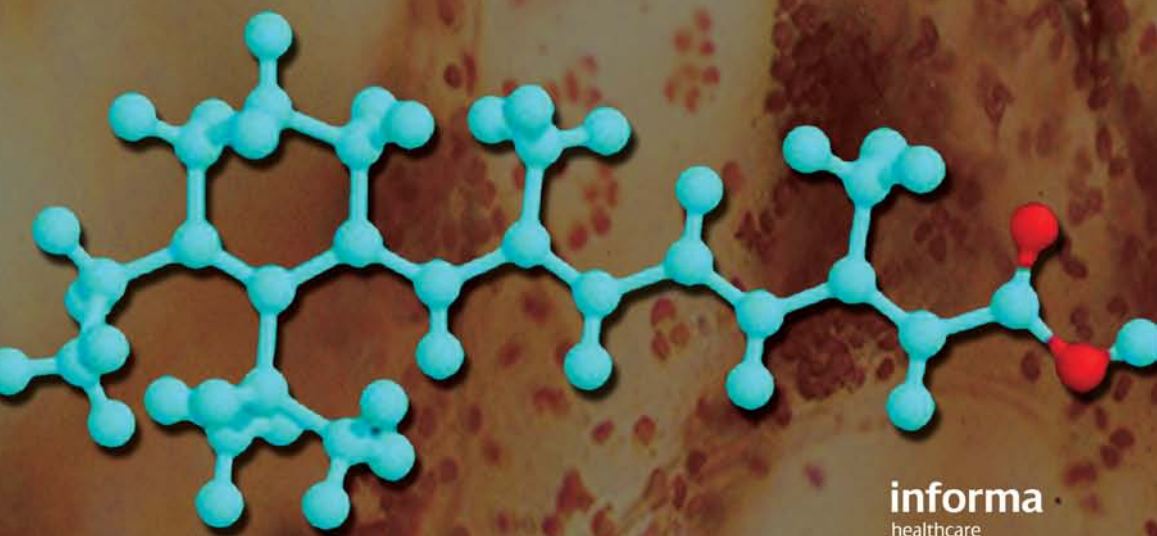
Retinoids and Carotenoids in Dermatology

Edited by

Anders Vahlquist
Madeleine Duvic



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Retinoids and Carotenoids in Dermatology

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To my wife Carin, for all the lost hours of companionship.
Anders Vahlquist

To Mike, Whitney, and Alice.
Madeleine Duvic

Introduction

During the past 25 years, there has been a vast explosion in new information relating to the art and science of dermatology as well as fundamental cutaneous biology. Furthermore, this information is no longer of interest only to the small but growing specialty of dermatology. Clinicians and scientists from a wide variety of disciplines have come to recognize both the importance of skin in fundamental biological processes and the broad implications of understanding the pathogenesis of skin disease. As a result, there is now a multidisciplinary and worldwide interest in the progress of dermatology.

With these factors in mind, we have undertaken this series of books specifically oriented to dermatology. The scope of the series is purposely broad, with books ranging from pure basic science to practical, applied clinical dermatology. Thus, while there is something for everyone, all volumes in the series will ultimately prove to be valuable additions to the dermatologist's library.

The latest addition to the series, volume 39, edited by Drs. Anders Vahlquist and Madeleine Duvic, is both timely and pertinent. The editors are internationally respected for their basic science and clinical expertise in the molecular biology and clinical applications of retinoids and carotenoids, and have assembled an outstanding group of contributors for this latest addition to our series. We trust that this volume will be of broad interest to scientists and clinicians alike.

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Preface

There are numerous reasons for publishing a book that jointly focuses on retinoids and carotenoids and on their relevance in skin biology and dermatology. Retinoids (i.e., retinol and its analogs) and carotenoids are both chemically and biologically related; the latter molecules are all basically polyprenoids (i.e., consist of repeated isoprene units) and are more or less omnipresent in nature (1,2). Some carotenoids (principally beta-carotene) can be converted to retinoids in mammals and fishes via enzymatic cleavage, making them important precursors of vitamin A in the diet. Furthermore, both groups of compounds are characterized by the high number of synthetic derivatives that have appeared since the advance of organic chemistry in the 1940s. Yet, from a biologic and therapeutic standpoint, retinoids and carotenoids are often regarded as separate entities, probably because they are used for different indications and have profoundly different dose-response curves.

Retinoid therapy, in the form of high-dose oral vitamin A, was initiated in the 1940s for hyperkeratotic skin diseases, but was later abandoned for toxicity reasons. Following the identification of all-trans retinoic acid as an active metabolite of vitamin A in the 1950s and the production of new retinoid derivatives in the 1970s with better therapeutic ratios in animal tumor models, some of these compounds (notably isotretinoin and acitretin) have since become a sine qua non for dermatology, especially in the field of acne, psoriasis, and keratinizing disorders. Subsequent to the discovery of retinoic acid receptors and their role in transcriptional regulation of important genes in the 1980s, a whole new paradigm has arisen where the strategy is to design specific ligands for the various retinoid receptors, aiming at fine-tuning the transcription machinery to mitigate various pathogenic mechanisms. As a direct consequence, new drugs and new indications for retinoid therapy have appeared, such as the use of oral bexarotene (targretin) in cutaneous lymphoma and alitretinoin (9-cis retinoic acid) in chronic hand eczema (3), and targretin gel has been used to treat chronic hand dermatitis and alopecia areata.

However, not all effects of retinoids are mediated by nuclear receptors, a fact that should not be overlooked when designing new drugs in this field.

Regrettably, throughout the process of developing new retinoids, toxicity problems (teratogenicity, etc.) have remained an insurmountable obstacle that necessitates strict precautions when prescribing oral formulations. Therefore, this book not only discusses the side effects and how to avoid them, but also focuses on useful knowledge about the pharmacology and various peculiarities of retinoid pharmacodynamics that underlie the untoward effects.

Carotenoids, on the other hand, are compounds that are much less toxic and are mainly known in human medicine for their antioxidant properties. They are prescribed by dermatologists to patients with various photosensitivity syndromes (e.g., protoporphyria), most often in the form of oral beta-carotene. Canthaxantin is another carotenoid that was popular in the 1980s as artificial skin pigmentation, but it was withdrawn from the market due to a hazardous accumulation in the retina after oral administration. More recently, a variety of other carotenoid molecules have been re-examined in the field of dermatology and may eventually emerge as approved drugs. Carotenoids are thought to play a significant part in the skin's natural antioxidant defense system and may also help prevent malignancy in other organs. This has led to an interest in monitoring the individual's carotenoid status, for example, by using such noninvasive techniques as Raman spectroscopy of the skin, showing a good correlation to the blood levels of carotenoids. Although the promising anti-tumor effects of carotenoids (and retinoids) originally observed in animal experiments have been somewhat disappointing when translated to the human situation, there are several indications that this may change in the future.

Although carotenoids in their capacity as lipid-soluble antioxidants and scavengers of free radicals seem to operate in human tissues mostly via non-genomic mechanisms, recent studies indicate that they may also affect more specific cellular functions. So, in this sense also, carotenoids and retinoids may again be merging and we may benefit from a combined approach when describing the mechanism of their action.

The primary objective of this book is to describe how retinoids and carotenoids function in the skin, and how they can be used as powerful agents to prevent and treat skin diseases. Although the emphasis of this book is on the clinical aspects of these compounds, several chapters are devoted to new basic research that is being done despite the adverse reactions that can especially characterize the retinoids. It goes without saying that this treatise is not strictly confined to dermatological aspects, but also describes important developments in other fields of retinoid and carotenoid research, especially in relation to cancer and immunology. Furthermore, the book provides a means for some readers to update their knowledge about biomedical issues outside the field of dermatology, such as general vitamin A nutrition, the role of antioxidants in aging, metabolic activation and degradation of polyprenoids, cellular signalling, inflammation, and the role of lipoproteins in atherosclerosis.

In organizing the book we included chapters written by internationally recognized authorities with widely different backgrounds, ranging from biochemistry and nutrition to molecular biology and clinical science. It is our hope that this broad approach attracts not only dermatologists, but also other clinicians and scientists with a general interest in retinoids, carotenoids, and the biology of the skin. We wish to express our sincere gratitude to all authors for their valuable contributions.

Anders Vahlquist
Madeleine Duvic

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From Carotenoids and Vitamin A to Retinoids

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INTRODUCTION

For more than 75 years, it has been known that vitamin A is critically important for growth and differentiation of epithelial cells, and it was soon realized that β-carotene is a provitamin that can replace vitamin A in the diet (1). As early as 1925, Wolbach and Howe (2) showed that vitamin A deficiency in rats led to the replacement of differentiated mature epithelium with squamous, keratinizing epithelial cells: Hyperkeratosis was observed in the skin, while hyperplastic and metaplastic changes were observed in epithelia of mucous membranes in vitamin A deficient rats. They concluded that vitamin A influenced the differentiation of epithelial cells, from the normal, simple, and pseudostratified phenotype to squamous, metaplastic lesions that start focally and spread throughout the epithelium. Shortly after, Nicholls (3) described phrynoderma, a distinct form of follicular hyperkeratosis, in African prisoners who also had night blindness and xerophthalmia (4). When treated with vitamin A containing cod live oil, both skin lesions and night blindness improved. In 1953, Fell and Mellanby (5) reported that the phenotype of chick epidermis in organ culture could be changed from keratinized to mucus-producing tissue by treatment with retinol or retinyl acetate. These

observations were followed by numerous studies focusing on the pharmacological action of retinoids and carotenoids in skin. This has ultimately resulted in the development of some thousand new synthetic compounds and the establishment of retinoids and carotenoids as treatment for various skin diseases (6–11). During the last years, the ability of retinoids to affect the gene expression and differentiation of epithelial cells in vivo and in vitro has been studied in great detail.

Today we know that vitamin A is essential for the life of all chordates, and has important functions, not only in maintenance of epithelial surfaces, but also in numerous other functions or processes such as vision, immune competence, reproduction, hematopoiesis, and embryonic growth and development. The major disciplines for vitamin A research include molecular-, cell- and developmental biology, dermatology, oncology, and public health, but potential roles are being explored in almost every field of biomedical research.

The aim of this introductory chapter is to describe relevant biochemical and cellular aspect of retinoid and carotenoid metabolism as a foundation for the succeeding chapters on specific topics related more directly to dermatology. A more comprehensive and neurobiology-related version of this chapter was recently published, and may serve as a source for further references and details (12).

NOMENCLATURE, STRUCTURE, AND CHEMICAL PROPERTIES

The term "vitamin A" is defined as the generic descriptor for all C_{20}-β-ionone derivatives that qualitatively exhibit the biological activity of all-*trans*-retinol. The term "provitamin A" is restricted for the carotenoids giving rise to vitamin A (Fig. 1) (13). Chemically, vitamin A belongs to the "retinoids," which are defined as a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner. Thus, all retinoids may be formally derived from a monocyclic parent compound containing five carbon–carbon double bonds and a functional terminal group at the terminus of the acyclic portion. By this definition, retinoids would include both the naturally occurring forms of vitamin A as well as the many synthetic analogs of retinol, with or without biological activity. One problem with this definition is the fact that several synthetic compounds, which do not fit into the definition of retinoids have been shown to be much more active than retinol or retinoic acid in several assays for vitamin A or retinoid activity. It was therefore proposed (14) that "a retinoid should be defined as a substance that can elicit specific biologic responses by binding to and activating a specific receptor or set of receptors." In practice, most researchers today use a combination of these two definitions, that is, the class of retinoids consists of retinol analogs (with or without biologic activity) but also of several compounds, which are not closely related to retinol but elicit biological vitamin A or retinoid activity.

Several thousand synthetic retinoids have been developed. Synthetic agents that specifically bind to retinoid X receptors (RXR) are called rexinoids (15), whereas synthetic compounds that have lost the ring structure are called acyclic retinoids or acyclic rexinoids.

