible that anti-angiogenic agents could be developed as long-term, chronic therapies rather than acute therapies, such as done for the treatment of high blood pressure or high cholesterol.

In support of this hypothesis, some chemotherapeutic drugs have been found to have anti-angiogenic activity, when used on metronomic dosing schedules. Kerbel and colleagues *(32)* called such chemotherapeutic drugs "accidental" anti-angiogenic drugs. For example, vinblastine and cyclophosphamide (CPP) were more efficacious when administered on a low, metronomic dose schedule as compared with a MTD schedule in mouse tumor models with significantly diminished toxicity, although complete tumor regression was not achieved *(33–35)*. It was later determined that the effect of CPP, with metronomic dosing, was due to increased TSP-1 expression *(36)*. TSP-1 is known to induce VECs apoptosis *(37)*, as will be discussed in detail in Section 6 below. Subsequently, other small molecule drugs have been shown to induce expression of anti-angiogenic proteins, including celecoxib, doxycycline, and rosiglitazone *(38)*. In addition, many more have been shown to have anti-angiogenic activity, including thalidomide, tamoxifen, captopril, D-penicillamine, *N*-acetylcysteine, and dexamethasone; however, the mechanisms of these are less clear *(39)*. Using chemotherapeutic agents on metronomic dosing schedules offers several advantages over traditional dosing schedules in that side effects are greatly reduced and tumor resistance is less likely. Although many of these agents are very effective in eradicating tumors in mice, whether or not the approach of using chemotherapeutic agents with anti-angiogenic activity or pure anti-angiogenic agents on metronomic dosing schedules is effective in cancer patients is still being evaluated.

Anti-angiogenic agents have been in clinical trials for many years. To date, however, many such agents, when used alone, even on metronomic scheduling, have not shown significant efficacy in the majority of patients or take an extended period of time to achieve significant tumor regression, even up to 1 year or more *(40)*. In animal models, even the chemotherapies used on metronomic schedules do not appear to achieve complete tumor regression when used as single agents. However, when these treatments are combined with a second agent, metronomic chemotherapies do achieve significant or complete tumor regression. Teicher and colleagues *(23–26)* were the first to pioneer this combined treatment modality and produced several convincing studies demonstrating synergy between various anti-angiogenic therapies and various cytotoxic therapies in mouse models. Her first study, reported in 1992, demonstrated synergy in treatments combining an anti-angiogenic steroid with one of several different chemotherapies or radiation therapy *(26)*. Studies by other researchers have supported her work. One caveat, however, is that when anti-angiogenic agents were used with chemotherapies on a MTD schedule, the combination produced some serious side effects *(41,42)*. Therefore, combining anti-angiogenic therapies with chemotherapies that have demonstrated anti-angiogenic function and efficacy on a low dose, metronomic schedule, rather than a MTD schedule, may prove to be the safest and most

effective approach. Supporting this approach, in a neuroblastoma model, the combination of vinblastine, on a low, metronomic dosing schedule, with DC101, an antibody against the VEGF receptor, was more effective in achieving tumor regression than either agents alone *(35)*. Similarly, metronomic CPP treatment was more effective against CPP-resistant lung and breast tumor models when TNP-470, an anti-angiogenic agent, was added to the CPP treatment, with the combined treatment completely eradicating the tumors *(43)*. In addition, we and others have shown that treatment with an angiostatin isoform, angiostatin4.5, is synergistic with radiation therapy *(44,45)* and chemotherapies *(46,47)* in achieving tumor regression in mouse models. Based on these animal studies, it appears that combining anti-angiogenic agents with traditional cytotoxic therapies may be the most promising treatment avenue, and this is being actively pursued in clinical trials, as discussed in Section 4 below.

4. EVIDENCE FOR ENDOTHELIAL CELL APOPTOSIS AS A PRIMARY EVENT IN ANTI-ANGIOGENIC THERAPY

To inhibit angiogenesis, an anti-angiogenic agent could target the VECs at any of the steps necessary for neovascularization, such as extracellular matrix degradation, migration, proliferation, or tube formation. For example, platelet factor-4 inhibits endothelial cell proliferation *(48)*, whereas tissue inhibitor of matrix metalloproteinases-2 inhibits extracellular matrix degradation, tube formation, and VEC proliferation *(49)*. However, in many tissues that have active tissue remodeling, such as in reproductive tissue cycles, wound healing and tumor growth, VEC apoptosis can be readily detected. This is in contrast to normal tissues where the VECs are normally long lived, with very low proliferation and apoptotic rates. Thus, it seems likely that both proliferation and apoptosis of VECs are necessary for active remodeling of the vasculature. Therefore, it is reasonable that an angiogenesis inhibitor functions by actively promoting apoptosis of VECs. *In vitro* studies have shown that many endogenous angiogenesis inhibitors do indeed induce apoptosis, including TSP-1 *(37)*, pigment epithelium-derived factor (PEDF) *(50)*, prolactin *(51)*, angiostatin isoforms *(52,53)*, endostatin *(54)*, tumstatin *(55)*, and canstatin *(56)*. Other anti-angiogenic agents and/or chemotherapeutic agents have also been shown to induce VEC apoptosis, such as 2-methoxyestradiol *(57)*, and doxorubicin *(58)*.

Many studies have demonstrated VEC apoptosis *in vivo* using animal tumor models, and in the prostate, in particular, the vasculature has been intensely studied for its involvement in prostatic growth regulation. In castrated rats, which have an atrophied prostate, testosterone treatment increases VEGF expression and stimulates angiogenesis prior to prostatic re-growth, and androgen ablation therapy also appears to target the vasculature *(59,60)*. In both the normal prostate and prostate tumor models, androgen ablation therapy increases VEC apoptosis, reduces blood flow, and results in vascular regression, and the VEC apoptosis precedes the epithelial cell apoptosis and prostate tissue regression *(61,62)*. In both rodent and human prostate tissues, in response to androgen ablation therapy in the normal and tumor epithelial cells as well as stromal cells, VEGF expression is decreased *(63,64)* and the expression of the angiogenesis inhibitors PEDF *(65)* and TSP-1 is increased (J. A. Doll and S. E. Crawford, unpublished observation). The changes in expression of VEGF, TSP-1, and PEDF, in response to androgen withdrawal, likely shift the angiogenic mediator balance thus triggering

VEC apoptosis and vascular regression. These studies support a critical role for angiogenic regulation in normal and malignant prostate growth and emphasize the role of VEC apoptosis in growth regulation.

In animal tumor models, VEC apoptosis has also been documented following treatments with anti-angiogenic agents. For example, in subcutaneous prostate tumors in nude mice, we have shown that VEC apoptosis occurs following PEDF treatment by coimmunostaining tumor tissue for factor VIII-related antigen, an endothelial cell marker, and TUNEL *(65)*. Browder and colleagues *(33)* showed that CPP, when used on a low dose metronomic schedule, induced VEC apoptosis *in vivo* in a neuroblastoma tumor model. The authors further demonstrate that the VEC apoptosis precedes the tumor cell apoptosis by four days *(33)*. These observations and additional studies showing that VEC apoptosis precedes tumor cell apoptosis, following anti-angiogenic therapy supports a key role for VEC apoptosis in the anti-tumor activity of anti-angiogenic cancer therapies.

5. THE ACTIVATED VEC: PRIMED FOR APOPTOSIS

It is well established that endothelial cell survival depends on the balance of both stimulatory and inhibitory signals in the extracellular milieu. When a predominance of inducers is present, the VECs become activated, with subsequent changes in gene and protein expression profiles. There are many angiogenic inducers now known (Table 1); however, of these, VEGF seems to be up-regulated in a wide range of tumor types and thus has been the most intensely studied in tumor biology *(21,66)*. Therefore, here we will focus on the pathways stimulated by VEGF in the VECs. It is important to note, however, that pathways stimulated by other growth factors and molecules overlap with those stimulated by VEGF. VEGF is a member of the VEGF family of related proteins. It is the original member and is denoted as VEGF-A or simply VEGF. It is produced as several isoforms resulting from alternative splicing, with the most abundant ones being $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. Of these, $VEGF_{165}$ is the most prevalent isoform and is over-expressed in many tumor types *(21,66)*.

There are several VEGF receptors on VECs, including receptors 1 and 2 (VEGFR-1 and VEGFR-2) and neuropilin-1. VEGFR-1 (also called flt-1) primarily plays a regulatory role in VECs, as a "decoy" receptor, rather than a signal transduction role *(67,68)*. Neuropilin-1 has signaling activity in other cell types; however, in VECs, intracellular signaling has not been demonstrated, but it may function to stabilize the VEGF/VEGFR-2 complex *(21,69)*. VEGFR-2 (also called flk-1 or Kinase insert domain receptor (KDR)) is expressed on the surface of most VECs and is primarily responsible for VEGF signal transduction. It is a transmembrane protein with seven immunoglobulin-like extracellular domains, a single transmembrane region and an intracellular tyrosine kinase domain, interrupted by a kinase insert domain *(21)*. VEGF binding to VEGFR-2 stimulates receptor dimerization and auto-phosphorylation of tyrosine residues in the cytoplasmic tail, which then triggers the phosphorylation of SH2-domain containing proteins *(69,70)*. This initiates multiple signaling cascades within the VECs ultimately supporting survival, mitogenesis, migration, and tube formation *(66)*. Cascades leading to vascular permeability are also initiated but will not be discussed here.

Survival and proliferation cascades are initiated through the phophatidyinositol-3 kinase (PI3K)/Akt pathway and Ras pathway, whereas migration is initiated through

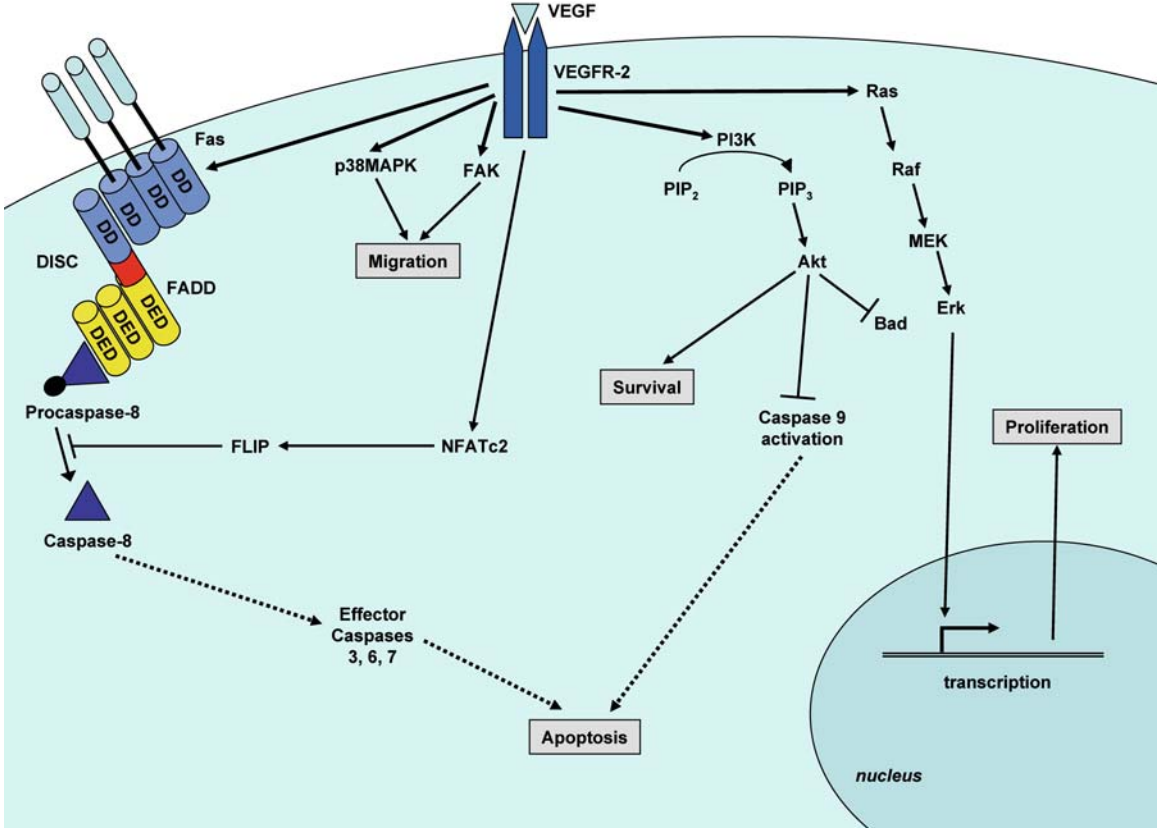

Fig. 2. Model of vascular endothelial growth factor (VEGF)-initiated signaling pathways in vascular endothelial cells (VECs). Solid arrows indicate pathways activated by VEGF signaling. Dashed arrows indicate pathways that become inactivated. Many of the pathways depicted involve additional factors that are not illustrated here for simplicity. Abbreviations not in text are as follows: DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; and DISC, death-inducing signaling complex. See text for references.

the p38 mitogen-activated protein kinase (p38MAPK) and FAK pathways *(66,69)*. VEGF also directly inhibits apoptosis through induction of expression of anti-apoptotic proteins such as bcl-2, survivin and A1, and repression of expression of pro-apoptotic proteins such as Bad, Bax, and caspase-9 *(70,71)*. In addition, through the PI3K/Akt pathway, VEGF also stimulates expression of the anti-apoptotic Flice-like inhibitor protein (FLIP) *(72)*. FLIP inhibits the activation of procaspase-8, and cells expressing elevated levels of FLIP are resistant to Fas-mediated apoptosis. Several studies have investigated the regulation of FLIP expression. One study demonstrated that elevated FLIP levels resulted from inactivation of the FOXO3a transcription factor, which suppresses FLIP expression; however, this study used serum stimulation of the VECs *(73)*. Another study showed that VEGF treatment activated NFATc2, another transcription factor *(74)*. NFATc2 has been shown to induce FLIP expression in VECs *(75)*. These VEGF-stimulated signaling cascades ultimately result in VEC survival and the necessary steps for angiogenesis, including degradation of the extracellular matrix, VEC proliferation, migration, and tube formation. Figure 2 presents a basic schematic of the signaling cascades initiated by VEGF binding to VEGFR-2 on the VECs.