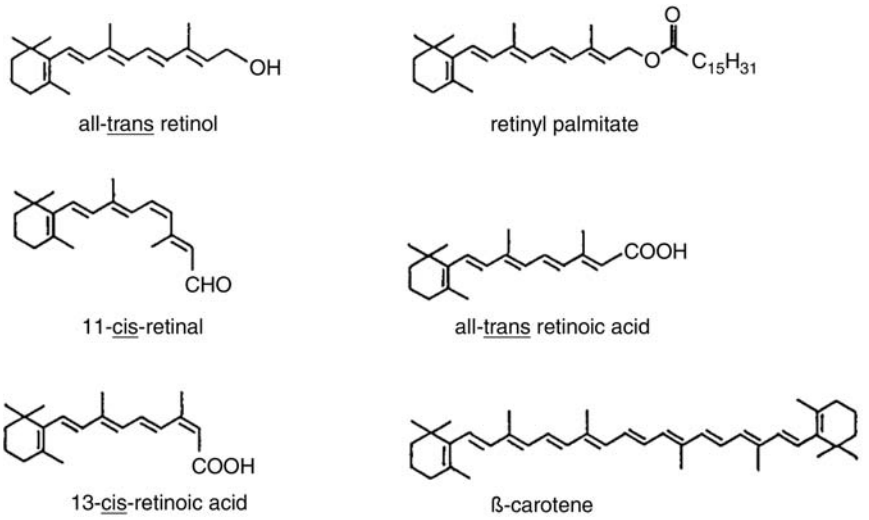

Figure 1 Structural formulas of some retinoids and β-carotene.

The parent retinoid compound all-*trans* retinol is a primary alcohol with molecular weight 286. In most animal tissues, the predominant retinoid is retinyl palmitate, but other fatty acid esters, such as retinyl oleate and retinyl stearate, are also found. Most of these metabolites occur in the all-*trans* configuration. The 11-*cis* aldehyde form, 11-*cis* retinal, is present in the retina of the eye, and several acid forms such as all-*trans* and 13-*cis* retinoic acid, are metabolites of retinol found in many tissues (Fig. 1). As discussed later, many more metabolites of retinol are detected in different cells and tissues. Still, too little is known about the role of these retinoids: they are presumably intermediates in the activation or degradation of retinol.

All-*trans* retinol and its derivatives are highly unstable in the presence of oxidants and light, which leads to their oxidative degradation or isomerization (16). These properties require tissues containing retinoids as well as retinoid standard solutions to be stored and handled experimentally in an inert atmosphere and under dim illumination (17,18).

Vitamin A (all-*trans* retinol) is a fat-soluble vitamin. Many binding proteins are therefore involved in transport of retinoids through hydrophilic phases such as plasma and extra- and intracellular fluids. Many retinoids, however, are also soluble to some extent in fluids such as plasma. The water solubility at room temperature and pH 7.3 of all-*trans* retinol, all-*trans* retinal and all-*trans*-retinoic acid is 60 nM, 110 nM, and 210 nM, respectively. This feature makes all-*trans* retinoic acid an ideal morphogen with ability to diffuse efficiently through water-soluble phases as well as hydrophobic membrane.

Originally, international units (IUs) (1 IU = 0.3 μg of all-*trans* retinol) were used for nutritional recommendations of vitamin A. Later, "retinol equivalents" (RE) were used to convert all sources of preformed retinol and provitamin A carotenoids in the diet into a single unit. When defining RE it was assumed that the absorption of provitamin A carotenoids was relatively efficient. Recent studies document, however, that absorption of carotenoids is much lower and appears to be quite variable. In addition, a number of factors such as protein-energy malnutrition, zinc-deficiency, dietary fat, alcohol, infections, and degree of food processing/food matrix affect the bioavailability and bioconversion of retinol and carotenoids. Based on these studies which are limited and not conclusive as yet, it is now generally assumed (19) that 1 retinol activity equivalent (RAE) is equal to 1 μg of dietary or supplemental preformed vitamin A (i.e., retinol), 2 μg of supplemental β-carotene, 12 μg of dietary β-carotene, or 24 μg of other dietary provitamin A carotenoids (e.g., α-carotene and β-cryptoxanthin).

DIETARY UPTAKE OF CAROTENOIDS AND VITAMIN A

No animal species are capable of de novo vitamin A synthesis. However, plants and some bacteria, algae and fungi can synthesize carotenoids, some of which can be converted to vitamin A in animals. The carotenoids represent a large group of pigments that are widespread in nature and responsible for the yellow, orange, red, or purple colors of many vegetables, fruits, and flowers (20). Many of these carotenoids can be absorbed and stored in animals, and often to such a degree that they give color to animal tissues. For example, lutein and zeaxanthin are concentrated in human macula, lycopene in human prostate, β-carotene in bovine corpus luteum and chicken egg yolk, astaxanthin and canthaxanthin in salmon flesh and flamingo feather (21). Animals and plants can cleave the carotenoids to form biologically active molecules, such as abscisic acid in plants, trisporic acid in fungi and retinoids in animals (22). Thus, carotenoids including α-carotene, β-carotene and β-cryptoxanthin are in animals like Drosophila, fish, chicken, mice, and humans, converted into retinal or apocarotenoids (which subsequently can be converted to retinoids), and animals can thereby obtain compounds with vitamin A activity from the diet.

As an alternative animals can obtain vitamin A from the diet by eating tissues from animals that already have converted the provitamin A carotenoids into retinoids. Thus, since retinyl esters, and to a lesser extent retinol, accumulate in fish-, avian-, and mammalian livers as well as in other animal tissues, these retinoids also contribute to the dietary intake of vitamin A. In Western countries, the intake of preformed retinyl esters or retinol typically account for 25% to 75% of the total vitamin A intake, with the rest being provided by provitamin A carotenoids (23). Retinoic acid from animal sources does not significantly contribute to the daily intake of vitamin A, since animal tissues typically contain only 3 to 15 μg retinoic acid per kg (24) (typical dietary retinol intake is about 1 mg/day for adults).

Intestinal Absorption of Carotenoids

Uptake of carotenoids by enterocytes in the small intestine is by passive diffusion. The efficiency of carotenoid absorption may however fall with increasing intake. Moreover, uptake in vitro by Caco-2 cells also indicates that a saturable process may be involved (25).

In 1930, Moore described (26) that β-carotene can be converted to retinoids in the small intestine. Shortly after, Karrer et al. (27) proposed a central cleavage mechanism at the 15,15′ carbon double bond. The biochemical characterization of this enzyme activity was independently described in the laboratories of Dewitt Goodman (28) and James Allen Olsen (29). Both laboratories were able to show that this cleavage yielded two molecules of retinal. As they observed that the enzymatic activity was dependent on molecular oxygen, but no other cofactors were required, the enzyme was termed β,β-carotene-15,15′-monooxygenase. The molecular cloning of this enzyme was however reported only recently. In 2000, von Lintig and Vogt (30) cloned the enzyme in Drosophila, and Wyss et al. (31) cloned the enzyme in chicken. More recently, several cDNAs encoding mammalian counterparts have been reported (32–35).

Another mechanism of carotene cleavage has also been identified. Both the original observation by Glover and Redfearn (36), as well as more recent data (37,38) suggest that an asymmetric cleavage occurs. This so-called eccentric cleavage leads to the formation of two molecules of β-apocarotenals with different chain length. The longer of the two can subsequently be shortened enzymatically into retinoic acid or retinal. The enzyme responsible for this eccentric cleavage was recently cloned by Kieder et al. (39). This enzyme, β,β carotene 9′,10′-dioxygenase, cleaves β-carotene but also other carotenoids such as lutein.

Retinal, formed in the intestine as a product of central or asymmetric cleaving of carotenoids, is subsequently reduced to retinol. Although several retinal reductases are able to catalyze this reduction in vitro, the in vivo intestinal retinal reductase activity remains to be identified (40).

Intestinal Absorption of Retinol and Retinyl Esters

Dietary retinyl esters are enzymatically converted to retinol in the intestinal lumen prior to uptake in the enterocytes (Fig. 2). Pancreatic triglyceride lipase and the intestinal brush border enzyme phospholipase B are responsible for this hydrolysis. The unesterified retinol is taken up by enterocytes by a saturable carrier-mediated process, but the proteins involved in this uptake have not been identified and characterized. After ingestion of pharmacological doses of retinol or retinyl esters, a nonsaturable diffusion-dependent process may also be involved. The mechanism involved in the absorption of dietary vitamin A has recently been reviewed by Harrison (23).

Secretion into Lymph and Portal Circulation

In enterocytes, retinol is bound to cellular retinol-binding protein type II (CRBP-II). CRBP-II is expressed primarily in the absorptive cells in the intestine and

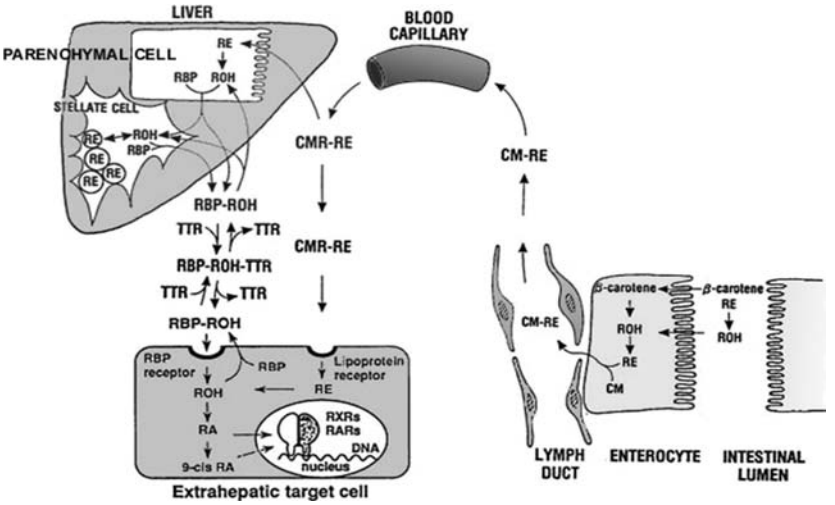

Figure 2 Major pathways for retinoid transport in the body. *Abbreviations*: CM, chylomicron; CMR, chylomicron remnant; RA, retinoic acid; RAR, retinoic acid receptor; RBP, retinol-binding protein; RE, retinyl ester; ROH, reinol; RXR, retinoid X receptor; TTR, transthyretin.

accounts for approximately 1% of the total soluble proteins in the mucosa (41). Studies with CRBP-II knockout mice have demonstrated that CRBP-II plays an important, but not essential, role in the intestinal absorption of vitamin A, since knockout mice maintained on a vitamin A-enriched diet have reduced their hepatic stores of retinyl esters (and thus presumably the absorption of vitamin A) by about 40% compared to wild type mice given the same diet (42).

Most of retinol is re-esterified with long chain fatty acids (mainly palmitate) in the enterocytes after absorption. Binding of CRBP-II to retinol facilitate retinol esterification by the enzyme lecithin:retinol acyl transferase (LRAT) (43). The role of CRBP-II is to solubilize the fat-soluble retinol, to protect retinol from degradation, and most importantly to direct retinol to the enzyme LRAT. As only trace levels of retinyl esters were found in tissues of LRAT knockout mice, this enzyme seems to be important for retinol esterification (44).

Retinyl esters and carotenoids are then incorporated into chylomicrons (45), and these large lipoprotein complexes (100–2000 nm in diameter) are subsequently secreted from the enterocytes into the intestinal lymph (46). Although most of the absorbed vitamin A is secreted into lymph as chylomicron retinyl esters, a significant amount is also secreted into portal circulation as unesterified retinol (23). The portal absorption is likely to be important for pathological conditions that affect secretion of chylomicrons, such as abetalipoproteinemia.

LIVER METABOLISM OF RETINOL

Following secretion of chylomicrons into the lymph these lipoprotein particles move into the general circulation, where several processes such as triacylglycerol hydrolysis and apolipoprotein exchange result in the formation of chylomicron remnants. Almost all retinyl esters present in the chylomicrons remain with the particle during conversion to chylomicron remnants (46). The chylomicron remnants are mainly cleared by the parenchymal cells of the liver (i.e., hepatocytes) (47,48). Extrahepatic uptake of chylomicron remnants may, however, also be important in the delivery of retinyl esters and carotenoids to tissues such as bone marrow, peripheral blood cells, spleen, adipose tissue, skeletal muscle, and kidney (49) (Fig. 2).