Interestingly, in addition to the pro-angiogenic/pro-survival cascades initiated, VEGF also induces expression of a protein that primes the pro-apoptotic cascades. VEC treatment with VEGF results in increased localization of the Fas (CD95) protein, a death receptor, to the cell surface *(72,76)*. This simultaneous stimulation of pro-survival pathways and the priming of apoptotic pathways poise the activated VECs to be extremely sensitive to apoptotic signals. In addition, as discussed in Section 2 above, the tumor vasculature, which lacks associated pericytes and basement membrane structure, does not receive the normal pro-survival signals provided by these structures *(20)*. Quiescent vessels, (with intact pericytes, basement membrane structures and that are not activated by VEGF) do not express Fas at high levels. Therefore, the quiescent VECs are resistant to the effects of circulating angiogenesis inhibitors, whereas the activated VECs are sensitized to respond to apoptotic stimuli. Therefore, if the balance shifts such that angiogenesis inhibitors predominate within the tumor microenvironment, apoptosis will be initiated in the activated VECs. The differences between quiescent and activated VECs provide the specificity that anti-angiogenic therapies have for activated VECs associated with the tumor vasculature.

6. APOPTOTIC INDUCTION IN ACTIVATED VECs

VEC apoptosis is essential during normal development and in the adult during phases of reproductive cycles and wound healing. As in other cell types, VEC apoptosis can occur through either the extrinsic (death receptor) or the intrinsic (mitochondrial) pathway. In addition to signals received by these pathways, Duval and colleagues *(20)* delineated four other essential signaling pathways that can regulate the fate of VECs, including paracrine, autocrine and endocrine factors (i.e. survival factor withdrawal), cell adhesion, blood flow and sheer stress, and the transcriptome and glycome. These pathways reflect the complex environment of the VECs. In respect to endogenous anti-angiogenic agents, the most studied of these pathways are the death receptor and mitochondrial-associated signaling pathways. For many endogenous inhibitors, including angiostatin, endostatin, TSP-1, and PEDF, caspase activation is necessary

for induction of VEC apoptosis *(76)*. Of these, TSP-1 and PEDF have been the most intensely studied, with intracellular pathways more defined; therefore, these will be the primary focus here.

TSP-1 binds to the CD36 receptor on the VEC surface *(77,78)*. PEDF also binds to the VEC surface; however, the surface receptor has not yet been identified. Following receptor binding, both TSP-1 and PEDF treatment stimulates an increase in Fas ligand (FasL/CD95 ligand) expression *(76)*. For a TSP-1 peptide, called DI-TSP, this up-regulation has also been shown *in vivo* in a mouse tumor model *(79)*. As VEGF stimulates Fas expression, the activated VEC is primed to respond to the FasL. PEDF treatment has also been shown to decrease FLIP expression through PEDF-stimulated inhibition of NFATc2 *(75)*. FLIP, as discussed in Section 5 above, inhibits activation of procaspase-8; therefore, decreases in FLIP are permissive to apoptosis. The PEDF-induced FLIP repression is dependent on c-Jun NH_2-terminal kinase (JNK) activity as a JNK inhibitor blocks the FLIP repression *(75)*. This repression is via JNK-1/2 stimulating NFATc2 nuclear export and JNK-2 retaining NFATc2 in the cytoplasm *(75)*. Decreased FLIP activity, as well as activation of Fas by the FasL, results in activated caspase-8 and initiation of the apoptotic cascade. These data, and the VEGF data presented above, suggest that the levels of Fas/FasL and FLIP may represent two check points in VECs that determine survival or apoptosis. Figure 3 illustrates how these two inhibitors affect the signaling pathways in activated VECs, as is currently understood.

Among the other endogenous angiogenesis inhibitors, the mechanism of action of angiostatin has also been intensely studied. However, its biology is more complex as angiostatin, a kringle-containing fragment of plasminogen, exists as several isoforms. Plasminogen has five kringle domains, and the angiostatin isoforms, generated by proteolysis, vary in their kringle domain content. Angiostatin isoforms can inhibit VEC migration, tube formation, and proliferation *(80–84)* and can also induce apoptosis of VECs *(52,53)*. And, for the angiostatin isoform containing kringles 1–3, caspase activation is necessary for the anti-angiogenic activity *(76)*. Using angiostatin4.5 (AS4.5), an angiostatin isoform containing kringles 1–4 and 85% of kringle 5, we and others identified specific activation of caspase-8, caspase-9 and caspase-3, which is necessary for apoptotic induction *(85,86)*. This suggests that both the intrinsic and extrinsic apoptotic pathways are activated by AS4.5 and may explain why AS4.5 is one of the most potent angiostatin isoforms and angiogenesis inhibitors identified to date. Chen and colleagues showed that treatment of VECs with an angiostatin isoform containing kringles 1–4 induced p53-mediated, Bax-mediated and t-Bid-mediated release of cytochrome c into the cytosol, thus initiating the intrinsic (mitochondrial) apoptotic pathway *(87)*. This treatment also up-regulated FasL mRNA, decreased FLIP, and activated caspase-3, thus also initiating the extrinsic apoptotic pathway *(87)*.

Another study using an angiostatin isoform containing kringles 1–3 showed down-regulation of cdk5, a cell cycle protein that is up-regulated in response to bFGF treatment *(88)*. In a study using just the kringle 5 domain, Gao et al. *(89)* showed that treatment of human retinal capillary endothelial cells down-regulated VEGF expression and up-regulated PEDF expression, thus tipping the autocrine loop toward apoptosis. This held even under hypoxic conditions *(89)*, which normally increases VEGF *(66)* and decreases PEDF *(65)*. These studies indicate that angiostatin isoforms may have multiple effects on VECs or that the different isoforms inhibit angiogenesis by different mechanisms. The latter seems more likely as one study found that kringle 5 treatment

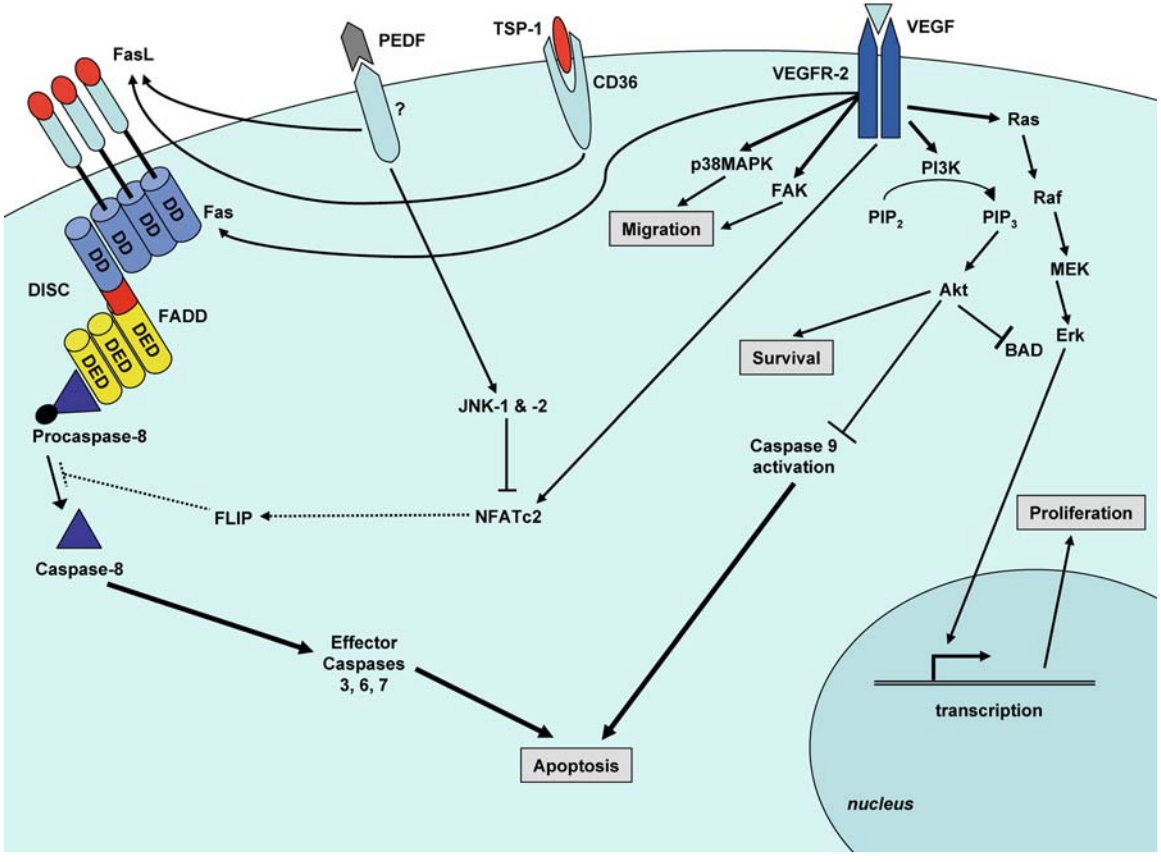

Fig. 3. Model of thrombospondin-1 (TSP-1)-induced and pigment epithelium-derived factor (PEDF)-induced vascular endothelial cell (VEC) apoptosis. Solid arrows indicate pathways stimulated by VEGF, TSP-1, or PEDF. Dashed arrows indicate pathways that become inactivated. Many of the pathways depicted involve additional factors that are not illustrated here for simplicity. Abbreviations not in text are as follows: DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; and DISC, death-inducing signaling complex. See text for references.

acted synergistically with AS4.5 treatment *(90)*. Thus, by elucidating the mechanism of action of the various angiogenesis inhibitors, we could more effectively design anti-angiogenic combination therapies, combining inhibitors with different mechanisms of action, therefore, potentially enhancing anti-angiogenic and anti-tumor activity.

7. CLINICAL TRIALS: DIRECT AND INDIRECT ANTI-ANGIOGENIC AGENTS

Many of the molecules regulating angiogenesis have now been identified, and we have begun to identify the unique properties of the tumor vasculature which distinguishes it from the normal vessels. As our basic understanding of tumor angiogenesis progresses, so does our ability to develop effective anti-angiogenic agents clinically, and a large number of anti-angiogenic agents are currently in clinical trials, as monotherapies and in combination with other anti-angiogenic or anti-cancer therapies. These agents vary in their molecular and cellular target(s) and are classified in different ways. The system used here classifies them as direct and indirect agents *(40,91)*. As a comprehensive review of all of these agents is beyond the scope of this review, more detailed information can be found in the reviews cited herein as well as on the NCI clinical trials website (http://www.cancer.gov).

7.1. Direct Angiogenesis Inhibitors

Direct angiogenesis inhibitors act to prevent the VECs from responding to pro-angiogenic stimuli or to directly induce VEC apoptosis. To prevent the VECs from responding to pro-angiogenic molecules, several different approaches have been taken, including sequestering the inducer in the tumor microenvironment, inhibition of binding of the inducer to its cognate receptor on the VECs, and inhibition of receptor signaling. Sequestration of inducers has been achieved using soluble receptors (decoy receptors) and inducer-binding antibodies. As VEGF is a potent angiogenic inducer and has been shown to be over expressed in many tumor types, it is the target for many agents of this type. Bevacuzimab (Avastin; Genentech, South San Francisco, CA, USA), a humanized anti-VEGF antibody, has recently gained FDA approval for treatment of some types of cancer. Bevacuzimab was first approved for use in colorectal cancer, and a recent phase III study combining Bevacuzimab with cytotoxic therapy showed improved survival in patients with metastatic colorectal cancer *(92,93)*. Additional studies in non-small-cell lung cancer and metastatic breast cancer are in progress *(94,95)*. The VEGF-trap (Regeneron, Tarrytown, NY, USA), also in clinical trials, is a soluble VEGF decoy receptor. It binds to VEGF, preventing binding to the VECs, and it is reported to have higher VEGF binding affinity than the monoclonal VEGF antibodies *(96)*. Many additional drugs targeting VEGF are also in development.

One theoretical disadvantage of many first generation agents of this class is that they target a single angiogenic inducer. Many tumors secrete multiple inducers, and cells within a given tumor can produce different angiogenic inducers; therefore, inhibiting one inducer may only select for the sub-population of tumor cells expressing different inducers or may not be effective against tumors that secrete multiple angiogenic inducers. Using these drugs in combination with other types of angiogenic inhibitors or with low dose (metronomic) chemotherapy, as do the Bevacuzimab studies mentioned above, should overcome this limitation *(30,33,35)*. Developing agents that inhibit multiple inducers

would also compensate for this shortcoming, as do some of the indirect agents, as discussed in Section 7.2 below. A more direct approach would be to develop agents that directly induce apoptosis of VECs as do many of the endogenous inhibitors identified thus far, including endostatin, angiostatin isoforms, TSP-1 and PEDF, as discussed in detail in Section 6 above. An advantage of this approach is that it would be effective against the activity of multiple inducers and thus multiple tumor types. Although the clinical development of these agents has lagged behind anti-inducer therapies because of the technical limitations of developing recombinant proteins or small molecule mimetics, some progress has been made. Of the endogenous inhibitors, a peptide of TSP-1, ABT-510 (Abbott Laboratories, Abbott Park, IL, USA), endostatin, and angiostatin isoforms have been tested in clinical trials. In addition, a trial of a drug cocktail to produce angiostatin4.5 *in vivo* from a patient's own plasminogen is also in progress *(97)*.

7.2. Indirect Angiogenesis Inhibitors

Indirect angiogenesis inhibitors target tumor cells to block the expression and/or secretion of pro-angiogenic proteins that stimulate tumor-associated VECs or to increase expression of angiogenesis inhibitors. These agents appear to hold much promise as they can potentially act to change the expression pattern of multiple angiogenic mediators. One of the most thoroughly studied of this class of inhibitors is Iressa (Gefitinib/ZD1839; Astrazeneca, Wilmington, DE, USA). Iressa is a small molecule inhibitor that targets the tyrosine kinase activity of the epidermal growth factor receptor (EGFR), a member of the ErbB family of cell surface receptors. Although this drug, as many in its class, was developed to directly inhibit the tumor cell, it has also been found to inhibit angiogenesis as blocking this signaling pathway inhibits tumor cell synthesis of pro-angiogenic VEGF, bFGF, transforming growth factor-α, and interleukin-8 *(98,99)*. This decrease in angiogenic inducer expression would result in a shift to an anti-angiogenic environment. Erbitux (IMC225; Imclone, New York, NY, USA), Tarceva (Erlotinib/OSI774; OSI Pharmaceuticals, Melville, NY, USA), and several other agents in clinical development also target the EGFR *(40)*.

Many indirect agents currently in development target several signaling pathways and thus are sometimes labeled multiple target agents. For example, SU11248 (Pfize, New York, NY, USA) inhibits the signaling activity of both the VEGF and PDGF receptors, and SU6668 (Pfizer, New York, NY, USA) targets these two receptors as well as the bFGF receptor. The advantage of this type of agent is that it may be effective against tumors with different types of mutations. In addition, some of these indirect agents can also act on the tumor-associated VECs if the targeted receptor is expressed in the VECs. Thus, the drug could potentially have a greater effect. For example, when VEGF binds to the VEGFR on the VECs, receptor signaling stimulates the release and/or production of several growth factors, which contributes to the pro-angiogenic stimuli present in the tumor microenvironment. If the receptor signaling is inhibited in the VECs, this autocrine loop will be shut off, further inhibiting angiogenesis.

8. CONCLUSIONS AND FUTURE POSSIBILITIES

Folkman first began championing his hypothesis that tumor growth was dependent on angiogenesis in 1971, and with the FDA approval of Avastin in 2004, anti-angiogenic therapies are finally a proven tool in the treatment of cancer. Through this long journey,

many of the mediators of this process have been identified and their mechanisms of action on the VECs elucidated. As our understanding evolves, so will our ability to design more effective anti-angiogenic agents. One of the challenges in trials of anti-angiogenic therapies has been in appropriate evaluation of efficacy at each stage of the clinical trials. With traditional therapies, phase I trials have focused on determining the MTD. However, as we have learned thus far with anti-angiogenic therapies, the MTD is not the optimal means of delivering these agents. Thus, we have had to re-evaluate the means by which we judge the efficacy of these drugs in our search for the optimal biologic dose. Looking for tumor mass or tumor marker responses, while appropriate in phase II and III trials, is not a fair measure in phase I dose determining studies, and evaluating microvessel density or angiogenic mediator expression in tumor biopsies is not practical. Urine and serum markers as well as accumulation of angiogenic mediators in platelets are being intensely studied in the hopes of identifying markers that are predictive of efficacy of anti-angiogenic agents. Identification of such cancer biomarkers will not only aid in the clinical development of anti-angiogenic agents and other novel drug therapies but also may provide additional means by which to detect cancer before it becomes symptomatic.

Another shift occurring in the treatment of cancer is in the design of therapies. Historically, cancer treatments were based on the tissue of origin of the tumor, with all patients with the same tumor type receiving the same therapy. Later treatments considered the histology of the tumor, pathological grade, and stage classifications. Around the turn of the century came a significant change to the traditional approaches. The successful development of Herceptin, an EGFR tyrosine kinase inhibitor, for therapy against ErbB2-positive breast cancer, gave birth to a new era in cancer drug design, tailoring therapies to the genetic profile of a given patient's tumor. Drugs that specifically target a signaling pathway known to be mutated in cancers are also called molecular targeting agents. Using genomic and/or proteomic screening, tumors could be profiled for genetic mutations and protein expression patterns, thus identifying the specific changes in angiogenic mediator expression. With this information, a tumor could be effectively treated with agents specifically targeting the identified culprits. Such designer therapies are the future of all cancer therapies and should prove to be much more effective than traditional approaches.

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27 Photodynamic Therapy-Induced Apoptosis

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Summary

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-malignant conditions, which employs a photosensitive drug followed by light in the visible range to produce an oxidative stress and cell death in the targeted tissue. The photosensitizers (PSs) are most commonly porphyrins or related hydrophobic macrocycles that localize in one or more intracellular membranes and that are activated by long-wavelength (red) visible light. Singlet molecular oxygen and other reactive oxygen species are the primary damaging species produced by PDT, and these oxidize cellular substrates, including lipids and proteins of the membranes in which the PS resides. PDT is an efficient inducer of apoptosis; this has been demonstrated in many cell types and with many different PSs. Once induced, apoptosis follows recognized paths but most commonly the intrinsic (mitochondrial) pathway, mediated by activated caspases and resulting in DNA fragmentation and morphological apoptosis. The aspects of apoptosis that are unique to PDT are the molecular targets, the types of initial cellular damage, and the immediate consequences of that damage. Many PSs target mitochondria, resulting in changes in the permeability transition pore complex, the apoptotic proteins Bcl-2 and Bcl-xL, and/or phospholipids, especially cardiolipin. Some PSs also target the endoplasmic reticulum (ER), damaging calcium pumps and resulting in the efflux of stored calcium into the cytosol and subsequently mitochondria. With PSs that localize in lysosomes, photoactivation damages the lysosomal membrane, causing release of cathepsins and other factors that can activate apoptosis mediators such as Bid that in turn promote mitochondrial apoptosis. Although the plasma membrane is not commonly a direct target of most PSs, it can be secondarily affected by the release of ligands of membrane receptors, such as Fas ligand. Evidence supporting the roles of each of the organelles in PDT-induced apoptosis are reviewed, and a model is proposed in which mitochondrial damage by PDT directly activates apoptosis and damage to the ER and/or the lysosome promotes the central mitochondrial pathway of apoptosis.

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1. PHOTODYNAMIC THERAPY: AN INTRODUCTION

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-malignant conditions, which employs a photosensitive drug followed by light in the visible range to produce an oxidative stress and cell death in the targeted tissue *(1–4)*. The US Food and Drug Administration (FDA) has approved PDT with the photosensitizer (PS) Photofrin® for treatment of advanced esophageal and early and late lung cancers *(5)*. Approvals for several other cancers exist in other countries, and clinical trials for bladder, brain, skin, head-and-neck, gastrointestinal, genitourinary, and other cancers are ongoing *(5)*. PDT is also FDA approved for the treatment of certain non-cancerous conditions: age-related macular degeneration using the PS Verteporfin®, actinic keratosis using Levulan®, and Barrett's esophagus using Photofrin® *(5)*. Numerous other PSs are being tested in pre-clinical models and in clinical trials *(5)*.

PDT is a three-component treatment *(1–3)*. The *PS* is delivered to the patient or animal (intravenously or topically) or cell culture (by addition to the culture medium), and after a suitable time to allow for accumulation of the dye in the desired compartment, the tissue or cell culture is exposed to non-thermal *visible light* of a wavelength absorbed by the PS. The third component of PDT is *molecular oxygen*. Upon absorption of a photon, the PS is activated to an excited triplet state (Fig. 1). In what is known as type II photochemistry, the triplet transfers energy to ground-state molecular oxygen ($^3O_2$), generating singlet oxygen ($^1O_2$), a non-radical but highly reactive form of oxygen thought to be the major cytotoxic mediator of PDT *(6,7)*. The ground-state PS is regenerated and is then available to accept another photon. In type I photochemistry, the triplet PS (^{3}PS) can also undergo electron transfer (i.e., an oxidation–reduction reaction) with nearby cellular constituents, generating free radicals *(6,7)*.

One of the advantages of PDT relative to other cancer therapies is the extreme specificity it offers in the localization of the photodynamic effect; most PSs accumulate somewhat preferentially in malignant or other abnormal tissue in comparison to the surrounding normal tissues, for reasons that still remain largely obscure, and the light can be precisely focused onto a selected region. Furthermore, because of the strong oxidative stress produced, the PDT response is unhindered by the usual modes of resistance to conventional cancer treatments. In addition, it can be used in combination with conventional treatments. And finally, but perhaps most importantly, both the PS and the light are inert by themselves, precluding most types of systemic toxicities *(1–3)*.

Limitations of PDT also exist. Despite attempts to elicit an immune or vaccine response with PDT *(8)*, at present it remains a local treatment. The PSs can distribute in a tumor unevenly, allowing some regions to escape photodynamic damage. The PSs can remain in the skin for up to several weeks, making the patient sensitive to sunlight; in practice, this is a problem only for Photofrin®. Finally, the penetration of photoactivating light through human tissue increases with wavelength and is most efficient for red and infrared light. The energy of the photon decreases with increasing wavelength, such that above approximately 800–850 nm, formation of the triplet PS state is inefficient. These factors confine the practical wavelength range for PDT to

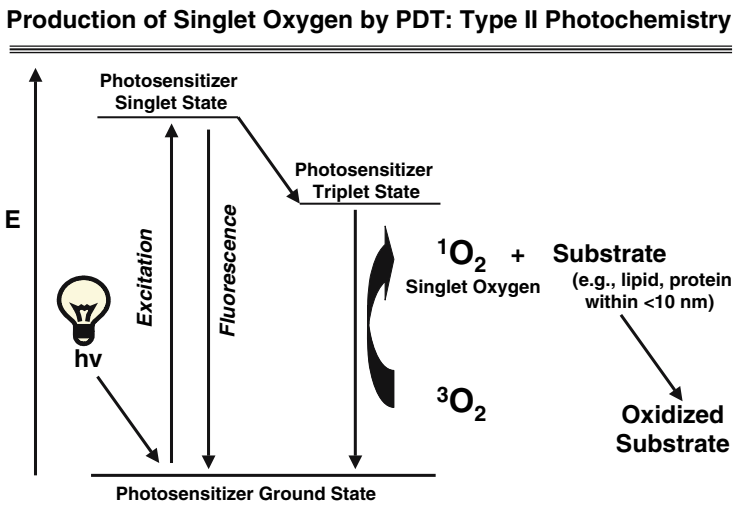

Fig. 1. A simplified energy diagram showing the conversion of light energy to cellular oxidative stress. The ground-state photosensitizer absorbs a photon and is energized to an excited singlet state, from which it can release energy as fluorescence or undergo intersystem crossing to the triplet state. The triplet photosensitizer transfers its energy to ground-state molecular oxygen (a triplet) to generate singlet molecular oxygen and the ground-state photosensitizer. Singlet oxygen reacts with nearby cellular targets. Because in cells, the photosensitizers are membrane localized, singlet oxygen formed in the membranes will oxidize unsaturated membrane lipids and proteins.

approximately 600–800 nm. Because Photofrin® and other simple porphyrins absorb poorly in this region ($\varepsilon \sim 5000$, $\lambda_{max} \sim 630$ nm), many newer PSs have been developed with higher extinction coefficients at wavelengths above 650 nm *(3,6,9,10)*.

In both animals and human patients, PDT can result in a complete tumor response within a few days. Three types of mechanisms are reported to contribute to the rapid PDT response *in vivo (1–3,6)*. First, PDT can directly damage and kill the malignant cells of the tumor, by either apoptosis or a non-apoptotic mechanism. Second, PDT may produce profound changes in the tumor vasculature, including blood flow stasis, vascular collapse, and/or vascular leakage, that can result in indirect killing of the malignant cells. And third, PDT can promote release of cytokines and other inflammatory mediators from treated cells that induce an inflammatory response and recruit additional host cells to the tumor. The contribution of each mechanism to the overall tumor response depends upon the PS, the tumor, and the treatment parameters [e.g., the dose of PS and the amount (fluence) of light]. One critical parameter is the fluence rate, that is, the rate at which photons impinge on the tissue. It has long been known that the delivery of oxygen *in vivo* to the treatment site can be a limiting factor in PDT. Molecular oxygen is consumed in the photodynamic process to generate singlet oxygen, which is further fixed in oxidized substrates. High light fluence rates can deplete the targeted tissue of oxygen, limiting the impact of further photoirradiation. Slowing the rate of light delivery can spare the available oxygen, reduce vascular blockage allowing more oxygen to be delivered through the circulation, and produce a greater overall response (more tumor cures) at a lower total light dose. Lower fluence rate irradiation also produces a higher level of apoptosis in the treated tumors *(11)*.