In hepatocytes, the retinyl esters are hydrolyzed. Unesterified retinol may associate with retinol-binding protein (RBP), a protein found in high concentration in hepatocyte endoplasmic reticulum (50). Binding of retinol to RBP apparently initiates a translocation of retinol-RBP from endoplasmic reticulum to the Golgi complex, followed by secretion of retinol-RBP into plasma (51). Additionally, a large portion of unesterified retinol in hepatocytes is also transferred to perisinusoidal stellate cells (52,53). In fact, in vitamin A-sufficient states, most of the chylomicron remnant retinyl esters taken up by hepatocytes are transferred as retinol to perisinusoidal stellate cells in the liver for storage.

In mammals, 50% to 80% of the body's total retinol is normally present in the hepatic stellate cells (54,55). More than 95% of the stellate cell vitamin A is present in the form of retinyl esters packed together in cytoplasmic lipid droplets. Retinyl esters account for 30% to 50% of the content of lipid droplets (Fig. 2).

CRBP-I (a cellular binding protein with homology to CRBP-II) and LRAT are highly expressed in hepatic stellate cells. These proteins play key roles in the storage of retinyl esters, since mice lacking CRBP-I or LRAT have impaired storage of stellate cell retinyl esters (56,57). The normal reserve of retinyl esters in stellate cells represent an adequate supply of vitamin A for several weeks or months (58). This extensive storage of retinyl esters in stellate cells ensures a steady blood plasma retinol concentration of about 1 to 2 μM. The mechanism responsible for retinol mobilization from stellate cells has not been elucidated in detail. Results from knockout mice indicate, however, that RBP is involved (59).

TRANSPORT OF RETINOIDS AND CAROTENOIDS IN PLASMA

Kanai et al. in Goodman's laboratory demonstrated that retinol in plasma is bound to a specific RBP (60). The plasma concentration of retinol-RBP is strictly regulated and maintained at about 2 μM despite fluctuations in daily dietary intake of vitamin A. Only in severe vitamin A-deficiency, that is, when liver retinyl esters are depleted, is the concentration of plasma retinol-RBP decreased. Approximately 95% of the plasma RBP is associated with transthyretin (TTR) (61), a binding that

reduces the glomerular filtration of retinol. The targeted disruption of the RBP gene result in mice with plasma retinol levels one-tenth of wild-types, reduced retinol and retinyl ester levels in retina, and increased liver stores of retinyl esters (presumably in stellate cells) (62). In such mice, impaired retinal function and visual acuity is observed during the first months of life. Surprisingly, adult RBP knockout mice fed a vitamin A-sufficient diet are phenotypically normal. However, when these mice are fed a vitamin A-deficient diet, impaired vision appears (63). The mechanism for cellular uptake of retinol has been an issue of great controversy (12,45,59), but the recent cloning of a cell-surface receptor for RBP is an important step in resolving the dispute (64).

A number of retinoids, in addition to retinol and retinyl esters, are present in plasma at nanomolar concentrations (about 5–10 nM). These include all-*trans* retinoic acid, 13-*cis* retinoic acid, 13-*cis*-4-oxo retinoic acid, all-*trans*-4-oxo retinoic acid, and all-*trans* retinoyl β-glucuronide (65,66). With the exception of all-*trans* retinoyl β-glucuronide, these retinoids are believed to be transported in plasma bound to albumin. The level of most of these retinoids is dependent on the intake of vitamin A and will typically increase two to four times after ingestion of a large amount of vitamin A (67).

Carotenoids are transported in plasma bound to lipoproteins. They are absorbed from the diet and enter the lymph in association with chylomicrons, and they follow the chylomicrons mainly to the liver. The carotenoids do not accumulate in liver cells, but are mobilized as components of the VLDL particles, which are converted to VLDL remnants and LDL in the circulation by a process resembling the chylomicron remnant formation (68). Since many cell types have the capability to synthesize retinol, retinal, or retinoic acid from provitamin A carotenoids, lipoprotein-mediated uptake of carotenoids may be important for cellular functions of vitamin A.

CELLULAR FORMATION OF RETINAL AND RETINOIC ACID

Most active retinoid metabolites are synthesized in target cells. The major source of this synthesis in higher vertebrates is all-*trans* retinol taken up from plasma. However, cellular uptake of lipoproteins containing retinyl esters, retinol, and carotenoids, as well as retinyl esters locally stored in lipid droplets in the target cells themselves or neighboring cells may also contribute to synthesis of active retinoid metabolites. Additionally, cellular uptake of all-*trans* retinoic acid or its metabolites from plasma may also contribute (Fig. 3).

All-*trans* retinoic acid is the major active cellular retinoid metabolite, and the synthesis of all-*trans*-retinoic acid from all-*trans* retinol occurs in a two-step reaction. The rate-limiting step in this process is the oxidation of retinol to retinal, and the final step is the oxidation of retinal to retinoic acid. Several cells are also able to catalyze the reverse reaction from retinal to retinol (Fig. 3). The major function of retinoic acid is to act as an activator of transcription factors (see subsequently).

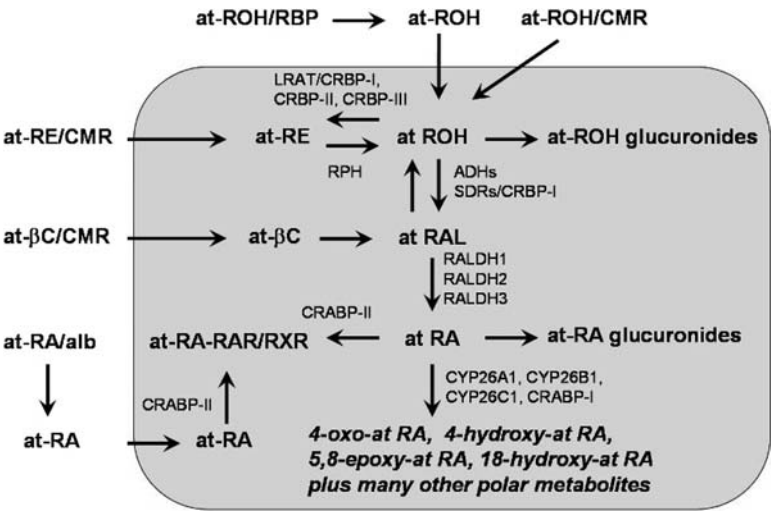

Figure 3 Major pathways for cellular retinoid metabolism in nonvisual cells. *Abbreviations*: βC, β-carotene; RA, retinoic acid; RAL, retinal; RAR, retinoic acid receptor, RE, retinyl ester; ROH, retinol; RXR, retinoid X receptor.

OXIDATION OF ALL-*TRANS* RETINOL TO ALL-*TRANS* RETINAL

Cytosolic Medium-Chain Alcohol Dehydrogenases

Ample data suggest that alcohol dehydrogenases (ADHs), such as ADH1, ADH3, and ADH4 are involved in oxidation of all-*trans* retinol forming all-*trans* retinal. They are all able to oxidize all-*trans* retinol in vitro, with ADH4 being the most efficient. Mice lacking ADH3 demonstrate reduced survival and a growth defect that can be rescued by dietary retinol supplementation, whereas the effect of loss of ADH1 and ADH4 is noticed only in mice subjected to vitamin A excess or deficiency, respectively (69). The ADH3 enzyme is ubiquitously expressed, whereas the expression pattern of ADH1 and ADH4 suggest a more tissue-specific function of these proteins. The ADHs do not seem to oxidize all-*trans* retinol bound to CRBP-I.

Membrane-Bound Short-Chain Dehydrogenase/Reductase

Oxidation of retinol to retinal appears also to be catalyzed by members of the short-chain dehydrogenase/reductase (SDR) family of microsomal enzymes including RDH1, RDH5, RDH11, CRAD1, CRAD2, CRAD3, and retSDR1 (69). The SDRs utilize all-*trans*-retinol bound to CRBP-I as substrates. They are expressed in many vitamin A sensitive cell types and are often coexpressed with CRBP-I. CRBP-I acts as a chaperone for retinol and retinal and directs them to these metabolizing enzymes (70) (Fig. 3).

Oxidation of All-*trans* Retinal to All-*trans* Retinoic Acid

It has been established that several enzymes are able to oxidize retinal to retinoic acid. Retinal dehydrogenase 1 (RALDH1, also called ALDH1A1, Adh-2, and ALDH1) is expressed at very high levels in the dorsal retina of embryos and in several adult epithelial tissues (71). Only minor effects are observed in the dorsal retina and its axonal projections in RALDH1 knockout embryos, suggesting that this enzyme is not essential for retinoic acid synthesis in most tissues (72). Instead it has been suggested that RALDH1 is involved in the catabolism of excess retinol.

RALDH2 (also called ALDH1A2 and V2) is expressed in many cell types, both of embryonic and adult tissue origin. In mice embryos RALDH2 is localized in mesenchymal cells including trunk mesoderm, proximal limb bud, and lung bud mesoderm, as well as in heart (73). RALDH2 null mice die in utero due to defects in heart development. Retinoic acid is able to rescue RALDH2 knockout embryonic development to a considerable extent (74). Thus, it appears that RALDH2 is responsible for the production of retinoic acid in several cell types during embryonic development.

RALDH3 (also called ALDH1A3, V1, and ALDH6) is expressed in mouse and chicken retina, lens, and olfactory pit, as well as ureteric buds and surface ectoderm over the developing forebrain. RALDH3 null mice die from defects in nasal development within 10 hours of birth, implying that it is essential for retinoic acid synthesis (75). Furthermore, this embryonic malformation can be prevented by a simple maternal treatment with retinoic acid. These data indicate different organs and cells use different mechanisms (i.e., RALDH1, RALDH2, or RALDH3) to meet their need for retinoic acid synthesis (76).

RALDH4 that is expressed in mouse liver and kidney, is about two-orders of magnitude more active in vitro with 9-*cis* retinal than with all-*trans* retinal (77).

SYNTHESIS OF RETINOIC ACID FROM CAROTENOIDS

Retinoic acid may also be synthesized from β-carotene in organs, such as intestine, liver, kidney, and lung, without prior oxidation of β-carotene to retinol (78,79). Thus, β-carotene and other carotenoids may be a source of retinoic acid particularly in species, such as humans that are capable of accumulating high concentrations of tissue carotenoids (Fig. 3).

CELLULAR CATABOLISM OF RETINOIDS

The catabolism of all-*trans*-retinoic acid is an important mechanism of controlling retinoic acid levels in cell and tissues. White et al. (80) first cloned a zebrafish cytochrome P450 enzyme (CYP26A1), which was able to degrade all-*trans* retinoic acid to polar metabolites, including 4-hydroxy retinoic acid, 4-oxo retinoic acid, 18-hydroxy retinoic acid, 5,6-epoxy retinoic acid and 5,8-epoxy retinoic

acid. The enzyme was subsequently cloned in human, mouse, rat, and chicken (81). CYP26A1 was shown to be present at the highest levels in the liver, duodenum, colon, and placenta and in some regions of the brain (82,83). The proximal upstream promoter region of the CYP26A1 gene contains a functional retinoic acid response element (RARE), and the transcripts are therefore induced by retinoic acid. Thus, these results imply a direct mechanism through which the CYP26A1 gene may sense the concentration of retinoic acid and regulate the oxidative metabolism of all-*trans*-retinoic acid accordingly.

A second cytochrome P450 enzyme, called CYB26B1, was subsequently cloned. This enzyme has a different tissue specific expression pattern than CYP26A1, but the two enzymes have a similar catalytic activity (84).