2. CHEMICAL AND BIOCHEMICAL PROPERTIES OF THE PHOTOSENSITIZERS USED IN PDT

Most PSs are porphyrins or related macrocycles, such as benzoporphyrins, chlorins, pheophorbides, purpurins, or phthalocyanines, whose hydrophobic ring systems tend to localize them to one or more intracellular membrane systems *(3,4,6,9)* (Fig. 2). Many analogs of the parental macrocycles have been produced, with varying hydrophilicity/hydrophobicity, pharmacokinetics, and affinity for cellular-binding and tissue-binding sites, through modifications of the ring systems and/or addition of substituents either on or axial to the ring *(3,9)*. Some PSs are not porphyrins; for example, hypericin, a PS derived from St. John's Wort, which is under investigation in Europe and Singapore *(10)*. Structures for a selection of the more widely studied PSs can be found in Fig. 2. Finally, some PDT protocols employ the heme biosynthetic precursor 5-aminolevulinic acid (ALA) to generate the natural PS protoporphyrin IX (PPIX), the penultimate intermediate of the heme biosynthetic pathway *(12,13)*. Upon administration of ALA to cells or patients (either systemically, orally, or topically), PPIX is generated over the first 2–4 h, reaches a maximum level, and then is lost, either by metabolic conversion to heme through the introduction of iron catalyzed by the enzyme ferrochelatase or by diffusion out of the cell. Although PPIX remains at the site of its formation in the mitochondria, it is an efficient PS and can also be used for fluorescence detection of tumors, which are often more efficient in the generation of

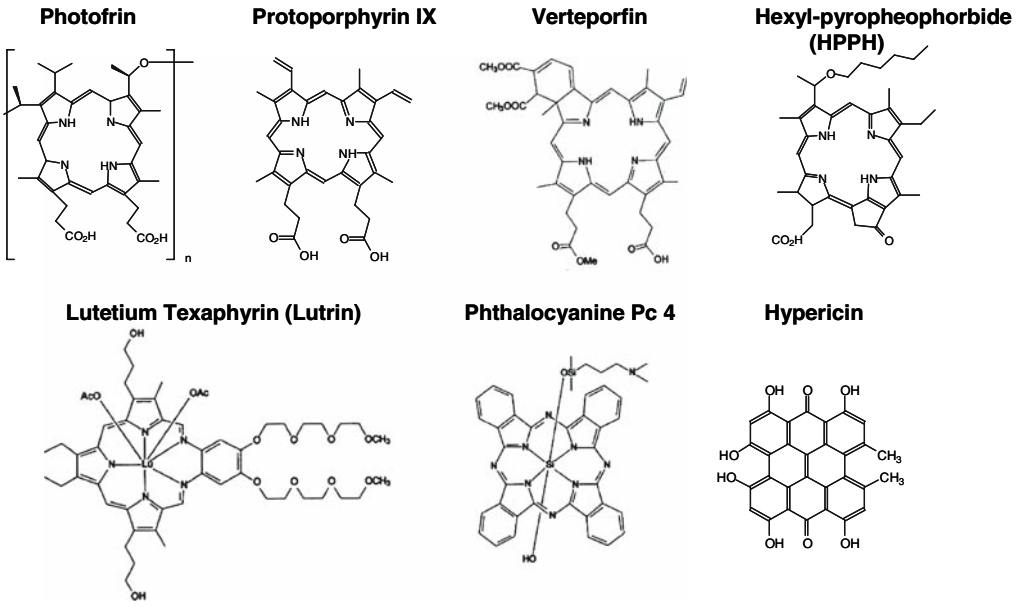

Fig. 2. Examples of photosensitizers used for photodynamic therapy. Photofrin is the first-generation photosensitizer now approved in the USA for treatment of lung and esophageal cancer and Barrett's esophagus and for numerous indications worldwide. As Photofrin is a complex mixture of porphyrin oligomers, a monomeric unit is shown. Protoporphyrin IX is the natural photosensitizer metabolically synthesized from exogenously applied 5-aminolevulinic acid or its esters. Verteporfin is Food and Drug Administration-approved for the treatment of age-related macular degeneration. All of the other photosensitizers shown here are in clinical trials.

PPIX than are normal tissues *(12,13)*. Various esters of ALA have also been employed to enhance cell uptake of ALA *(14)*.

3. PDT-INDUCED APOPTOSIS

The literature on this topic has grown markedly, since the first report of the induction of apoptosis by a PS and light *(15)*. Several reviews have appeared, the most recent of which *(3,4,6,16–19)* have provided a comprehensive view of the knowledge to date. There are now a plethora of examples of the induction of apoptosis by the photoactivation of many different PSs in numerous *in vitro* and *in vivo* systems. This chapter will summarize our current understanding of the apoptosis pathways triggered by PDT and then focus on recent mechanistic insights. The reader is referred to the previous reviews for lists of cell and tumor types studied and pathways modulated with particular PSs. These reviews also provide a compilation of the many signaling pathways that are activated by PDT, some of which can modulate the extent or timing of apoptosis. The purpose of this chapter is to emphasize what is unique about PDT, most particularly the initial damages, and to distinguish mechanisms of PDT from those that are common to other cell stresses.

To make these comparisons, we will reserve the term "molecular target" to indicate molecules that are directly altered by the reactive oxygen species (ROS) that PDT generates. In most cases, the ROS is singlet oxygen, but as a result of oxidation–reduction reactions, other ROS can be formed, especially superoxide anion ($O_2^-\bullet$). Thus, a pathway that is triggered as a result of photooxidation of a target is a response but not itself a target. The preferred targets of singlet oxygen are unsaturated fatty acids and cholesterol of membranes, certain amino acids within proteins (cysteine, histidine, methionine, tyrosine, and tryptophan), and guanine bases within nucleic acids *(20)*. However, because of its high reactivity, $^1O_2$ reacts within very short range (< 10 nm) of its site of formation, which must be at the site of binding of the PS *(21)*. As mentioned above, owing to the hydrophobicity of their ring systems, the PSs tend to localize in membranes, where the initial photodynamic targets are located. DNA damage, when it occurs at all, is not relevant to the killing of cells by PDT *(6)*.

PDT is an efficient inducer of apoptosis *(3,15)* both in cultured cells and *in vivo*. As with other toxic agents, for example, hyperthermia, PDT causes necrosis at higher doses than those inducing apoptosis, indicating that one factor in determining the pathway for the final demise of the cell is the overall amount of damage. A transition from apoptosis to necrosis as the overall dose is raised has been found to occur with PDT sensitized by numerous PSs, for example, hypericin, dimethyl tetrahydroxyhelianthrone, phthalocyanines, and methylene blue derivative *(22–27)*. Another factor that can affect the final cell death mechanism is the site of localization of the PS. For some, variable incubation times can result in different localization patterns and culminate in different modes of cell death *(28)*. Moreover, the initial binding site of some PSs may be different from its preferred damage site in PDT *(29)*. In addition, different reactive species produced at the same sites can have markedly different cellular effects *(30)*, and singlet oxygen produced by PDT at the plasma membrane can still induce apoptosis *(31)*. Differential sensitivity to PDT occurs among different cell types, *(32,33)* and PDT-induced apoptosis can occur in any stage of the cell cycle *(34,35)* and even despite cell-cycle arrest *(36)*.

PDT activates many of the same signaling pathways turned on by other types of stress. These include the p38 and SAPK/JNK family of MAP kinases; NF-κB, fos, jun, and other components of transcription factors; and phospholipases, cyclooxygenases, and other lipid-metabolizing enzymes that generate lipid-derived signaling molecules (such as ceramide, phosphoinositides, prostaglandins, thromboxanes, and leukotrienes). The specific conditions of PSs, cells, and overall doses that elicit these responses have been reviewed in detail *(3,16,17)*. Many of these can promote cell death or act in a protective mode, as is true for their activation by other stresses.

4. CELLULAR TARGETS OF PDT: MITOCHONDRIA

Investigations of a variety of PDT systems found that upon photoirradiation, PSs that are localized to mitochondria kill cells more efficiently than do those targeted to the plasma membrane or lysosomes *(37)*. Hence, the mitochondrion has been considered the most sensitive target for directly triggering PDT-induced apoptosis *(3,38)*. Photodynamic damage to this organelle results in generation of ROS within it, loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), and release of pro-apoptotic factors (e.g., second mitochondrial activator of caspases (SMAC), apoptosis-inducing factor (AIF) and cytochrome c) from the mitochondrial intermembrane space into the cytosol. Subsequent activation of the caspase cascade results in expression of the characteristic features of morphological apoptosis *(3,18,39)*.

As the initial damage produced by PDT with mitochondrion-targeted PSs is in that organelle, it is necessary to ask whether the full apoptosis pathway is essential for the cells to die or whether the mitochondrial damage is lethal irrespective of the pathway for elimination of the cell. We examined this issue first in a pair of MCF-7 human breast cancer cells *(40)*. The parental cells lack procaspase-3, and the two derivative cell lines were created by C. Froehlich of Northwestern University by transfection of either human procaspase-3 or the empty vector. When treated with Pc 4-PDT, only the cells with the stably overexpressed transgene were capable of efficiently activating caspase-3 and caspase-9, cleaving poly(ADP-ribose) polymerase (PARP), fragmenting DNA, and condensing their chromatin to generate morphological apoptosis. However, cytochrome c release was equally sensitive in the two cell lines, and they were equally sensitive to Pc 4-PDT when assayed for survival by clonogenicity, indicating that the damage that initiates apoptosis at the sites of Pc 4 binding is lethal, and the later caspase-dependent steps are necessary for apoptosis but not for cell death *(40)*.

Further analysis of critical steps was carried out with cells that are deficient in Bax, the pro-apoptotic Bcl-2 family member that migrates from the cytosol to the mitochondria during apoptosis to mediate cytochrome c release. In DU145 cells, which lack Bax because of a mutation, the cells are incapable of undergoing all the steps in apoptosis, beginning with the release of cytochrome c. [Although the cells express Bak, it appears not to substitute for Bax in this situation, in contrast to the case of murine embryonic fibroblasts *(41)*.] Cells could be induced to undergo apoptosis by transient transfection of a plasmid containing Bax cDNA, and the level of apoptosis in the transfected cells was further increased by PDT. Bax-deficient DU145 cells were as sensitive as other (Bax-replete) cell lines to killing by PDT, as judged by clonogenic assay *(42)*. These studies have led us to a model wherein Pc 4-PDT damages intracellular membranes, primarily mitochondria, producing lethal damage.

Then, apoptosis is a convenient mechanism for disposing of a dead cell, not the process that determines life or death of the cell. Although it is likely that these conclusions apply to other PSs with localization properties similar to Pc 4, the relevant experiments remain to be done. Recent reports have demonstrated that in addition to apoptosis, PDT also activates the cell death process of autophagy, as demonstrated by the generation of autophagosomes within the cytoplasm of treated cells and by the autophagy-associated conversion of the protein LC3-I to LC3-II *(43–45)*. Notably, autophagy was observed in Bax-deficient cells and may provide a mechanism for the elimination of lethally irradiated cells that are unable to undergo apoptosis. It will be important in future work to determine the relationship between these two death pathways in response to PDT.

Inhibition of respiration and oxidative phosphorylation is also a common early response with mitochondrion-localized photosensitization *(23,46,47)*. Photofrin-PDT inhibited electron transport components, including succinate dehydrogenase and cytochome c oxidase, and disrupted the mitochondrial electrochemical gradient *(48–51)*. Strikingly, the inhibition of respiration caused by Pc 4-PDT in L5178Y-R murine lymphoma cells could largely be restored by addition of exogenous cytochrome c to the permeabilized cells *(47)*, supporting the notion that the inhibition of respiration in that situation primarily resulted from cytochrome c release rather than from damage to the major enzyme complexes of the electron transport chain.

Although it is clear that PDT damage to the mitochondrion can efficiently induce the intrinsic pathway of apoptosis in cells that express all of the requisite intermediates, the exact order of the known steps can vary. For example, after PDT with different PSs, cytochrome c release has been observed to occur simultaneously with the loss of $\Delta\Psi_m$ *(52)*, delayed after loss of $\Delta\Psi_m$ *(39)*, simultaneously or delayed depending on the dose *(53)*, or in the absence of any changes in transmembrane potential *(54)*. For example, in mouse lymphoma L5178Y-R cells treated with Pc 4-PDT, high fluences caused a rapid dissipation of membrane potential concurrent with cytochrome c release, whereas lower fluences produced cytochrome c release without any measurable loss of transmembrane potential *(53)*. As a result of these disparate observations, the mechanism by which cytochrome c is released from mitochondria following PDT remains unclear, some data suggesting that it is due to opening of the permeability transition (PT) pore, swelling of the inner membrane, and lysis of the outer membrane, whereas other data are consistent with opening of Bax-dependent channels on the outer membrane *(55)*.

4.1. The Permeability Transition Pore Complex

One model for the movement of solutes into and out of the mitochondrion envisions a large conductance channel known as the permeability transition pore complex (PTPC), formed by association of proteins of the outer [voltage-dependent anion channel (VDAC) and peripheral benzodiazepine receptor (PBR)] and inner [adenine nucleotide translocator (ANT) and cyclophilin D] mitochondrial membranes *(56,57)*. The components of the PTPC have received much attention as potential targets of PDT. One such component is the 18-kDa PBR, which was suggested to be the critical molecular target for PPIX and related porphyrins *(58–60)* based on a strong correlation between a porphyrin's binding affinity to the PBR and cellular photocytotoxicity. Support for the PBR as a PDT target was obtained in studies of ALA/PPIX *(61)* and pyropheophorbide

ethers *(62)*. In contrast, the phthalocyanine Pc 4 bound to the PBR only at concentrations much higher than those needed for efficient photosensitization *(63)*. As well, two positional isomers of PPIX (PPIII and PPXIII) with similar uptake and mitochondrial binding and the same ability to photosensitize L1210 leukemia cells as PPIX (as measured by a clonogenic assay) had significantly lower binding affinity to the PBR than PPIX *(64)*. Thus, the relationship between PBR binding and photosensitizing efficacy may be limited to certain PSs but not apply to others.

In isolated rat liver mitochondria, hematoporphyrin photosensitization strongly inhibited the 30-kDa ANT and PTPC opening *(65)*. Verteporfin-PDT permeabilized ANT-containing proteoliposomes, a response that could be inhibited by the ANT ligands, ADP and ATP *(66)*. Although intriguing, these observations do not prove that ANT is the cellular target of PDT with either PS. Among the proteins associated with the PTPC is hexokinase, which was observed to be released into the cytosol and its activity inhibited by hypericin-PDT *(67)*. Unfortunately, inhibitors of the PTPC have provided contradictory results. The loss of $\Delta\Psi_m$ after hypericin-PDT was not prevented by cyclosporin A or bongkrekic acid *(68)*. Cyclosporin A produced a partial inhibition of PTPC opening in human epidermoid carcinoma A431 cells exposed to Pc 4-PDT, but cyclosporin A combined with trifluoperazine strongly inhibited PTPC opening and apoptosis, supporting a transient opening of the PTPC *(39)*. The dissipation of $\Delta\Psi_m$ in isolated mitochondria exposed to PDT with Victoria Blue dyes was insensitive to cyclosporin A and was proposed to result from direct effects of the dyes on inner membrane permeability to protons rather than on the mitochondrial PT *(69)*. Thus, the diverse responses suggest that damage to components of the PTPC may contribute to the permeabilization of the mitochondria, allowing release of pro-apoptotic factors, such as cytochrome c, SMAC, and AIF.

4.2. Photodamage to Bcl-2 and Bcl-xL

A unique feature of PDT with PSs that target mitochondria and endoplasmic reticulum (ER) is "photodamage" to Bcl-2 and Bcl-xL *(25,70–72)*, anti-apoptotic proteins which reside in the outer mitochondrial membrane, ER, and nuclear membrane, where they contribute to the resistance of tumors to many types of cancer treatment. PDT-generated "photodamage" has been observed on western blots as loss of the native protein in a dose-dependent but not time-dependent manner. This photodamage occurs immediately upon photoirradiation, for cells treated in the cold, for cells missing caspase-3 or Bax, and for cells given a variety of protease inhibitors; therefore, the response is a result of photochemical damage, not proteolytic or other enzymatic reactions. Bcl-2 now seems to be a target of PDT with all PSs formerly considered to target mitochondria but not those that specifically target lysosomes. Bcl-2 photodamage has been observed in every human cancer cell line in which we have looked and in both endogenous and overexpressed Bcl-2. Photodamage was found irrespective of estrogen or androgen receptor status of the cells, p53 status, or other factors that can contribute to the resistance to various cancer treatments *(3)*. Bcl-2 photodamage correlated well with the ability of Pc 4-PDT to kill cells *(73)*, and the membrane-anchoring regions of Bcl-2 were found to be critical for this sensitivity *(74)*. Studies of deletion and point mutants of Bcl-2 suggest that a secondary membrane-anchorage site of Bcl-2 formed by the α-5 and α-6 helices that occur between the BH1 and BH2 domains is essential to form the photosensitive target for PDT-induced photodamage; the results support the

notion that PDT initiates Bcl-2 complex formation with nearby proteins and/or lipids to form large aggregates *(75)*. These two alpha helices have been proposed to form a secondary membrane-binding site that is important for pore formation *(76)*. However, in one study, the α5/α6 region was found within the membrane only after apoptosis was initiated *(77)*. Bcl-2 and Bcl-xL interact with VDAC, the major outer mitochondrial membrane component of the PTPC *(78)*, yet VDAC is not photodamaged in the same manner as Bcl-2 and Bcl-xL *(72)*.

One hypothesis for the importance of the α5/α6 region of Bcl-2 is that when the region is inserted in the membrane, amino acids that are preferred targets of $^1O_2$ and oxygen radicals are oxidized by PDT. However, when all photooxidizable amino acids were substituted with amino acids having similar hydrophobicity but reduced ability to interact with $^1O_2$, the mutants responded to Pc 4-PDT like wild-type Bcl-2, indicating that the most $^1O_2$-sensitive amino acids in the α5/α6 region are not the primary targets *(75)*. These data suggest that the α5/α6 region is important for targeting of Bcl-2 by Pc 4-PDT, but it is not directly photooxidized, implying that its role is to interact with other membrane components to form the photosensitive target.

Additional insights into the role of photodamage to Bcl-2 in the induction of PDT-induced apoptosis were provided by Kessel et al. *(79)*. They found that the bile acid ursodeoxycholic acid (UDCA) greatly potentiated apoptosis after PDT sensitized by 9-capronyloxytetrakis (methoxyethyl) porphycene (CPO) or tin etiopurpurin (SnET2), PSs that tend to bind to multiple cytoplasmic membranes. UDCA can bind to mitochondrial membranes and protect cells from apoptosis induced by a number of cytotoxic agents, including more hydrophobic bile acids *(80)*. In contrast, UDCA *enhanced* PDT-induced apoptosis through potentiation of mitochondrial membrane potential loss, cytochrome c release, and activation of caspase-3. UDCA did not enhance the intracellular accumulation of these PSs; hence, the authors suggest that UDCA acted to sensitize mitochondrial membranes to photodamage.

Castelli et al. *(81)* recently studied the mechanism by which UDCA augments PDT-induced apoptosis. UDCA potentiates apoptosis triggered by PDT, but only with PSs that cause photodamage to Bcl-2. UDCA also potentiates apoptosis with the non-peptide Bcl-2/Bcl-xL antagonist HA14-1, which binds in the hydrophobic pocket created by folding of the BH1, BH2, and BH3 domains of Bcl-2, the pocket which normally binds the BH3 domain of the pro-apoptotic proteins Bax or Bak. These observations suggest that the bile acid causes a conformational change in the membrane housing Bcl-2, which enhances the effects of both HA-14-1 and PDT. Using fluorescence polarization of fluorescein-tagged Bak-BH3 peptide (flu-Bak), they showed that HA14-1 competed with flu-Bak for its binding site on Bcl-2, and UDCA enhanced the binding affinity of HA14-1. Castelli et al. *(81)* also measured fluorescence resonance energy transfer (FRET) between Bcl-2 and the PS CPO in a liposomal system; UDCA enhanced the FRET signal, which was interpreted as showing that UDCA causes a conformational change in Bcl-2 that both increases its affinity for HA14-1 and interferes with its anti-apoptotic function. Thus, for the class of PSs that cause photodamage to Bcl-2 and Bcl-xL, UDCA may offer a means to promote the efficacy of PDT with minimal adverse side effects.

4.3. Photodamage to Cardiolipin

As mentioned in Section 1, the localization of hydrophobic PDT PSs in membranes ensures that singlet oxygen is generated within the membranes, sufficiently close to its preferred targets, cholesterol and the unsaturated fatty acids of phospholipids. The significance of lipid oxidation in PDT-induced cell death has been given much attention by Girotti and co-workers, with analysis of lipid hydroperoxides (LOOHs) by high-performance liquid chromatography. For example, as a consequence of PDT photosensitized by merocyanine 540, LOOHs generated in L1210 leukemia cells were predominantly those of cholesterol (ChOOH) and phosphatidylcholine (PCOOH) *(82)*. These hydroperoxides were detected at a PDT dose that resulted in approximately 90% cell death, thus lending support to the notion that photoperoxidative damage to membrane lipids is important in overall cell killing. Kriska et al. *(83)* showed that ALA-PDT of COH-BR1 breast cancer cells induced apoptosis when PPIX was located in the mitochondria, but induced necrosis when PPIX had diffused to peripheral sites. Under apoptotic but not necrotic conditions, cytochrome c was released from the mitochondria into the cytosol, and caspase-3 was activated. Furthermore, a related cell line that stably overexpressed the selenium-dependent phospholipid hydroperoxide glutathione peroxidase (PHGPx) in mitochondria was resistant to the accumulation of LOOHs in the mitochondria and to apoptosis, as the enzyme reduced LOOH to LOH.

The most recent evidence suggests that a likely target for the initial lethal damage of PDT with some mitochondrion-bound PSs is cardiolipin (CL), the phospholipid found uniquely in the mitochondrial inner membrane and at the mitochondrial contact sites *(84)*. CL is a dimeric phospholipid; each CL molecule contains two negatively charged phosphates and four fatty acids composed of 95% linoleic acid (C18:2) *(85)*, making it highly sensitive to oxidation and an attractive candidate for initial singlet oxygen attack. CL is important both structurally and functionally to mitochondrial integrity. It forms the attachment site and is an integral component of many proteins that are crucial to the mitochondria, such as the respiratory complexes, cytochrome c, and components of the PTPC *(86,87)*. Two populations of cytochrome c have been demonstrated in rat liver mitochondria, tightly and loosely bound pools, anchoring to the inner membrane by electrostatic and hydrophobic interactions *(88)*. According to current models, cytochrome c release occurs when the oxidation of CL generates a soluble pool of the protein, which can then pass into the cytosol in a Bax-dependent process to initiate apoptosis. In cell-free assays, Nomura et al. *(89)* demonstrated that the binding affinity of cytochrome c for CL is markedly reduced upon peroxidation of CL (to CLOOH), but the binding could be restored if CLOOH were reduced to CLOH by the detoxifying enzymatic action of PHGPx. Similarly, photoperoxidation of CL in PPIX-sensitized liposomes could disrupt the binding of cytochrome c, which was reversible by treatment with GSH and PHGPx *(90)*.

Continuing work with the PHGPx-overexpressing and parental lines of COH-BR1 cells, Kriska et al. *(90)* found that the presence of the enzyme in mitochondria made the cells resistant to ALA-PDT-induced apoptotic cell death but not to necrotic cell death. Of all the measured LOOHs, CLOOH accumulation was preferentially inhibited by PHGPx overexpression and only when the PS PPIX was localized in mitochondria. These data demonstrate that enzymatic reduction and detoxification of mitochondrial LOOHs confers resistance to PDT-induced apoptosis. Moreover, the data suggest a specific mechanism whereby PDT with mitochondrion-targeting PSs can initiate

apoptosis: photooxidation of CL, resulting in the loosening of cytochrome c anchorage to CL, followed by release of cytochrome c from mitochondria in a Bax-dependent process.

Additional evidence for CL as a target of PDT is based on the apparent high affinity for binding of certain PSs at or near CL. Release of a zinc phthalocyanine (ZnPc) from liposomes to rat liver mitochondria was reduced when the liposomes contained CL, implying a higher affinity of ZnPc for CL than for other phospholipids *(91)*. Other studies used the fluorescent dye nonyl-acridine orange (NAO), a specific probe of CL. As assayed in phospholipid monolayers, the affinity of NAO for CL was 50 times greater than for other negatively charged phospholipids (phosphatidyl serine and phosphatidyl inositol), and there was virtually no interaction of NAO with zwitterionic phospholipids (phosphatidyl choline and phosphatidyl ethanolamine) *(92)*. The high-affinity binding may result from insertion of the nonyl chain into the bilayer at the hydrophobic surface created by the four fatty acid chains *(93)*. NAO has been used to characterize a number of mitochondrial properties with respect to CL, including imaging by confocal microscopy *(94)*, measurements of mitochondrial mass *(95)*, and quantification of CL in the leaflets of the mitochondrial inner membrane *(96)*. NAO competitively inhibited the uptake of Photofrin into mitochondria, as judged by confocal microscopy, suggesting that these two species were competing for the same site at CL *(97)*. However, in cells, the specificity of NAO for CL is limited to low (nanomolar) concentrations and requires that the mitochondrial membrane potential be intact *(94)*. Morris et al. *(98)* provided direct evidence for the binding of a PS, Pc 4, to sites on or near CL based on FRET between NAO and Pc 4. Co-localization by confocal microscopy and the observed FRET confirm that both species reside in the same mitochondrial regions and suggest that CL is a likely initial target for the initiation of apoptosis by Pc 4-PDT. FRET has also been observed between NAO and PPIX, strengthening the evidence for CL as a phototarget of PDT with that PS (Morris et al., unpublished).