More recently, a third enzyme, CYP26C1 was cloned (85). Transiently transfected cells expressing CYP26C1 convert all-*trans* retinoic acid to polar water-soluble metabolites similar to those generated by CYP26A1 and CYP26B1. However, CYP26C1 can catabolize 9-*cis* retinoic acid much better than CYP26A1 and CYP26B1. CYP26C1 is not widely expressed in the adult but seem to be inducible by retinoic acid. The expression pattern of CYP26A1, CYP26B1, and CYP26C1 is generally nonoverlapping suggesting individual roles for each of the CYP enzymes in the catabolism of retinoic acid (86).

Also glucuronides may be formed from retinol and retinoic acid, probably destined for excretion in bile and urine (87). Notably, several of the putative degradation products, including 4-hydroxy retinoic acid, 4-oxo retinoic acid, 18-hydroxy retinoic acid, and retinoyl beta-glucuronide are still biologically active (88,89).

Cellular retinoic acid binding protein type I (CRABP-I) seems to be involved in regulating retinoic acid degradation. Specifically, Boylan and Gudas (90,91) showed that overexpression of the CRABP-I protein in transfected F9 stem cell lines resulted in a higher degradation of retinoic acid and thus lower sensitivity to retinoic acid compared with untransfected cells.

NUCLEAR RETINOIC ACID RECEPTORS ARE LIGAND INDUCIBLE TRANSCRIPTION FACTORS

Six different genes coding for nuclear retinoid receptors have been cloned to date: three retinoic acid receptors (RAR) and three RXR. These receptors belong to the family of steroid/thyroid hormone receptors (92–96), which all are ligand-dependent transcription factors. The nuclear RARs function as heterodimers (one of the RARs complexed with one of the RXRs), and possibly also as a homodimer of two RXRs, binding to DNA sequences called RAREs or retinoid X response elements (RXREs) located within the promoter of target genes. RAREs consist of direct repeats of the consensus half-site sequence "(a/g)g(g/t)tca" separated most commonly by five or two nucleotides (DR5 and DR2, respectively), whereas RXREs are typically separated most commonly with one nucleotide spacing (DR1) (97,98).

In vitro binding studies have demonstrated that all-*trans* retinoic acid and 9-*cis* retinoic acid, but not 13-*cis* retinoic acid, are high affinity ligands for RARs, whereas only 9-*cis* retinoic acid binds with high affinity to RXRs (97). The most important physiological ligand for the RAR-RXR heterodimer seems to be all-*trans* retinoic acid binding to the RAR heterodimer partner. The physiological role of 9-*cis* retinoic acid has been questioned.

Important information about the role of the various RARs and RXRs has been obtained from studies where one or several of the receptor genes have been deleted from mice. Interestingly, many, but not all symptoms of the vitamin A deficiency syndrome can be recapitulated in such mice (99). When mutations only affect one receptor, the mice survive and the abnormalities are limited, suggesting a functional redundancy between various receptors and isoforms. In double mutant mice lacking either two RAR subtypes or both RAR and RXRα, the animals do not survive and the abnormalities are more pronounced (100).

Over the last quarter century, more than 500 genes have been suggested to be regulatory targets of retinoic acid. In some cases, the regulation of these genes has been shown to be direct, driven by a liganded RAR-RXR heterodimer bound to a DNA response element. In many cases, however, the gene regulation appears to be indirect, reflecting the actions of intermediate transcription factors, nonclassical associations of receptors with other proteins, or even more distant mechanisms. Twenty-seven genes are unquestionably direct targets of the classical RAR-RXR-RARE pathway in permissive cellular contexts. About 100 other genes appear as good candidates, but firm conclusions must await further investigations (101).

EFFECTS OF RETINOIC ACID-RAR/RXR COMPLEXES ON GENE TRANSCRIPTION UNRELATED TO RARES

RARs modulate transcription through several distinct mechanisms, which include both activation and repression activities. These activities can be genomic, mediated through RARE or non-RARE binding sites, or nongenomic, through mechanisms that are either ligand-dependent or ligand-independent. Interestingly, we recently identified more than 100 target genes to be indirectly regulated, that is, not through binding to classical RAREs (102).

Within the last few years it has been shown that retinoic acid-RAR/RXR complex can regulate gene expression independently of an RARE, that is, through regulating the transactivation of other transcription factors. For example, retinoids exhibit antineoplastic activities that may be linked to retinoid receptor-mediated transrepression of activator protein 1 (AP1), a heterodimeric transcription factor composed of fos- and jun-related proteins. It has been observed that liganded RAR-RXR complexes inhibit c-jun DNA binding (103), and sequester and thereby limit the availability of CBP for AP1 (104). The transactivation of RARE and transrepression of AP1 can be dissociated: Chen et al. observed (105) that synthetic retinoids that specifically induce transactivation by RAR-β and

antagonized transactivation by RAR-α and RAR-γ, all allow all three RAR types to repress AP1 activity. Similar observations have been done in different experimental systems (106). Additionally, RAR mutants with altered transactivation ability have retained their AP1 transrepression properties (107).

The transcription factor nuclear factor kappa-B (NF-κB) has also been shown (108,109) to be modified by retinoic acid. A transient induction of the NF-κB family of transcription factors is essential for an appropriate immune- and inflammatory reaction. Thus, dysregulation of NF-κB activity is a feature of chronic inflammatory diseases, atherosclerosis and some cancers (110). Several reports have indicated that vitamin A deficiency promotes inflammatory reactions (111). Furthermore, retinoic acid has recently been shown to inhibit several types of inflammatory reactions in experimental model systems (112–114). Inappropriate inflammatory reactions could therefore explain several of the symptoms associated with the vitamin A deficiency syndrome. We have recently generated a transgenic NF-κB-reporter mouse model, that enables noninvasive molecular imaging of NF-κB in vivo (115). Using this model, we have shown that administration of retinoic acid to vitamin A deficient or vitamin A sufficient mice, transiently represses NF-κB activity by about 40%, and that basal and UV-B induced NF-κB activity is elevated in vitamin A deficient mice (116). We have also observed that retinoic acid inhibits lipopolysacarides (LPS)-induced NF-κB activation, and that vitamin A deficiency potentiates LPS-induced NF-κB activation (unpublished data). Thus, the defective immune- and inflammatory reaction observed in vitamin A-deficiency is likely to involve dysregulation of NF-κB.

NONGENOMIC MECHANISMS OF ACTION OF RETINOIC ACID

All-*trans*-retinoic acid modulates the activity of protein kinase C (PKC), a protein that regulates fundamental cellular functions including proliferation, differentiation, tumorigenesis, and apoptosis. Based on crystal structure analysis and binding experiments it was suggested that all-*trans*-retinoic acid decreased PKCα activity through direct binding to PKC isozymes (117). More recently, Ochoa et al. demonstrated that all-*trans* retinoic acid binds to the regulatory C2-domain of PKCα, and that competition between binding of all-*trans* retinoic acid and acidic phospholipids to PKC is a possible mechanism by which all-*trans* retinoic acid modulates PKCα activity (118).

MECHANISM OF ACTION OF RETRO-RETINOIDS, RETINOL, AND OTHER RETINOIDS

Most retinoids contain five double bonds located between carbon atoms 5-6, 7-8, 9-10, 11-12, and 13-14. The "standard" position of the double bonds may be shifted under certain conditions, however, forming biologically active retinoids called retro-retinoids. The first retro-retinoid identified was anhydroretinol, a major metabolite formed when various types of cells are incubated with retinol

(119). Several retro-retinoids seems to have biological activity that is distinct from that of retinoic acid (120).

Buck and Hammerling et al. observed that retinol, but not retinoic acid, is an essential cofactor for growth of B lymphocytes and for activation of T lymphocytes (121). They demonstrated that the retinol metabolite 14-hydroxy-4,14-retro-retinol (14-HRR) is responsible for the effects, and that anhydro-retinol reversed the effects of 14-HRR (122,123). Similar effects have been observed in fibroblasts and promyelocytes: 14-HRR is a growth factor, whereas anhydro-retinol is an antagonist of the growth-promoting effect of 14-HRR.

Nearly all cells in the body have the ability to convert all-*trans* retinol to HRR, and this biochemical capacity seem to be conserved from insects to humans. The cellular targets mediating the effects of HRR and another nonacidic retinol metabolite, 13,14-dihydroxy-retinol (124) were recently suggested to be members of the serine/threonine kinase family, cRaf and PKC, which all bind retinoids with nanomolar affinity (125).

In dermatology, the binding of specific retinoids to RARs or RXRs does not match their therapeutic efficacy. Thus it is believed that nongenomic effects of retinoids may explain many of the activities in the skin (126).

RETINOYLATION OF PROTEINS

An additional mechanism for retinoid action has been suggested (127–129). Retinoic acid may be covalently linked to certain regulatory proteins via, for example, a thioester bond. The number of retinoylated proteins are rather small, but include important proteins like the regulatory subunits of cAMP-dependent protein kinase, cytokeratins, and ribonucleotide reductase (130–135). It is interesting to note that other members of the isoprenoid family (which include farnesol, geranylgeranol, and retinoids) have also been found covalently linked to regulatory proteins such as yeast mating factors, G-proteins, and *ras* oncogenes, and to affect the function of these proteins (136,137).

PHYSIOLOGICAL FUNCTIONS OF RETINOIC ACID

Although there are many indications that all-*trans* retinoic acid does not mediate every effect of vitamin A, the role of all-*trans* retinoic acid as a ligand for nuclear RARs is by far the best studied nonvisual mechanism of action of vitamin A. Retinoic acid is a key factor in the development of different vertebrate tissues and organs, due to its ability to promote differentiation (138,139) and regulate apoptosis (140,141) and its role in the specification of positional information of cells and tissue patterning (142). Retinoic acid was the first morphogen to be identified in vertebrates (143), and numerous studies have elucidated the important role of retinoic acid in many physiological processes including limb development (144), lens development and regeneration (145), lung development and regeneration (146), development of the central nervous system (147–149) reproduction (150), hematopoiesis (151) as well as

pathological conditions such as cancer (97,152,153), skin diseases (154,155), premature birth (156), rheumatoid arthritis (157), and osteoporosis (158).

TOXICITY OF RETINOIDS

The classical signs of hypervitaminosis A that occurs following an excessive dietary intake or as a consequence of an intake of drugs containing large doses of specific retinoids, may occur in skin, nervous system, musculo-skeletal system, circulation, internal organs as well as the fetus (159–163). It has also been suggested that intake marginally above the recommended dietary intake is associated with embryonic malformations (164), reduced bone mineral density and increased risk for hip fracture (165). Retinoid intoxication, especially from pharmaceutical drugs, may also have additional manifestation. Recently, it was observed that 13-*cis* retinoic acid, which is a naturally occurring retinoids, but also is used in several drugs (isotretinoin) may inhibit the visual cycle as well as reducing spatial memory learning.

13-*cis* Retinoic Acid May Inhibit the Visual Cycle

Vertebrate phototransduction is initiated by a photochemical reaction where 11-*cis*-retinal bound to its opsin moiety is isomerized to all-*trans*-retinal producing conformation changes in opsin. In vertebrates, restoration of a photosensitive receptor conformation (return to the dark state) requires the formation of 11-*cis*-retinal from all-*trans*-retinal via the retinoid cycle (Fig. 4). The retinoid

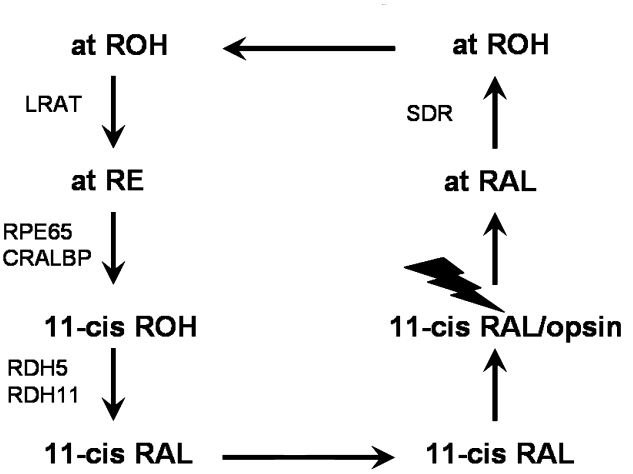

Figure 4 The retinoid visual cycle. *Abbreviations*: RAL, retinal; RE, retinyl ester; ROH, reinol.

cycle that involves reactions in preceptor cells as well as retina pigment epithelial cells (RPE), has recently gained much attention owing to the fact that multiple genes encoding components of this cycle have been identified, cloned and linked to retinal diseases.

In RPE, all-*trans* retinol is esterified in an LRAT-mediated reaction, which transfers an acyl group from lecithin to retinol (166,167). The all-*trans* retinyl esters are mobilized by RPE65, an abundant protein in the RPE that bind the esters with high affinity (168). RPE65 also binds 13-*cis* retinoic acid. Interestingly, high doses of 13-*cis* retinoic acid are used in the treatment of dermatological diseases and cancer, and are known to cause night blindness. The binding of the 13-*cis* retinoic acids to RPE65 is competitive with all-*trans*-retinyl ester binding, and this competition inhibits visual cycle function (169).

Effect of 13-*cis* Retinoic Acid on Spatial Memory Learning

In the adult brain, retinoic acid has been shown to be involved in synaptic plasticity of the hippocampus. Retinoic acid is important for acquisition of spatial and relational memory (170–175), for maturation of learned behaviours (e.g., song learning in birds) (176,177), in the control of dopamine signaling in mesolimbic and mesostriatal neurons, as well as in the survival of nigrostriatal dopaminergic neurons (178,179). Recently, it was observed that long-term exposure of adult mice to pharmacological doses of 13-*cis* retinoic acid, reduces cell proliferation in the subventricular zone and in the hippocampus (180), and thereby reduces the generation of neurons and decreased ability of spatial memory learning.

CONCLUSION

During the last 25 years tremendous progress has been made in our understanding of retinoid and carotenoid physiology and biochemistry, as well as cellular and molecular biology. We have learned that retinoids and carotenoids stand out as exceptional dietary compounds with more complexity and multiplicity than other micronutrients and phytochemicals. Although the groundbreaking discoveries in retinoid and carotenoid research give great promise for future progress in several clinical areas including dermatology, we predict that the emerging field of systems biology will revolutionize our conception of the role of retinoids and carotenoids in human health and disease.

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Animal Models for Retinoid Receptor Research: Implications for Epidermal Homeostasis, Skin Barrier Function, Wound Healing, and Atopic Dermatitis

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MOUSE AS A MODEL TO UNVEIL THE FUNCTIONS OF RETINOID RECEPTORS

The mouse, whose genome is similar to that of humans both in size and gene number, is considered as an excellent animal model for defining the functions of human genes as it exhibits reproductive, developmental, physiological, and pathological similarities to man. The generation of targeted mutations in the mouse genome is expected to play a crucial role in the elucidation of the physiological functions of mammalian genes and in the characterization of genetic factors involved in human pathologies (1). The introduction of targeted mutations in the germ line of the mouse has markedly advanced our understanding of the roles played by a number of gene products during development and adult life. However, this approach has some inherent limitations, as the lack of a protein that serves essential functions during development may result in early lethality, possibly in utero, thus precluding analysis of its possible functions at subsequent stages. Furthermore, numerous genes exert multiple functions in distinct cell types during ontogeny and in postnatal life (pleiotropy). Germline ablation of such genes may result in complex phenotypes, in which it may be impossible to distinguish events

that involve cell-autonomous effects from those more complex that involve cell-nonautonomous effects. The outcomes of a germ line mutation may also be compensated during development, thus preventing the appearance of an abnormal phenotype at subsequent stages. In addition, in the case of closely related genes belonging to a multi-gene family, the mutation of a given member of the family may not result in an abnormal phenotype, due to functional redundancies among the family members (possibly artefactually generated by the mutation), thus precluding the identification of the function of individual members of the family and requiring the generation of compound mutants to reveal the function(s) played by the gene family. Defining the role of individual family members may become even more difficult when the family is involved in highly pleiotropic signalling pathways, such as those of nuclear receptors, for example, retinoid receptors (2–5). Other potential effects confounding conventional germline knockouts include the risk of impaired fertility and generalized, systemic disorders (6,7). In many instances, these limitations in targeted germ line mutagenesis prevent the determination of the function of a given gene product in a defined subset of tissue/cell-type at any given time during the animal's life. Moreover, germline knockouts prevent engineering of mouse models for human diseases that are caused by somatic mutations, particularly when these diseases result, as in most cancers, from a combination of somatic mutations (8).

To overcome these limitations, methods to achieve spatial or temporal conditional gene inactivation have been developed, based on tissue/cell-type specific or inducible expression of the bacteriophage P1 site-specific Cre recombinase that can efficiently excise a DNA segment flanked by two *lox*P sites (floxed DNA) in animal cells (9). Spatially- (10–13) or temporally-controlled (14) somatic mutations were thus obtained by placing the Cre gene under the control of either a tissue/cell-specific or an inducible promoter, respectively. However, these conditional gene targeting systems still have a number of limitations, as they are either spatially- or temporally-controlled (15–17). Ideally, one would like to have a system that allows generation of somatic mutations targeted to defined genes, at a given time in the life of the animal, and in a specific cell-type. The first step in this direction was achieved a decade ago with the generation of chimera fusion proteins, in which the Cre recombinase was fused to the ligand binding domain (LBD) of the human estrogen receptor alpha (ERα), yielding a recombinase (CreERα), whose activity was specifically induced upon addition of estrogen (18,19). The replacement of the wild-type ERα LBD by mutant forms that bind tamoxifen (TAM), but not estrogens, yielded the chimera proteins CreER^{T} (20) and CreER^{T2} (21), whose Cre recombinase activity was efficiently induced in the mouse upon TAM administration (20,22). A number of transgenic mice specifically expressing CreER^{T} or CreER^{T2} in a variety of tissue/cell-type was subsequently generated, allowing the introduction of targeted cell type-specific temporally-controlled somatic mutations in floxed genes (23,24).

In this chapter, we illustrate how germline and conditional mutagenesis, combined with pharmacological approaches, allowed us to uncover some of the

physiological functions that are controlled by retinoid nuclear receptors in the postnatal mouse skin.

IS THE SKIN A TARGET TISSUE FOR RETINOIDS?

The skin is composed of the epidermal layer and its appendages (hair follicles), which are separated from the dermal layer by a basement membrane. The epidermis, a stratified epithelium made principally of keratinocytes, is a highly dynamic structure (25). The inner most basal layer that is attached to the basement membrane is a proliferative layer, from which keratinocytes periodically withdraw from the cell cycle and commit to terminal differentiation, while migrating outward into the next layers (suprabasal layers), known as the spinous and granular layers. Terminally differentiated (squamous) keratinocytes form the cornified layer or stratum corneum. Some squamous keratinocytes are lost daily from the surface of the skin, and are continuously replaced by differentiating cells vectorially moving toward the skin surface. Hair follicles that develop through a series of mesenchymal–epithelial interactions during embryogenesis are also dynamic structures. They are mostly composed of keratinocytes, and their outer root sheath (ORS) is contiguous with the epidermal basal layer. Once formed, hair follicles periodically undergo cycles of regression (catagen), rest (telogen), and growth (anagen), through which old hairs are eventually replaced by new ones (26–29). Ligand deprivation studies both in vitro (cell in cultures) and in vivo have indicated that the active retinoid derivatives [retinoic acids (RAs)] of vitamin A can play functions in growth, differentiation and maintenance of mammalian epidermis and hair follicles (30,31). For example, depleting a secretory epithelium of retinoids in vivo causes its transformation into a squamous epithelium resembling epidermis (32). Along these lines, the response of epidermal keratinocytes to retinoids is different depending on experimental conditions. In vitro, removal of retinoids from the culture medium induces keratinocyte terminal differentiation, whereas their addition suppresses differentiation (33). In vivo, vitamin A-deficiency exacerbates differentiation yielding hyperkeratosis (34), whereas topical treatment of normal skin with RAs or synthetic active retinoids reduces differentiation and induces epidermal hyperplasia (30). It is noteworthy that even though the pharmacological effects of retinoids have been studied more in epidermis than in any other tissue, the cell-type in which and the mechanism by which RA regulates gene expression in epidermis has been elusive until recently.

The generation of the RA signal from vitamin A (retinol) is tightly spatiotemporally controlled throughout life. At any time the RA level is under the control of complex metabolic machinery that involves enzymes required for RA synthesis and catabolism. The local conversion of vitamin A to RA involves two sequential oxidation steps: (*i*) generation of retinaldehyde from vitamin A that is essentially carried out by the ubiquitously expressed alcohol dehydrogenase type 3 (35) and (*ii*) synthesis of RA from retinaldehyde that is catalyzed by retinaldehyde dehydrogenases (RALDH). At least three isotypes (RALDH1 to RALDH3) are

able to synthesize RA (36). Using antibodies, RALDH2 has been detected in the mouse interfollicular epidermis and RALDH3 in the hair follicle during anagen (37). The RALDH-mediated irreversible conversion of retinaldehyde to RA creates a situation in which RA is committed either to activate receptors to regulate RA-responsive genes or to be degraded into inactive forms. This catabolism is performed by cytochrome P450 hydroxylases belonging to the CYP26 family (38). Actually, the spatio-temporally controlled patterns of RALDHs and CYP26s expressions generate areas with active and inactive RA signaling pathways, respectively (39). In mouse epidermis, both CYP26A1 and CYP26B1 can be detected by reverse transcription of RNA coupled to polymerase chain reaction (unpublished data), while only CYP26B1 can be visualized by in situ hybridization in the interfollicular epidermis (40). In addition, inside cells vitamin A binds to cellular retinol binding proteins (CRBPs) that interact with enzymes involved in retinoid metabolism, such as the esterifying lecithin–retinol acyl transferase enzyme (LRAT) playing altogether an important role in the balance between storage and utilization of vitamin A (41–45). RA can bind to cytoplasmic RA binding proteins (CRABPI and II), which have been proposed to act in retinoid signaling either through sequestering RA in the cytoplasm and directing it towards catabolism (46) or, on the contrary, shuttling RA to the nucleus for retinoic acid receptor (RAR) activation (47). However, null mutants for CRABPs are viable and do not display obvious defects related to altered RA signaling pathways (48,49), therefore making unlikely that CRABPs are physiologically involved in RA signaling, as assumed from in vitro studies (46,47). In any events, all CRBP isotypes, LRAT, and CRABPII are present in mouse epidermis (46; unpublished data). Thus, epidermis appears to express the complete machinery necessary for storage (CRBPs, LRAT), oxidation (RALDH2, RALDH3) and degradation (CYP26A1, CYP26B1) of retinoids. Analysis, at the electron microscope and molecular levels, of the epidermis phenotype of mutant mice bearing germline null mutations of CRBPs (42–44), CRABPs (48,49), and LRAT (45), as well as Cre-mediated keratinocyte-restricted ablations of conditional alleles of RALDHs (50–52) or CYP26s (unpublished data) should reveal whether RA is synthesized and how RA concentration is tightly controlled in epidermis.

The physiological effects of RAs are mediated by members of two families of nuclear receptors (NRs), the RARα, β, and γ isotypes, and the RXRα, β, and γ isotypes. RARs bind both all-*trans* and 9-*cis* RA, whereas only 9-*cis* RA stereoisomer binds to retinoid X receptor (RXRs). As RXR/RAR heterodimers, these receptors control the transcription of RA-target genes through binding to RA-response elements (RAREs) (3,4). Transcriptional corepressor complexes associated with histone deacetylase activity, are recruited by unliganded or retinoid antagonist-bound RAR of RXR/RAR heterodimers, resulting in chromatin condensation and transcriptional silencing. Upon retinoid agonist binding to RAR of RXR/RAR heterodimers, corepressors are released and recruitment of coactivator complexes exhibiting histone transacetylase activity results in chromatin remodeling and transcription of RA-targets gene (53). RXRs also heterodimerize

with a number of additional nuclear receptors, including notably vitamin D3 receptor (VDR), thyroid hormone receptors (TRs), liver X receptors (LXRs) as well as peroxisome proliferator activated receptors (PPARs) (3,4,54). The phenotypic analysis of embryos and fetuses bearing germline mutations in all RAR and RXR genes allowed us to demonstrate that during prenatal development: (*i*) the RA signal is transduced by RXR/RAR heterodimers; (*ii*) in these heterodimers, functional redundancies may exist both within the RARs and the RXR partners; (*iii*) within RXRα/RAR(α, β, or γ) heterodimers, the ligand-dependent transcriptional activation function (AF-2) of RXR is often required to mediate the pleiotropic effects of RA on morphogenesis and organogenesis (2,5).