5. CELLULAR TARGETS OF PDT: LYSOSOMES

Whereas the emphasis of PDT research has been on the triggering of apoptosis by mitochondrion-localized photodamage, there is now mounting evidence that damage to other organelles can elicit apoptotic cell death. Kessel, Reiners, and their colleagues have identified a number of PSs that bind preferentially to lysosomes and upon photoactivation can induce apoptosis *(99–101)*. Photoactivation of NPe6-loaded murine hepatoma 1c1c7 cells caused a delayed apoptotic response, marked by rapid destruction of the lysosomes, Bid cleavage to generate truncated Bid (tBid), cytochrome c release from mitochondria, and activation of caspase-3 and caspase-9 *(100)*. They further determined that lysosomal extracts contained proteolytic activity that was capable of cleaving Bid *(100)*, in agreement with a similar finding of Stoka et al. *(102)*. Although cathepsins are known to reside in lysosomes and to be released upon lysosomal damage, inhibitors of cathepsins B, D, and L were unable to block apoptosis triggered by NPe6-sensitized PDT *(100)*. To further define the role of lysosomal proteases in PDT-induced apoptosis, the aryl hydrocarbon receptor (AhR)-deficient cell line, Tao, derived from 1c1c7 cells, was shown to be relatively resistant to the induction of apoptosis by NPe6-PDT, as judged by a colony-forming assay, despite a slightly better uptake of the

PS into its lysosomes *(101)*. When compared at equal levels of cell killing, apoptosis induction in Tao cells was considerably delayed relative to the induction in 1c1c7 cells, an observation that could be attributed to lower levels of cathepsins and other protease activities in the endosomal/lysosomal compartment of the cells. The authors proposed that AhR has a role in the processing and uptake of cathepsins and other proteases into lysosomes. To test this idea, and because 1c1c7 cells had been mutagenized to produce Tao cells, they compared cell lines in which the AhR level had been altered by molecular techniques. In every case tested, sensitivity to NPe6-PDT tracked with AhR level. Thus, cells with higher levels of AhR, such as 1c1c7 cells, had higher levels of lysosomal proteases available for release, more rapid and extensive cleavage of Bid, activation of caspase-3 and caspase-9, and more rapid and extensive development of morphological apoptosis.

One unique response has been reported for ceramide signaling after PDT. Separovic and co-workers found that ceramide levels increase in a variety of different cells with a PDT dose–response similar to that for induction of apoptosis *(34,103)*. Evidence favors the hypothesis that elevated ceramide promotes apoptosis, as has been proposed for other cell stresses *(104)*. When cells are exposed to ionizing radiation and certain chemotherapeutic drugs, the increase in cellular ceramide has been shown to be due to activation of lysosomal and other sphingomyelinases, enzymes that cleave sphingomyelin to generate ceramide and phosphorylcholine *(104)*. In contrast, when PDT sensitized by Pc 4 is the triggering agent, either no change or a decrease in sphingomyelinase activity was observed. Instead, the enhanced level of ceramide following PDT results from inhibition of two enzymes that utilize ceramide for the synthesis of complex sphingolipids, sphingomyelin synthase (SPS), and glucosylceramide synthase (GCS) *(105)*. Still, data on pyropheophorbide-a methyl ester (PPME)-sensitized PDT in human colon cancer HCT-116 cells suggest the involvement of lysosomal acid sphingomyelinase in the generation of ceramide in response to PDT, although in that case, a contribution of SPS and GCS was not investigated *(106)*. Furthermore, lymphoblasts from Niemann–Pick patients, which are genetically devoid of acid sphingomyelinase, were unable to either synthesize ceramide or undergo apoptosis in response to PDT, suggesting an important role for acid sphingomyelinase in this system *(107)*.

6. CELLULAR TARGETS OF PDT: ER

There have been a number of observations of apparent photodamage to certain proteins of the ER membranes, a phenomenon similar to that found with Bcl-2 and Bcl-xL, that is, loss of the native protein observed on western blots (Section 4.2.). The sarcoplasmic/ER calcium (SERCA) pump may be subject to photodamage from Verteporfin-sensitized PDT in HeLa cells *(66)*, and several peptides, including calreticulin precursor, p58 microsomal protein, and protein disulfide isomerase, are lost from HL60 cells immediately upon a high dose of PDT sensitized by ALA-PPIX *(108)*. With Pc 4, PDT causes photodamage to the SERCA pump, but not the related plasma membrane calcium pump, and to ERp57, but not ERp72 (unpublished observations). The photosensitivity of these ER proteins is in keeping with the binding of the PSs to ER membranes. Damage to these membrane constituents and likely others not yet identified may explain the strong ability of PDT with many PSs to stimulate the rapid efflux of ER Ca^{2+} into the cytosol *(109)*.

Intracellular Ca^{2+} is maintained by Ca^{2+} transporters on the plasma membrane and the membranes of the organelles. Several of these Ca^{2+} transporters have thiol groups

that are required to be in the reduced state to function properly. Considering that PDT induces profound oxidative damage to the membrane proteins, it is not surprising that dysregulation of intracellular Ca^{2+} occurs during PDT. Indeed, several studies report an increase in cytosolic free Ca^{2+} in various cell types induced by PDT with a wide range of PSs *(110–122)*. The increased cytosolic Ca^{2+} may be due to influx of Ca^{2+} from the extracellular space or release from the internal stores, such as ER, or from mitochondria. Also, the sources contributing to increased cytosolic Ca^{2+} may vary depending on the PS used, which reflects its subcellular targets. Whether the increase in cytosolic Ca^{2+} contributes to PDT-induced cell death or whether it is merely a consequence of irreversible photodamage to the cells is still unclear.

Under physiological conditions, cytosolic Ca^{2+} is approximately 100 nM compared with extracellular Ca^{2+} of 1 mM, ER Ca^{2+} of approximately 500 μM, and Golgi apparatus of approximately 300 μM. A wide range of PSs perturb intracellular Ca^{2+} regulation and increase intracellular Ca^{2+} in different cell types *(111,114,115,118,121)*. The intracellular calcium chelator BAPTA-AM inhibits the release of cytochrome c, caspase activation, and apoptotic death of cancer cells photosensitized with pheophorbide or 2,4,5,7-tetrabromorhodamine 123 bromide, suggesting that Ca^{2+} plays a role in PDT-induced apoptosis *(123,124)*. Increased cytosolic Ca^{2+} after PDT may originate from the influx of Ca^{2+} through ion channels in the plasma membrane *(115,121)*, release of Ca^{2+} from sequestered internal stores *(111,114)*, including ER and Golgi *(109,111,113,114,118,125)*, or activation of ion transport/exchange mechanisms *(117)*.

Low cytoplasmic Ca^{2+} concentrations are maintained by high-affinity Ca^{2+}-ATPases and lower affinity Na^+/Ca^{2+} exchangers on the plasma membrane *(126)*. Direct photodamage of the Ca^{2+} transporters would potentially result in Ca^{2+} overload, provided that the PS of interest binds to the plasma membrane. Low doses of tetrasulfonated aluminum phthalocyanine ($AlPcS_4$), when photoactivated, induce intracellular Ca^{2+} increase mainly because of Ca^{2+} influx, whereas higher doses cause Ca^{2+} release from internal stores *(111)*. The PS Pc 4 does not bind to the plasma membrane, as assessed by confocal microscopy, suggesting that direct damage to the plasma membrane Ca^{2+} transporters is probably not contributing to Pc 4-PDT-induced killing *(39)*. Plasma membrane Ca^{2+} ATPases contain several possible caspase-3 cleavage sites *(127)*. A recent study by Bano et al. *(128)* reports that the plasma membrane Na^+/Ca^{2+} exchanger is cleaved by Ca^{2+}-activated proteases, calpains, in neurons during excitotoxicity resulting in Ca^{2+} overload. As the effector caspases are frequently activated during PDT, the inactivation of the plasma membrane Ca^{2+}-ATPases by caspases may contribute to the subsequent delayed increase in cytosolic Ca^{2+}, resulting in Ca^{2+} overload and Ca^{2+}-dependent necrotic cell death, which often occurs during PDT.

The ER is a major intracellular source of Ca^{2+}. The intracellular release of inositol trisphosphate (IP3) from the plasma membrane causes IP3-induced Ca^{2+} release from the ER to the cytosol through the IP3 receptors. Ca^{2+} is returned to the ER lumen through SERCA pumps. Coordinated regulation of Ca^{2+} release and uptake mechanisms maintains the ER free-Ca^{2+} pool at approximately 500 μM in physiological conditions. Any perturbations of the release and uptake mechanisms eventually affect cytosolic Ca^{2+} concentrations. In HeLa cells, PDT photosensitized by Verteporfin released Ca^{2+} from the ER, which was associated with a rapid caspase-independent inhibition of SERCA-2 *(118)*. In rat liver microsomes, Ca^{2+} uptake was impaired after photosensitization with hematoporphyrin or protoporphyrin *(129)*. The SERCA pump may be important in PDT-induced cell death with those PSs that

localize to the ER membranes, such as Pc 4 *(39)*. In rat pancreatic acini, PDT with gadolinium porphyrin-like macrocycle B or $AlPcS_4$ induced cytosolic Ca^{2+} oscillations by triggering ER Ca^{2+} release through IP3 receptors *(113,114)*. Similarly, AlPc-PDT induced a rapid transient release of Ca^{2+} from the ER in mouse L5178Y cells *(109)*. A phospholipase C inhibitor, U73122, blocked the transient increases in both IP3 and cytosolic Ca^{2+} and subsequent fragmentation of nuclear DNA *(109)*. In other experimental systems apart from PDT, the thiol-specific agent, thimerosal, causes increased intracellular Ca^{2+} in HeLa cells by sensitizing the IP3 receptor to the basal level of IP3 in the cell *(130)*. Oxidized glutathione alters the binding properties of IP3 to its receptor by revealing IP3-binding sites *(131)*. Cytochrome c released from mitochondria during apoptosis has been shown to bind to IP3 receptors amplifying calcium-dependent apoptosis *(132)*. Thus, with PSs such as Pc 4 that localize in mitochondria and ER *(39)*, the IP3 receptor may be a target for PDT-induced oxidative damage, causing dysregulation of intracellular Ca^{2+} and resulting in cell death. Another recent study shows that CPO localizes predominantly in the ER membranes in murine leukemia L1210 cells *(133)*. Although CPO induced increased cytosolic Ca^{2+}, the chelation of intracellular Ca^{2+} with BAPTA did not protect cells from apoptosis, suggesting that elevated cytosolic Ca^{2+} levels were not required for cell death in that case.

The mitochondrial matrix free Ca^{2+} concentration is maintained by a balance between uptake and efflux mechanisms. A negative membrane potential across the inner mitochondrial membrane provides the driving force for Ca^{2+} influx through a Ca^{2+} uniporter *(134)*. Ca^{2+} efflux involves $3Na^+/2Ca^{2+}$ and Na^+/H^+ exchange driven by the mitochondrial membrane potential and pH gradient *(135–140)*. Initially, it was proposed that mitochondria with their large capacity for Ca^{2+} uptake might regulate cytosolic Ca^{2+} concentration. Our current understanding is rather that cytosolic Ca^{2+} regulates mitochondrial Ca^{2+}. In pathological situations, when mitochondrial Ca^{2+} increases, Ca^{2+} efflux through $3Na^+/2Ca^{2+}$ exchange becomes saturated. Such Ca^{2+} overloading may lead to the mitochondrial PT, causing Ca^{2+} to be released from mitochondria through the mitochondrial PT pores. Presumably, two Ca^{2+}-binding sites exist on the matrix side of the PT pore, and Ca^{2+} binding to these sites induces PT pore opening *(141)*. Indeed, in isolated mitochondria, increased matrix rather than extramitochondrial Ca^{2+} induces the PT, resulting in mitochondrial depolarization *(142)*. Mitochondrial ROS formation occurred within minutes after Pc 4-PDT. This was followed by inner membrane permeabilization, mitochondrial depolarization and swelling, cytochrome c release, and apoptotic death *(39)*. As increased mitochondrial matrix Ca^{2+} is required for the mitochondrial PT to occur, these results suggest that mitochondria are exposed to microdomains of high cytosolic Ca^{2+} that may exceed the concentrations of bulk cytosolic Ca^{2+}. Microdomains may result from a close interaction between the mitochondria and the ER Ca^{2+} channels *(143,144)*. Thus, the dynamic interplay between the ER and mitochondria can contribute to Ca^{2+}-mediated mitochondrial dysfunction, which leads to release of proapoptotic proteins from mitochondria and activation of the apoptotic cascade. The weight of the evidence suggests that PDT with many different PSs can efficiently trigger apoptosis through dysregulation of calcium homeostasis.