The keratinocytes of the epidermis of both humans and mice contain predominantly RARγ and RXRα, while RARα and RXRβ are expressed at a much lower level, and RARβ and RXRγ are undetectable (30,55,56). However, the epidermis of mice bearing germline null mutations for RARα (57) or RARγ (58) displays a normal aspect on histological sections, suggesting that these retinoid receptors could be dispensable for epidermis homeostasis. Due to functional redundancy amongst RARs (2,4,5,59–61), some functions of RARα and/or RARγ in epidermis could have been overlooked. This possibility could not be investigated because compound germline disruptions of RARα and RARγ lead to embryonic lethality (62), thus precluding any epidermis analysis. Similarly, it was not possible to investigate the effect of the germline RXRα-null mutation on mouse epidermis, as it leads to fetal lethality (63,64), at the onset of epidermal morphogenesis. To circumvent these limitations, we undertook the selective ablation of RARs and RXRs genes in mouse epidermis, using the keratinocyte-specific, temporally-controlled somatic mutagenesis approach (23,24,55,56,65,66).

NONE OF THE RETINOID RECEPTOR RXR/RAR HETERODIMERS IS INDISPENSABLE FOR EPIDERMIS FORMATION

The skin of newborn mice selectively lacking RXRα or RARγ in epidermal keratinocytes since the onset of their differentiation during embryonic development (i.e., RXR$\alpha^{ep-/-(c)}$ and RAR$\gamma^{ep-/-(c)}$ mutants, respectively; Table 1) is apparently normal (although somewhat shinier, see subsequently), both morphologically (at the optical microscopy level) and functionally (no obvious defects in barrier function) (65,66). To explore a possible functional redundancy between RXRα and RXRβ, and between RARγ and RARα, which are also expressed in newborn epidermis (30,55,56), compound mutants have been generated lacking both RXRα and RXRβ or RARγ and RARα in epidermal keratinocytes (i.e., RXR$\alpha\beta^{ep-/-(c)}$ and RAR$\alpha\gamma^{ep-/-(c)}$ mutants, respectively; Table 1). Their epidermis is not histologically (light microscopy) different from that of RXR$\alpha^{ep-/-(c)}$ and RAR$\gamma^{ep-/-(c)}$ newborns, indicating that there is little, if any, functional redundancy between RXRα and RXRβ or between RARγ and RARα in keratinocytes. As RXRγ and RARβ are not expressed in skin, these data clearly demonstrate that none of the retinoid receptors is indispensable in keratinocytes for epidermis formation

Table 1 Generation of Mutant Mice Bearing Somatic Mutations in Epidermis

*lox*P-flanked genes and associated mutations	Cre, CreER^{T2} or CreER^{T} transgenes	Genotypes	Mutant names	Time of gene excision
Gene excision in basal keratinocytes				
Rarg (149)	K14-Cre (65)	K14-Cre$^{(tg/0)}$/*Rarg*$^{L2/L2}$	RAR$\gamma^{ep-/-(c)}$	From E9.5 onwards (65)
Rara (150) and *Rarg* (149)	K14-Cre (65)	K14-Cre$^{(tg/0)}$/*Rara*$^{L2/L2}$/*Rarg*$^{L2/L2}$	RAR$\alpha\gamma^{ep-/-(c)}$	From E9.5 onwards (65)
Rxra (56)	K14-Cre (65)	K14-Cre$^{(tg/0)}$/*Rxra*$^{L2/L2}$	RXR$\alpha^{ep-/-(c)}$	From E9.5 onwards (65)
Rxra (560)	K14-CreER^{T2} (22)	K14-CreER$^{T2(tg/0)}$/*Rxra*$^{L2/L2}$	RXR$\alpha^{ep-/-}$	Adulthood, after TAM injection (24,56)
Rxra (56) and *Rxrb* (119)	K14-Cre (65)	K14-Cre$^{(tg/0)}$/*Rxra*$^{L2/L2}$/*Rxrb*$^{L2/L2}$	RXR$\alpha\beta^{ep-/-(c)}$	From E9.5 onwards (65)
Rxra (56) and *Rxrb* (119)	K14-CreER^{T2} (22)	K14-CreER$^{T2(tg/0)}$/*Rxra*$^{L2/L2}$/*Rxrb*$^{L2/L2}$	RXR$\alpha\beta^{ep-/-}$	Adulthood, after TAM injection (24,56)
Rxra (56) and *Rxra af2o* mutation (61)	K14-Cre (65)	K14-Cre$^{(tg/0)}$/*Rxra*$^{af2o/L2}$	RXR$\alpha^{epaf2o(c)}$	From E9.5 onwards (65)
Rxra (56) and *Rxra af2o* mutation (61)	K14-CreER^{T2} (22)	K14-CreER$^{T2(tg/0)}$/*Rxra*$^{af2o/L2}$	RXRα^{epaf2o}	Adulthood, after TAM injection (24,56)
Rxra (56) and *Rxra af2o* mutation (61); *Rxrb* (119) and *Rxrb af2o* (89) mutations	K14-CreER^{T2} (22)	K14-CreER$^{T2(tg/0)}$/*Rxra*$^{af2o/L2}$/*Rxrb*$^{af2o/L2}$	RXR$\alpha\beta^{epaf2o}$	Adulthood, after TAM injection (24,56)
Ppard (66)	K14-CreER^{T2} (22)	K14-CreER$^{T2(tg/0)}$/*Ppard*$^{L2/L2}$	PPAR$\beta(\delta)^{ep-/-(c)}$	Adulthood, after TAM injection (24,56)
Gene excision in suprabasal keratinocytes				
Rarg (149)	CMV-CreER^{T} (20) and K10-CreER^{T2a}	CMV-CreER$^{T(tg/0)}$/*Rarg*$^{L2/L2}$ and K10-CreER$^{T2(tg/0)}$/*Rarg*$^{L2/L2}$	RAR$\gamma^{sb-/-}$	Adulthood, after TAM injection (24,56)
Ppard (66)	CMV-CreER^{T} (20) and K10-CreER^{T2a}	CMV-CreER$^{T(tg/0)}$/*Ppard*$^{L2/L2}$ and K10-CreER$^{T2(tg/0)}$/*Ppard*$^{L2/L2}$	PPAR$\beta(\delta)^{sb-/-}$	Adulthood, after TAM injection (24,56)

Note: The numbers indicate the references in which are described the *lox*P–flanked alleles (L2), the *af2o* mutations, the transgenes coding for the recombinases, the time point of gene excision, or the TAM treatments required to induce gene excision.

[a]Our unpublished data.

Abbreviations: CMV, cytomegalovirus promoter; E9.5, embryonic day 9.5; K10 and K14, keratin 10 and 14 promoters, respectively; PPAR, peroxisome proliferator activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; TAM, tamoxifen; tg/0, hemizygote for the transgene.

(55,65). In keeping with this observation, the epidermis of the few compound RARα/RARγ-null mutant fetuses that could be examined at gestation day 18.5 did not appear histologically altered (67–69).

CELL-AUTONOMOUS REQUIREMENT OF BOTH RXRα/RARγ AND PPAR$\beta(\delta)$/RXRα HETERODIMERS FOR THE GENERATION OF LAMELLAR GRANULES IN SUPRABASAL KERATINOCYTES

Repressing Activity of RXRα/Unliganded RARγ Heterodimers

That RXRα and RARγ could nevertheless exert some postnatal function in the interfollicular epidermis was suggested by the glossy appearance of RXR$\alpha^{ep-/-(c)}$ and RAR$\gamma^{ep-/-(c)}$ newborns (65). To elucidate the origin of the epidermis glossiness, was further investigated by electron microscopy examination (66). Late during granular keratinocyte differentiation, ovoid organelles named lamellar granules (LGs) are formed from the tubular branched portion of the Golgi apparatus. LGs contain lipids (e.g., phospholipids and cholesterol), hydrolytic enzymes (e.g., lipases and proteases) and other proteins [e.g., corneodesmosin (CDSN)] that are organized as lamellae. At the apical pole of the outermost granular keratinocytes, LGs release their content**,** which is then assembled into multi-lamellar lipid sheets surrounding the corneocytes, and also contributes to the formation of corneodesmosomes and of a continuous ribbon of neutral lipids evenly distributed on top of the cornified layer, thereby participating to the generation of the skin permeability barrier (70). In the mouse, the keratinocyte-selective genetic ablation of either RARγor RXRα impairs the formation of both these multi-lamellar lipid sheets and the corneodesmosomes, and alters the even distribution of neutral lipids on top of the cornified layer. That concomitantly, the mutant granular keratinocytes contain vesicles, whose size and localization are similar to those of LGs, but are unable to fuse to the membrane of the outermost keratinocytes, leaves little doubt that the primary defect(s) in these keratinocytes lie(s) in LG biogenesis (66).

The finding of identical LG and stratum corneum abnormalities (hereafter referred to as "LG and related defects") in RARγ-null and in mice selectively lacking RARγin suprabasal keratinocytes (i.e., RAR$\gamma^{kb-/-}$ mutants; Table 1), unambiguously demonstrates that RARγacts cell-autonomously in suprabasal keratinocytes. As a topical treatment with a panRAR antagonist (BMS493) has no effect on epidermis structure, whereas a treatment with all-*trans* RA or with a RARγ-selective agonist (BMS961) generates "LG and related defects" identical to those resulting from RARγ ablation, indicates that the defects exhibited by RARγ and RXRα loss-of-function mutants most likely reflect the artefactual expression of one or several genes, whose expression is normally repressed by unliganded RXRα/RARγ heterodimers. Furthermore, these defects are worsened by cotreatment with the RARγ-selective ligand and a panRXR agonist (BMS649), which on its own has no effect on LG structure (66). This synergism indicates that the RARγagonist-induced LG defects result from activation of RXRα/RARγ

heterodimers, previously shown to operate in suprabasal keratinocytes (55) and in which transactivation by RXR is subordinated to the binding of an agonist ligand to RARγ.

That heterodimers between unliganded RARγ and RXR mediate transcriptional repression events in epidermal keratinocytes is in keeping with (*i*) the expression of nuclear receptor corepressors (NCoR) in mouse epidermal keratinocytes and (*ii*) the effect of a topical treatment with the histone deacetylase inhibitor trichostatin A that relieves nuclear receptor-mediated transcriptional repression (71) and results in epidermis defects very similar, if not identical to those displayed by RARγ loss-of-function mutants (66). It is also in keeping with the apparent lack of RA available in epidermis to activate RARs (72,73) and with the lack of LG abnormalities in vitamin A-deficient mice (66). We therefore conclude that RXRα/RARγ-mediated repression events are cell-autonomously mandatory for LG biogenesis in suprabasal keratinocytes (Fig. 1A). When this repression is relieved, which can be achieved with doses of retinoids lower than those required for triggering the RXRα/RARγ-mediated proliferation of basal keratinocytes (55,66), the production of LGs is impaired due to the artefactual derepression of target gene(s) whose identity and function remain to be determined.

Activation of Transcription Mediated by Heterodimers Between PPAR$\beta(\delta)$ and Transcriptionally Active RXRα

According to the above repression scenario, impairment of the AF-2 ligand-dependent transcriptional activation functions of RXRα should not affect LG structure. However, either topical treatment with a panRXR AF-2 antagonist BR1211 (74) or impairment of the AF-2 of RXRα by deletion of the helix 12 AF-2 core (*af2o* mutation; 61) in epidermal keratinocytes (i.e., RXR$\alpha^{ep af2o(c)}$ mutants; Table 1), which both should not relieve the repression exerted by RARγ/RXR heterodimers in which RARγ is unliganded (75), also result in the characteristic "LG and related defects" (66). These observations suggested that the generation of LGs requires, in addition to an unliganded RARγ/RXRα-mediated repression, either a RXRα homodimer-mediated activation, or a RXRα-mediated activation in which RXRα AF-2 would synergistically act with a heterodimerization partner distinct from RARγ.

Two lines of evidence strongly support the conclusion that heterodimers between PPAR$\beta(\delta)$ and a transcriptionally active RXRα, cell-autonomously activate the expression of target genes that are required for LG biogenesis in suprabasal keratinocytes (Fig. 1B). Firstly, ablation of PPAR$\beta(\delta)$ in suprabasal keratinocytes (i.e., PPAR$\beta(\delta)^{sb-/-}$ mutants; Table 1) results in "LG and related defects" very similar to those generated by RXRα ablation. Secondly and importantly, a topical application of the PPAR$\beta(\delta)$ agonist L165041 (76) can rescue "LG and related defects" in mice that express RXRα lacking AF-2 (RXR$\alpha\Delta$AF-2 protein) in their keratinocytes (RXR$\alpha^{ep af2o}$ mice; Table 1), which supports the conclusion that PPAR$\beta(\delta)$ and RXRα synergistically contribute to LG biogenesis

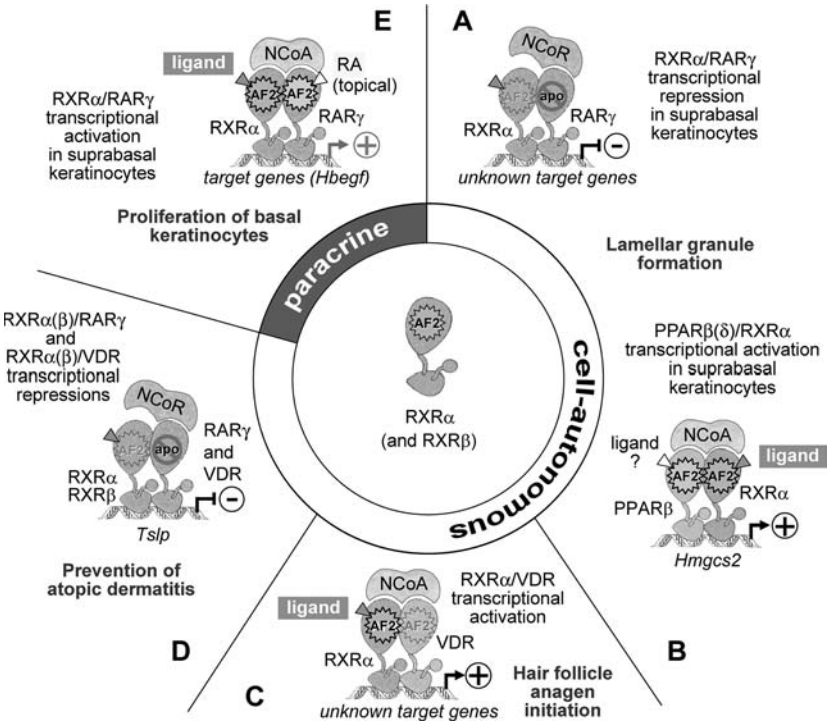

Figure 1 (*See color insert*) Germline and conditional mutagenesis approaches, combined with pharmacological approaches, have revealed some of the physiological roles of the retinoid nuclear receptors in homeostasis of mouse skin epidermis. Through its multiple nuclear receptor partnerships, RXRα plays multiple important functions in epidermis physiology. (**A**) Heterodimers between unliganded RARγ and RXRα, cell-autonomously repress events mandatory for LG biogenesis in suprabasal keratinocytes. (**B**) Heterodimers between PPARβ(δ) and a transcriptionally active RXRα (AF-2 required), cell-autonomously activate the expression of target genes (e.g., *Hmgcs2)* that are required for LG biogenesis in suprabasal keratinocytes. (**C**) Heterodimers between unliganded VDR and RXRα, in which the AF-2 ligand-dependent activation function is required, play a crucial role in keratinocytes during initiation of the anagen stage of the hair follicle cycle. (**D**) RXRα and RXRβ, heterodimerized with unliganded RARγ and unliganded VDR in keratinocytes, prevents the appearance of an atopic dermatitis-like Th2-type inflammatory reaction, through repression of the expression of thymic stromal lymphopoietin (TSLP) cytokine. This repression does not require the ligand-dependent AF-2 activation functions of RXRα and RXRβ. (**E**) Heterodimers between RARγ and RXRα in suprabasal keratinocytes are required for topical RA to induce the synthesis of HB-EGF paracrine signal in suprabasal keratinocytes (*Hbegf* gene), which in turn causes proliferation of basal keratinocytes. *Abbreviations*: AF-2, ligand-dependent activation function-2; LG, lamellar granuie, NCoA, coactivator; NCoR, corepressor; NR, yet unidentified nuclear receptor; PPAR, peroxisome proliferator activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D3 receptor.

(66). In this respect, it is worth stressing that deleting helix 12 of RXRα impairs neither the heterodimerization with PPAR$\beta(\delta)$, nor the ligand-dependent activity of the PPAR$\beta(\delta)$ partner in vitro, indicating that the RXRα *af2o* mutation abrogates the RXRα-dependent transcriptional activity without silencing that of PPAR$\beta(\delta)$ within the heterodimers (66). This conclusion is in keeping with our earlier in vivo observations showing that embryos expressing both RXR$\alpha\Delta$AF-2 and RXR$\beta\Delta$AF-2 survive until gestation day 14.5 (5), while null mutants for RXRα and RXRβ (77), for PPAR$\beta(\delta)$ (78), or PPARγ (79) all die at embryonic day 9.5 from highly similar, if not identical placental defects characterized by the absence of formation of the labyrinthine zone of the chorioallantoic placenta. Therefore, PPAR$\beta(\delta)$ and PPARγ in heterodimers with RXRα and RXRβ lacking AF-2 allow the early step of placentation to take place normally, clearly indicating that an *af2o* mutation does not convert an RXR into a potent transcriptional repressor that silence its PPAR partner in vivo.

The gene encoding 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase 2 is likely to be one of the targets controlled by activating PPAR$\beta(\delta)$/RXRα heterodimers, as both *Hmgcs2* expression and cholesterol levels are reduced in the epidermis of PPAR$\beta(\delta)^{ep-/-(c)}$ and RXR$\alpha^{ep-/-(c)}$ newborns. In addition, *Hmgcs2* expression is increased in WT adult epidermis topically-treated with either the PPAR$\beta(\delta)$-selective agonist L165041 or the panRXR agonist BMS649, whereas it is decreased by the panRXR antagonist BR1211 (66). These observations, the presence of functional PPAR response element in the *Hmgcs2* gene (80), the decrease of its expression in the skin of mutant mice selectively lacking AF-2 of RXRα in their keratinocytes (RXRα^{epaf2o} mice; Table 1), and the enhancement of its expression by the PPAR$\beta(\delta)$ agonist L165041 in these RXRα^{epaf2o} mice (66), all strongly support, at the molecular level, the conclusion that PPAR$\beta(\delta)$/RXRα heterodimers, in which RXRα is transcriptionally active, control *Hmgcs2* expression. That inhibition of cholesterol synthesis by lovastatin induces LG defects (81), whereas topical administration of cholesterol can cure the "LG and related defects" displayed by PPAR$\beta(\delta)^{ep-/-(c)}$ adults (66) further add to the notion that the PPAR$\beta(\delta)$/RXRα-controlled synthesis of cholesterol is instrumental to LG biogenesis.

A RAR–ACTIVATING RETINOIC ACID CANNOT BE THE PHYSIOLOGICAL RXR–ACTIVATING LIGAND IN MOUSE EPIDERMAL SUPRABASAL KERATINOCYTES

The biogenesis of LGs in mouse granular keratinocytes offers an interesting integrated model system in vivo to unveil the molecular mechanisms, which allow two nuclear receptor signaling pathways—that of RARγ, which is repressing and that of PPAR$\beta(\delta)$, which is activating—to concomitantly keep their identity, while sharing the same RXRα heterodimerization partner, even though this partner is bound to an agonistic ligand (66; see section "Activation of Transcription Mediated by Heterodimers Between PPAR$\beta(\delta)$ and Transcriptionally Active

RXRα"). The solution of this conundrum lies in the so-called subordination mechanism through which the transcriptional activity of an agonist-bound RXR is subordinated, within an RXR/RAR heterodimer, to the binding of an agonistic ligand to its RAR partner. To prevent an agonist-bound RXRα to transactivate on its own within a RARγ/RXRα heterodimer, a corepressor has to bind RARγ in order to block the transcriptional activity of its RXRα partner (4,53,82,83). Thus, there should be very little, if any, RARγ-activating RAs in suprabasal keratinocytes in order to allow the binding of a corepressor to RARγ, and to achieve concomitantly RXRα/RARγ-mediated repression and PPAR$\beta(\delta)$/RXRα-mediated activation of target genes that are instrumental to LG biogenesis (Fig. 2A). Several lines of evidence actually support the lack of retinoid available to activate RAR in epidermis under homeostatic conditions (72,73). That an active retinoid is not present in mouse keratinocytes is further supported by the findings that, in the mouse, topical skin treatments with (*i*) a RXR-specific agonist has no effect on its own on keratinocyte proliferation (55,84); (*ii*) the panRAR antagonist BMS493 does not reduce the homeostatic rate of basal keratinocyte proliferation (55); (*iii*) the panRAR antagonist BMS493 or the panRXR antagonist BR1211 both fail to decrease *Cyp26a1* and *Crabp2* expressions that are otherwise inducible by RA (66). In addition, it is known that 9-*cis* RA, which unlike all-*trans* RA can bind to RXRs, (*i*) is nearly as efficient as all-*trans* RA for binding and activating the RARs, and (*ii*) binds less efficiently to RXRs than to RARs (85). Thus, if 9-*cis* RA would be the RXRα-activating ligand, its presence in suprabasal keratinocytes could also activate RARγ within RXRα/RARγ heterodimers, thereby relieving RXRα/RARγ-mediated repression and inducing "LG and related defects" (Fig. 2B). We conclude that 9-*cis* RA cannot be the RXRα-activating ligand in suprabasal keratinocytes.