7. CELLULAR TARGETS OF PDT: PLASMA MEMBRANE

The most common pathway for apoptosis in response to PDT usually involves the release of cytochrome c followed by activation of caspase-3 and caspase-9; however,

other pathways can contribute, especially those through caspase-8, in situations where the dominant pathway is suppressed or when the cells respond by secreting ligands of plasma membrane death receptors *(3)*. The death receptor complexes can play a role in PDT-induced apoptosis, for example, expression and secretion of tumor necrosis factor-α (TNF-α) and FasL *(145–149)*. Nevertheless, combined effects of death receptor-targeted therapies and PDT remain inconclusive; for example, whereas treatment with anti-Fas antibody enhanced apoptosis induced by Verteporfin-PDT *(150)*, recombinant human TNF-α had no effect on the ability of Pc 4-PDT to induce apoptosis *(151)*. In a study of murine embryonic fibroblasts from wild-type FADD or FADD knock-out mice, the role of these plasma membrane death receptors was further delineated *(152)*. Both cell lines were equally sensitive to apoptosis (TUNEL-positive cells and caspase-3 activity) after Pc 4-PDT, but the FADD knock-out cells were resistant to the induction of apoptosis by TNF-α; hence, Pc 4-PDT-induced apoptosis does not require functional FADD. Taken together, the role of plasma membrane death receptors in PDT-induced apoptosis appears limited.

8. A MODEL FOR ORGANELLE PARTICIPATION IN APOPTOSIS FOLLOWING PDT

A model for the induction of apoptosis by PDT is presented in Fig. 3. The contact sites where the inner and outer mitochondrial membranes interact may offer a convergence site for the critical proteins and lipids involved in PDT-induced cell death,

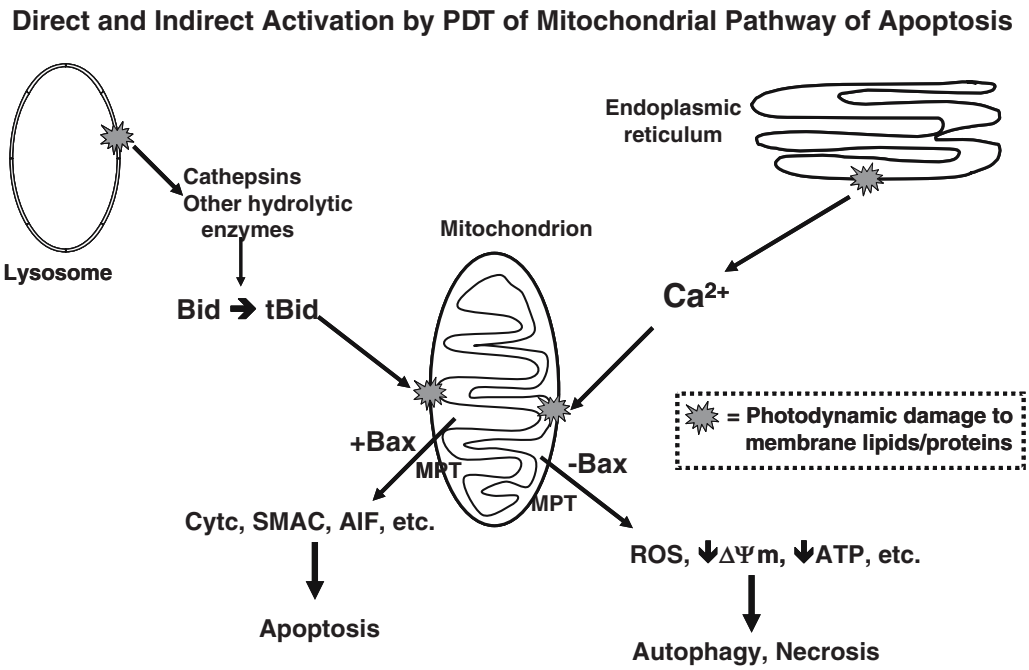

Fig. 3. A model for the induction of apoptosis by photodynamic therapy with photosensitizers localized in various subcellular compartments. Depending upon the photosensitizer, the relative level of damage to these organelles will vary.

especially with mitochondrion-targeting PSs. Enriched in both CL and Bcl-2/Bcl-xL, the contact sites make an attractive candidate for cytotoxic attack by singlet oxygen. As PSs typically bind to multiple organelles, we propose a model in which concomitant photodynamic damage to lysosomes or ER or both can promote and enhance mitochondrial pathways for cell death.

The pro-apoptotic Bcl-2 family member t-Bid is made available by photodynamic damage to lysosomes, and Ca^{2+} released from photodynamically damaged ER promotes mitochondrial swelling and release of cytochrome c. Depending on the level of damage, either apoptosis or necrosis can be initiated. In the absence of a complete pathway for apoptosis, autophagy may also contribute to the removal of lethally damaged cells. Further work will be required to evaluate this model and to provide the mechanistic details.

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28 Modulation of TRAIL Signaling for Cancer Therapy

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Summary

Apoptosis, the cell's intrinsic death program, is a key regulator of tissue homeostasis, and an imbalance between cell death and proliferation may result in tumor formation. Also, killing of cancer cells by cytotoxic therapies such as chemotherapy, γ-irradiation, or ligation of death receptors is predominantly mediated by triggering apoptosis in target cells. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that induces apoptosis upon binding to its receptors. TRAIL is of special interest for cancer therapy, as TRAIL has been shown to predominantly kill cancer cells, while sparing normal cells. Importantly, combined treatment with TRAIL together with chemotherapy or γ-irradiation synergized to achieve antitumor activity in tumor cell lines and also in tumor models *in vivo*. However, failure to undergo apoptosis in response to TRAIL treatment may result in tumor resistance. Understanding the molecular events that regulate TRAIL-induced apoptosis and their deregulation in resistant forms of cancer may provide new opportunities for cancer therapy. Thus, novel strategies targeting tumor cell resistance will be based on further insights into the molecular mechanisms of cell death, for example, triggered by TRAIL.

Key Words: Apoptosis; TRAIL; drugs; cancer therapy.

1. INTRODUCTION

Apoptosis, a distinct, intrinsic cell death program, occurs in various physiological and pathological situations and has an important regulatory function in tissue homeostasis and immune regulation *(1,2)*. Apoptosis is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation, and membrane blebbing *(1)*. Various stimuli can trigger an apoptosis response, for example, withdrawal of growth factors, or stimulation of cell-surface receptors *(1)*. Also, killing of tumor cells by diverse cytotoxic strategies such as

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anticancer drugs, γ-irradiation, or immunotherapy has been shown to involve induction of apoptosis in target cells *(3–5)*. In addition, T cells or NK cells may release cytotoxic compounds such as granzyme B, which can directly initiate apoptosis effector pathways inside the cell *(1)*. Proteolytic enzymes called caspases are important effector molecules of different forms of cell death *(6)*. Apoptosis pathways are tightly controlled by a number of inhibitory and promoting factors *(7)*. The antiapoptotic mechanisms regulating apoptotic cell death have also been implicated in conferring drug resistance to tumor cells *(5)*. Importantly, combinations of anticancer agents together with death-inducing ligands have been shown to synergize in triggering apoptosis in cancer cells and may even overcome some forms of drug resistance *(8)*. Further insights into the mechanisms controlling tumor cell death in response to death receptor ligation will provide a molecular basis for novel strategies targeting death pathways in apoptosis-resistant forms of cancer.

2. THE CORE APOPTOTIC MACHINERY

In most cases, anticancer therapies eventually result in the activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death *(6)*. Caspases are synthesized as inactive proforms, and upon activation, they cleave next to aspartate residues. The fact that caspases can activate each other by cleavage at identical sequences results in the amplification of caspase activity through a protease cascade. Caspases cleave a number of different substrates in the cytoplasm or nucleus leading to many of the morphologic features of apoptotic cell death *(1)*. For example, polynucleosomal DNA fragmentation is mediated by cleavage of inhibitor of caspase-activated DNase (ICAD), the inhibitor of the endonuclease CAD that cleaves DNA into the characteristic oligomeric fragments *(9)*. Likewise, proteolysis of several cytoskeletal proteins such as actin or fodrin leads to loss of overall cell shape, whereas degradation of lamin results in nuclear shrinking *(1)*.

Activation of caspases can be initiated from different angles, for example, at the plasma membrane upon ligation of death receptor (receptor pathway) or at the mitochondria (mitochondrial pathway) *(8)*. Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors results in the activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 *(10)*. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), Smac/Diablo, Omi/HtrA2, endonuclease G, caspase-2, or caspase-9 from the mitochondrial intermembrane space *(11)*. The release of cytochrome c into the cytosol triggers caspase-3 activation through the formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex *(12)*. Smac/Diablo and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to inhibitor of apoptosis proteins (IAPs) *(11)*.

Links between the receptor and the mitochondrial pathway exist at different levels. Upon death, receptor triggering activation of caspase-8 may result in cleavage of Bid, a Bcl-2 family protein with a BH3 domain only, which in turn translocates to mitochondria to release cytochrome c thereby initiating a mitochondrial amplification loop (13). In addition, cleavage of caspase-6 downstream of mitochondria may feed back to the receptor pathway by cleaving caspase-8 *(14)*.

3. TRAIL AND ITS RECEPTORS

TRAIL/Apo-2L was identified in 1995 based on its sequence homology to other members of the TNF superfamily *(15,16)*. TRAIL is a type II transmembrane protein, the extracellular domain of which can be proteolytically cleaved from the cell surface. TRAIL is constitutively expressed in a wide range of tissues. Comprising five different receptors, the complexity of the TRAIL receptor system is unprecedented. TRAIL-R1 and TRAIL-R2, the two agonistic TRAIL receptors, contain a conserved cytoplasmic death domain motif, which enables them to engage the cell's apoptotic machinery upon ligand binding *(17–21)*. TRAIL-R3 to TRAIL-R5 are antagonistic decoy receptors, which bind TRAIL, but do not transmit a death signal *(22–26)*. TRAIL-R3 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein, which lacks a cytoplasmic tail, whereas TRAIL-R4 harbors a substantially truncated cytoplasmic death domain. In addition to these four membrane-associated receptors, osteoprotegerin is a soluble decoy receptor, which is involved in the regulation of osteoclastogenesis *(27)*.

Similar to CD95L, TRAIL rapidly triggers apoptosis in many tumor cells *(28–30)*. The TRAIL ligand and its receptors are of special interest for cancer therapy, as TRAIL has been shown to predominantly kill cancer cells while sparing normal cells. The underlying mechanisms for the differential sensitivity of malignant versus non-malignant cells for TRAIL have not exactly been defined. One possible mechanism of protection of normal tissues is thought to be based on the set of antagonistic decoy receptors, which compete with TRAIL-R1 and TRAIL-R2 for binding to TRAIL *(31)*. However, screening of various tumor cell types and normal cells did not reveal a consistent association between TRAIL sensitivity and TRAIL receptor expression. Therefore, susceptibility for TRAIL-induced cytotoxicity has been suggested to be regulated intracellularly by distinct patterns of pro- and antiapoptotic molecules.

4. TRAIL SIGNALING

Similar to the CD95 system, ligation of the agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, by TRAIL or agonistic antibodies results in receptor trimerization and clustering of the receptors' death domains *(28,30,32–34)*. This leads to recruitment of adaptor molecules such as FADD through homophilic interaction mediated by the death domain. FADD in turn recruits caspase-8 to the activated TRAIL receptor complex. Oligomerization of caspase-8 at the activated TRAIL receptor complex drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-3. Also, activated caspase-8 can cleave Bid, which then translocates to mitochondria to induce cytochrome c release. In addition to caspase-8, caspase-10 is recruited to the TRAIL DISC *(33)*. However, the importance of caspase-10 in the TRAIL DISC for apoptosis induction has been controversially discussed *(35,36)*.

5. DEFECTIVE TRAIL SIGNALING IN CANCERS

Signaling by agonistic TRAIL receptors is regulated at various levels along the signaling pathway. Importantly, cancer cells have evolved numerous ways to evade induction of apoptosis triggered by the death ligand TRAIL, resulting in TRAIL resistance as outlined below.

5.1. Trail Receptors

For example, loss of expression of the agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, may account for TRAIL resistance. Both receptors are located on chromosome 8p, a region of frequent loss of heterozygosity (LOH) in tumors *(28)*. In a small percentage of cancers, for example, non-Hodgkin's lymphoma, colorectal, breast, head and neck, or lung carcinoma, deletions or mutations were found, which resulted in loss of both copies of TRAIL-R1 and TRAIL-R2 *(37–41)*. In addition, loss of TRAIL-R1 or TRAIL-R2 expression may be caused by epigenetic alterations such as promotor hypermethylation, for example, in neuroblastoma *(42)*.

5.2. c-FLIP

TRAIL signaling can also be negative by intracellular proteins that associate with the cytoplasmatic domain of TRAIL receptors such as c-FLIP *(43)*. c-FLIP has homology to caspase-8 and caspase-10 but lacks protease activity. By binding to FADD, c-FLIP prevents the interaction between the adaptor molecule FADD and procaspase-8. c-FLIP exists as a long (c-$FLIP_L$) and a short (c-$FLIP_S$) isoforms, both of which can inhibit death receptor-induced apoptosis. Interestingly, c-$FLIP_S$ was identified in a screen for genes that could confer resistance to TRAIL-induced apoptosis *(44)*. High c-FLIP expression has been detected in many tumors and has been correlated with resistance to TRAIL-induced apoptosis, for example, in melanoma. However, a consistent correlation between c-FLIP expression and TRAIL resistance has not always been found *(45)*. Importantly, downregulation of FLIP expression by inhibition of protein synthesis or RNA translation, cytotoxic drugs, proteasome inhibitors, PPARγ ligand, or siRNAI for c-FLIP restored sensitivity for TRAIL-induced apoptosis in several cell types *(46–48)*.

5.3. Bcl-2 Proteins

Bcl-2 family proteins play an important role in the regulation of the mitochondrial pathway, as these proteins localize to intracellular membranes such as the mitochondrial membrane *(13)*. They comprise both antiapoptotic members, such as Bcl-2 or Bcl-X_L, and proapoptotic molecules, such as Bax or Bid. Altered expression of Bcl-2 family proteins have been reported in various human cancers. Imbalances in the ratio of anti- and proapoptotic Bcl-2 proteins may favor tumor cell survival instead of cell death. Overexpression of Bcl-2 or Bcl-X_L blocked TRAIL-triggered apoptosis in many tumor cell lines, for example, prostate carcinoma, pancreatic carcinoma, neuroblastoma, or glioblastoma cells *(49,50)*. In addition, gene ablation studies showed that Bax was absolutely required for TRAIL-induced apoptosis in colon carcinoma cells *(44,51)*. However, overexpression of Bcl-2 or Bcl-X_L did not interfere with TRAIL-induced apoptosis in some cell types, for example, Jurkat or CEM T-cell leukemia cells *(52)*. Thus, the contribution of the mitochondrial pathway to TRAIL-induced apoptosis may depend on the cell type.

5.4. IAPs

The family of endogenous caspase inhibitors IAPs are highly conserved throughout evolution and comprise the human analogs XIAP, cIAP1, cIAP2, survivin, and livin

(ML-IAP) *(53)*. IAPs have been reported to directly inhibit active caspase-3 and caspase-7 and to block caspase-9 activation. In addition to regulation of apoptosis, IAP members such as survivin are involved in the regulation of mitosis *(54)*. The activity of IAPs are controlled at various levels, for example, by the transcription factor NFκB that has been reported to stimulate expression of cIAP1, cIAP, and XIAP *(53)*. In addition, Smac/Diablo and Omi, which are released from mitochondria upon apoptosis induction, neutralize IAPs through binding to IAPs thereby displacing them from their caspase partners *(11)*. Increased IAPs expression is detected in many tumors and has been correlated with adverse prognosis *(45,53)*. Importantly, overexpression of XIAP blocked TRAIL-triggered apoptosis in several cellular systems *(55,56)*.

5.5. NFκB

The transcription factor NFκB has been connected with multiple aspects of oncogenesis, including cell proliferation or inhibition of apoptosis *(57)*. NFκB is composed of hetero- or homodimers of NFκB/Rel family of proteins, which mediate protein dimerization, nuclear import, and specific DNA binding. In most cell types, NFκB is sequestered in the cytoplasm by its interaction with IκB proteins and therefore remains inactive. Upon stimulation, IκB becomes phosphorylated following activation of the IKK complex and is degraded through the proteasome thereby releasing NFκB to translocate into the nucleus for transcription of target genes. NFκB target genes include several antiapoptotic proteins, for example, cIAP1, cIAP2, TRAF1, TRAF2, Bfl-1/A1, Bcl-X_L, and FLIP. Interestingly, promotor activation of certain proapoptotic molecules of the TRAIL system including TRAIL-R1, TRAIL-R2, or TRAIL is also controlled by NFκB consistent with reports that NFκB can promote apoptosis under certain circumstances *(58,59)*. NFκB is constitutively active in certain tumor types such as Hodgkin's lymphoma or pancreatic carcinoma *(57)*. Also, NFκB activity is induced in response to various stimuli, for example, in response to cellular stress or anticancer agents. In addition, TRAIL can activate NFκB, which is mediated by TRAIL-R1, TRAIL-R2, or TRAIL-R4 through a TRAF2-NIK-IKKα/β-dependent signaling cascade *(18,60)*. Interestingly, TRAIL has been reported to activate NFκB especially under conditions, when apoptosis is blocked *(61,62)*. Inhibition of NFκB signaling, for example, by proteasome inhibitors, which prevent Iκ Bα degradation, or by overexpresison of non-degradable IκBα mutants, has been reported to sensitize tumor cells for TRAIL-induced apoptosis, at least in some contexts *(63)*.

6. TRAIL AND CANCER THERAPY

6.1. Safety of TRAIL Administration

The idea to specifically target death receptors to trigger apoptosis in tumor cells is attractive for cancer therapy as death receptors have a direct link to the cell's death machinery *(10)*. In addition, apoptosis upon death receptor ligation has been reported to occur independent of the p53 tumor suppressor gene, which is deleted or inactivated in more than half of human tumors *(64)*. However, the clinical application of CD95 ligand or TNFα is hampered by severe toxic side effects *(30)*. Systemic administration of TNFα or CD95 ligand causes a severe inflammatory response syndrome or massive liver cell apoptosis. In contrast, TRAIL appears to be a relatively safe and

promising candidate for clinical application, particularly in its non-tagged, zinc-bound homotrimeric form *(28)*. Studies in non-human primates such as chimpanzees and cynomolgus monkeys showed no toxicity upon intravenous infusion, even at high doses *(65)*. In addition, no cytotoxic activity of TRAIL was reported on various normal human cells of different lineages including fibroblasts, endothelial cells, smooth muscle cells, epithelial cells, or astrocytes *(66)*. However, some concerns about potential toxic side effects on human hepatocytes or brain tissue have also been raised *(67,68)*. The loss of tumor selectivity may be related to the TRAIL preparations used in these studies. TRAIL preparations, which are antibody crosslinked or not optimized for Zn content, have been reported to form multimeric aggregates thereby overpassing the threshold of sensitivity of normal cells *(28)*.

6.2. Antitumor Activity of TRAIL

Recombinant soluble TRAIL-induced apoptosis in a broad spectrum of cancer cell lines, including colon carcinoma, breast carcinoma, lung carcinoma, pancreas carcinoma, prostate carcinoma, renal carcinoma, thyroid carcinoma, malignant brain tumors, Ewing tumor, osteosarcoma, neuroblastoma, leukemia, and lymphoma *(28,29)*. Also, TRAIL exhibited potent tumoricidal activity *in vivo* in several xenograft models of colon carcinoma, breast carcinoma, malignant glioma, or multiple myeloma. Furthermore, monoclonal antibodies that engage the TRAIL receptors, DR4 or DR5, also demonstrated potent antitumor activity against tumor cell lines and in preclinical cancer models *(69,70)*.

6.3. Combination Therapy with TRAIL

Although these studies provided ample evidence of the potential of TRAIL for cancer therapy, many tumors remain refractory toward treatment with TRAIL, which has been related to the dominance of antiapoptotic signals. Importantly, numerous studies have shown that TRAIL synergized together with cytotoxic drugs or γ-irradiation to achieve antitumor activity in various cancers including malignant glioma, melanoma, leukemia, breast, colon, or prostate carcinoma *(71–76)*. Remarkably, TRAIL and anticancer agents also cooperated to suppress tumor growth in different mouse models of human cancers. The molecular mechanisms, which account for this synergistic interaction, may include transcriptional upregulation of the agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, which occurred in a p53-dependent or p53-independent manner *(77,78)*. Of note, p53 has also been shown to transcriptionally activate the antagonistic TRAIL receptors, TRAIL-R3 and TRAIL-R4 *(79)*. The synergistic interaction between cytotoxic drugs and TRAIL may also be mediated by downregulation of antiapoptotic proteins such as Bcl-2, Bcl-X_L, or FLIP upon drug treatment *(80)*. In addition, anticancer agents may sensitize tumor cells for TRAIL treatment by upregulating proapoptotic molecules including caspases or FADD *(81)*. Caspase-8 expression is frequently impaired by hypermethylation in several tumors including neuroblastoma, Ewing tumors, malignant brain tumors, or lung carcinoma *(82,83)*. Importantly, restoration of caspase-8 expression by gene transfer or by demethylation treatment sensitized resistant tumor cells for TRAIL-induced apoptosis *(83)*. Also, biological response modifiers such as IFNγ strongly enhanced the cytotoxic activity of TRAIL by upregulating caspase-8 expression in a STAT-1-dependent manner *(84)*.

As TRAIL demonstrated synergistic interaction with anticancer agents or irradiation, TRAIL may be most effective in combination with conventional cancer treatments. In addition, small molecules may serve as molecular therapeutics to specifically target tumor cell resistance toward TRAIL in resistant forms of cancer. To this end, Smac agonists have recently been reported to potentiate the efficacy of TRAIL treatment by antagonizing the inhibitory effect of IAPs, which are overexpressed in many tumors *(85)*. Importantly, Smac peptides synergized with TRAIL to eradicate malignant glioma in an orthotopic mouse model without any detectable toxicities to the normal brain tissue *(85)*.

Aberrant architecture of the chromatin has been implicated in tumor formation and progression *(86)*. Posttranslational modifications of histone proteins, in particular their acetylation status, control gene expression by regulating the accessibility of transcription factors to DNA *(86)*. Histone acetylation is governed by the relative activities of histone acetyltransferases (HATs), which favor decompaction of the chromatin structure for efficient transcription, and histone deacetylases (HDACs), which remove acetyl groups from histone tails, thereby suppressing transcription *(86)*. HDAC inhibitors (HDACIs) have been reported to cause growth arrest, differentiation, and/or apoptosis by altering the transcription of a small and finite number of expressed genes *(87)*. Interestingly, HDACIs were also found to significantly enhance the efficacy of TRAIL in various human cancers including breast, colon, lung or prostate carcinoma, mesothelioma, melanoma, as well as acute or chronic leukemia *(88–101)*. The molecular basis for the HDACI-mediated sensitization for TRAIL has been linked to upregulation of TRAIL-R1 and/or TRAIL-R2 expression in a SP1- or NFκB-dependent manner *(88–92,94,97,100)*. In addition, the cooperative interaction of HDACI and TRAIL has been reported to involve redistribution of agonistic TRAIL receptors to membrane lipid rafts *(99)* or increased efficacy of TRAIL-induced death-inducing signaling complex (DISC) formation *(89,91)*. Moreover, increased mitochondrial damage *(96,97)*, enhanced expression of proapoptotic molecules *(96,97)*, or downregulation of antiapoptotic proteins, for example, Bcl-X_L, Bcl-2, Mcl-1, FLIP, XIAP, or survivin *(89,90,95,97)*, have been identified to lower the threshold for apoptosis induction by TRAIL in the presence of HDACI. Although HDACI in combination with TRAIL have been described to exert no or little toxicity on non-transformed cells such as peripheral blood lymphocytes or human mammary epithelial cells *(93,94,97)*, there is recent evidence that HDACI may revert the lack of toxicity of TRAIL on non-transformed cells, for example, human renal proximal tubule epithelial cells *(100)*. Thus, the question whether HDACI sensitize for TRAIL-induced apoptosis in a cancer-selective fashion has not yet been answered.

Most studies investigating the TRAIL sensitivity of tumor cells have so far been performed *in vitro (29)*. Despite many limitations of cell-culture experiments, *in vitro* studies may reflect some of the important features of tumor treatment *in vivo*. Importantly, experiments performed on primary cancer cells ex vivo in most cases yielded similar results. For example, combined treatment of primary cancer cells with TRAIL together with anticancer agents synergized in antitumor activity.

6.4. Tumor Surveillance

There is also mounting evidence for an important role of TRAIL in tumor surveillance, for example, from studies with TRAIL knockout mice *(102,103)*. Although the

biology of the TRAIL system may differ significantly between mice and humans, as there is only one TRAIL receptor in mice, which is homologous to both TRAIL-R1 and TRAIL-R2, the phenotype of these knockout mice is informative with respect to the physiological function of TRAIL *in vivo*. Importantly, TRAIL-deficient mice were more susceptible to tumor metastasis than wild-type mice *(103)*. These data are in accordance with studies showing an important role of NK cells, which constitutively express TRAIL, in the control of tumor metastasis *(104,105)*. In addition, tumor formation induced by carcinogens was found to be enhanced in the presence of antagonistic TRAIL antibodies *(104)*. Thus, TRAIL may play an essential role as innate effector molecule in immune surveillance during tumor formation and progression.

7. CONCLUSIONS

Over the decade key elements of the TRAIL signaling pathway have been identified, and numerous studies provided substantial insights into the molecular mechanisms regulating TRAIL-induced apoptosis. The death ligand TRAIL is of special interest for cancer therapy because of its differential toxicity toward transformed versus normal cells. Importantly, TRAIL strongly synergized together with chemotherapy or irradiation in antitumor activity, even against some resistant forms of cancer.

However, several points remain to be addressed in future studies. As the tumor selective toxicity of TRAIL is presently not fully understood, further preclinical safety testing is necessary to assess the potential toxicity of TRAIL or agonistic TRAIL receptor antibodies on non-malignant tissues. In addition, the possible toxicity of TRAIL on normal tissues under conditions of combined treatment with anticancer agents or irradiation remains to be determined. Also, the molecular mechanisms leading to activation of survival pathways such as NFκB or AKT upon treatment with TRAIL and their significance for cancer therapy are only partially understood. Moreover, the promise of TRAIL alone or in combination protocols remains to be tested in clinical settings. Future studies on the role of TRAIL in individual tumors both *in vitro* and *in vivo* in tumor cells of patients under chemotherapy, for example, by DNA microarrays or proteomic studies, may provide the basis for "tailored" tumor therapy with TRAIL and may identify new targets for therapeutic interventions.

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