In view of the almost ubiquitous presence of RARα and RXRα in a variety of cell-types (86–88), one can wonder whether 9-*cis* RA could ever be an RXR agonistic ligand in vivo. In this respect, we recently reported that cholesterol efflux from sertoli cells involves activating LXRβ/RXRβ heterodimers in which RXRβ AF-2 is transcriptionally active, whereas VAD has not affect on this efflux, which strongly suggests that, in this case also, 9-*cis* RA is most unlikely to be the RXRβ-activating ligand (89). Potential low affinity RXR agonistic ligands that do not activate RARs include phytol metabolites (90), docosahexaenoic acid (91) and several unsatured fatty acids (92), whose impaired synthesis result in LG defects reminiscent of those described in the present study (93). As fatty acids activate not only RXRs, but also PPARs, it is tempting to speculate that the same ligand could activate the two partners of PPAR$\beta(\delta)$/RXRα heterodimers. We have proposed elsewhere that low affinity agonist ligands bound to the RXR partners of the heterodimers could "sensitize," through synergistic effects, the transcriptional activity of these heterodimers to discrete variations in the concentration of the nuclear receptor cognate ligands (4). In the present case, it is interesting to note that the level of the "sensitizing" RXR ligand is high enough in suprabasal keratinocytes to synergistically reach, upon topical addition of 3 nmoles of the

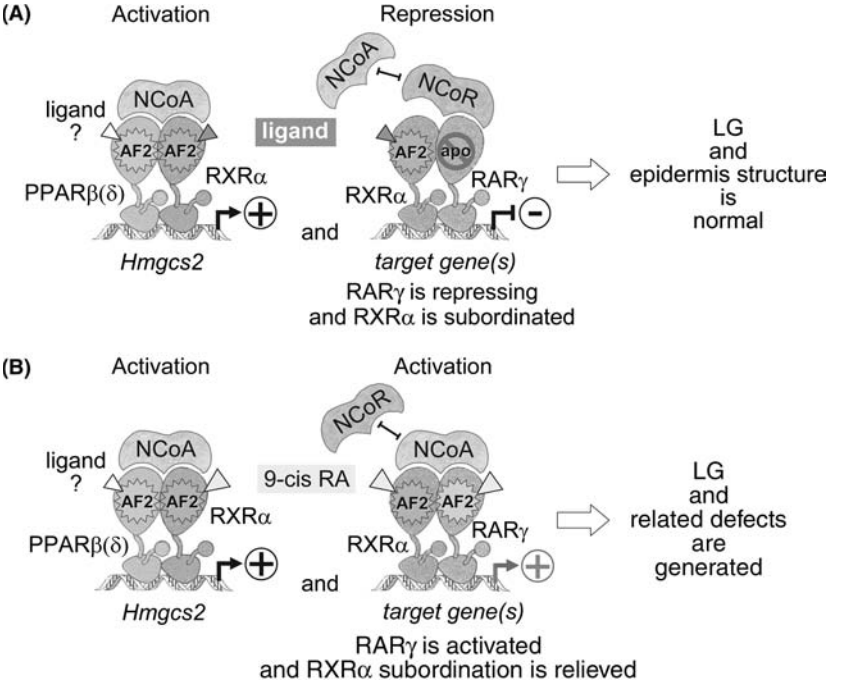

Figure 2 (*See color insert*) The concomitant occurrence of RAR/RXR-mediated repression and RXR/PPAR$\beta(\delta)$-mediated activation events observed in mouse epidermis suprabasal keratinocytes rules out the possibility that the ligand activating RXR AF-2 could be 9-*cis* RA. (**A**) Scenario A: The RXR-activating ligand is not 9-*cis* RA. Within activating PPAR$\beta(\delta)$RXRα heterodimers the AF-2 of RXRα is required to activate gene expression, notably that of *Hmgcs2*. Thus, RXRα most likely binds an agonistic ligand, and PPAR$\beta(\delta)$/RXRα heterodimers interact with coactivators (NcoAs/SRCs) (*left panel*). On the other hand, within repressing RARγ/RXRα heterodimers, RXRα cannot be transcriptionally active, due to subordination of its transcriptional activity to that of its repressing RARγ partner, which has to be in its unliganded apo-form in order to bind corepressors (NcoR/SMRT) that block the transcriptional activity of RXRα (*middle panel*). It follows that there should not be any retinoic acids in suprabasal keratinocytes, and therefore that the RXR-activating ligand cannot be 9-*cis* RA. Under these conditions, LG formation and epidermis ultrastructure are normal (*right panel*). (**B**) Scenario B: The RXR-activating ligand is 9-*cis* RA. In this case, RXRα binds 9-*cis* RA and PPAR$\beta(\delta)$/RXRα heterodimers interact with coactivators (NcoAs/SRCs) (*left panel*). However, 9-*cis* RA also binds to RARγ, thus relieving RXRα subordination and activating RARγ/RXRα heterodimers that no longer repress gene expression (*middle panel*). Under these conditions, LG biogenesis is impaired (*right panel*). As actually this is not the case, the possibility that 9-*cis* RA could be the ligand activating RXR AF-2 is ruled out. *Abbreviations*: AF-2, ligand-dependent activation function-2; LG, lamellar granuie; NCoA, coactivator; NCoR, corepressor; NR, nuclear receptor; PPAR, peroxisome proliferator activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SMRT, silencing mediator of retinoid and thyroid; SRCs, steroid receptor coactivators.

RARγ-selective ligand (BMS961), the threshold level sufficient to relieve the repression exerted by RXRα/RARγ heterodimers, but not to trigger in these cells the paracrine events that induce the proliferation of basal keratinocytes, which necessitates an additional coreatment with the selective RXR ligand BMS649 (55,66).

RXRα/VDR HETERODIMERS, IN WHICH AF-2 OF RXRα IS ACTIVE, ARE INVOLVED IN HAIR FOLLICLE CYCLING

Normal morphogenesis and cycling of hair follicles in mouse is dependent on a series of mesenchymal–epithelial interactions (26,27,94,95). The first follicle growth phase that is initiated in utero persists until around postnatal day 14, when the follicle enters the first catagen stage. Within three days, this involution stage is completed and after a short three day telogen resting phase, the second cycle starts. The intervals between the cycles that are repeated throughout the life of the mouse, increase with age to become erratic with prolonged periods of telogen, while the cycle stage is predictable at specific anatomic sites during the first two cycles (94,96).

In RXR$\alpha^{ep-/-(c)}$ mice, selectively lacking RXRα in epidermis keratinocytes from the onset of their differentiation during development (Table 1), the primary hair growth is somewhat delayed (by about four to five days), but the first hair coat appears normal and no defects are observed in the hair follicle at the end of the first hair cycle (65). The RXR$\alpha^{ep-/-(c)}$ mutants subsequently lose progressively their hair and develop, by four to six weeks of age, an alopecia, which is extensive by 10 to 12 weeks of age. This hair loss, that is reproduced upon ablation of RXRα in adult keratinocytes (RXR$\alpha^{ep-/-}$ mice; Table 1), results from a keratinocyte-autonomous requirement of RXRα at the anagen initiation stage of the hair cycle (65), and its progression likely reflects a partial functional redundancy between RXRα and RXRβ. Such a redundancy may also account for the greater severity of alopecia in females than in males, which have higher levels of RXRβ in the skin (56). Under the skin surface of hairless regions, many cysts are visible. With increasing age, they become larger, and spread all over the body. Histological analysis of hairless ventral skin has shown that these cysts displayed the typical features of degenerated hair follicles: actually many skin surface-connected ampuliform structures, which exhibited a dilatation of the piliary canal, a lack of hair shaft and are filled with horny cells (i.e., "utriculi") (96,97) are observed both in RXR$\alpha^{ep-/-(c)}$ (65) and RXR$\alpha^{ep-/-}$ mice (56).

The VDR is differentially expressed during distinct hair cycle stages in both the ORS keratinocytes and dermal papilla of mouse hair follicle (98). Interestingly, VDR mutations in humans (99) and *Vdr* gene knockouts in mice (100–103) result in alopecia, which, in mice, closely resembles the one exhibited by RXR$\alpha^{ep-/-(c)}$ (65) and RXR$\alpha^{ep-/-}$ mice (56). Actually, both in RXRα- and

VDR-null mutants the onset of alopecia appears to be secondary to a cell-autonomous defect occurring in keratinocytes at the anagen phase of the second hair cycle (56,103–107). Thus, heterodimers between RXRα and VDR in keratinocytes likely play an important role in the initiation of the anagen during the hair follicle cycle (Fig. 1C).

Studies in humans and animals have failed to demonstrate the presence of alopecia in vitamin D3 deficiency (99). Furthermore, neither humans nor animals with mutations in the enzyme required for hormone activation, the 25-hydroxy-vitamin D3-1α hydroxylase, manifest alopecia (108–112). These observations suggested that hair loss could be a hallmark reflecting impaired actions of an unliganded VDR. In keeping with this proposal, it has recently been demonstrated that the keratinocyte-restricted expression of a VDR bearing a mutation, which abolishes only the vitamin D3 binding can restore normal hair cycling in VDR-null mice (113). This finding further adds to the notion that the effects of the VDR on the hair follicle are ligand-independent. Following the example of the closely related thyroid hormone and retinoid receptors, unliganded VDR can repress the expression of some target genes (114). However, unlike TRs and RARs, VDR interacts only weakly with classical corepressors such as NCoR and silencing mediator of retinoid and thyroid (SMRT) hormone receptor (115,116). On the other hand, recent investigations have demonstrated that VDR interacts with hairless that functions as a corepressor (117,118), and mice bearing mutations at the hairless (*hr*) locus exhibit a postnatal alopecia, with utriculi and dermal cysts (97), that are similar to those of VDR-null, RXR$\alpha^{ep-/-(c)}$ and RXR$\alpha^{ep-/-}$ mutants. These observations raised therefore the interesting possibility that RXRα/VDR heterodimers, bound to hairless, could have been required to repress genes whose deregulation in mutant mice yielded hair follicle degeneration through a block at the anagen stage. However, the main failure in hair follicles of *hr*/*hr* mice occurs during the first catagen stage, and yields a rapid and sharply demarcated alopecia occurring in less than three weeks after birth (97), in marked contrast to the alopecia developed by RXRα and VDR loss-of-function mutants (56,65,100–103). Thus, it appears unlikely that unliganded RXRα/VDR heterodimers are responsible for the repression events that control normal hair follicle homeostasis.

As an alternative, the activity of these heterodimers could be induced by ligand(s) that bind(s) the RXRα partner. In keeping with this proposal, it is noteworthy that mutant mice expressing RXRα lacking the AF-2 ligand-dependent activation function (RXRα^{epaf2o} mice) exhibit an alopecia closely resembling that of RXR$\alpha^{ep-/-(C)}$, RXR$\alpha^{ep-/-}$, or VDR-null mice (119). Therefore, the anagen step of hair follicle cycling may be controlled by VDR/RXRα heterodimers in which the RXRα AF-2 is transcriptionally active (Fig. 1C). If one assumes that the requirement of RXRα AF-2 activity reflects the binding of a ligand, then the latter is unlikely to be a retinoid since vitamin A deficiency does not induce an alopecia (32,34; and unpublished data).

ABLATION OF RXRα AND RXRβ IN KERATINOCYTES GENERATES AN ATOPIC DERMATITIS TRIGGERED BY THYMIC STROMAL LYMPHOPOIETIN

The selective ablation of RXRα in basal keratinocytes (RXR$\alpha^{ep-/-(c)}$, RXR$\alpha^{ep-/-}$ mutants; Table 1) results in a hyperproliferation and an abnormal differentiation of interfollicular epidermal keratinocytes. The increased number of suprabasal layers is correlated with increased proliferation of mutant epidermal basal keratinocytes as assessed by bromo-deoxyuridine (BrdU) incorporation, whereas the perturbation of terminal differentiation is reflected by aberrant expression of keratins in the hyperproliferative epidermis, notably of K6 that is normally confined to hair follicles and of profillagrin that is normally restricted to the uppermost granular keratinocytes (65). Most interestingly, these abnormalities have not been observed in VDR-null mutants (56,103,107), even though the hair follicle defects are highly similar in these mutants and our RXRα loss-of-function mutants (see previously). The hyperproliferation of interfollicular epidermal keratinocytes in RXR$\alpha^{ep-/-(c)}$ and RXR$\alpha^{ep-/-}$ adults is associated with an inflammatory infiltrate, which is characterized by the presence of dilated dermal capillaries and a marked increase in epidermal Langerhans cells, but which does not appear to correspond to an immune response to microbial antigens, as there is no evidence for bacterial or fungal infection as assessed by Gram and periodic acid–Schiff reagent staining (65). Along these lines, no B lymphocytes infiltrates could be detected using antibodies to B lymphocyte IgM and CD45R (65). As it is the case for keratinocyte hyperproliferation, this immune response does not appear to be secondary to hair follicle defects, as there is very little inflammatory reaction in VDR-null mice (56,103,107). These observations therefore suggest that RXRα may exert some functions in proliferation/differentiation of keratinocytes and in the immune response that both are distinct from those mediated through RXRα/VDR heterodimers in hair-follicle cycling.

Atopic dermatitis (AD), a chronic skin inflammatory disease with a strong genetic component that affects children (10–20%) and adults (1–3%), is characterized by pruritic and eczematoid skin lesions, associated with systemic immunological abnormalities, including peripheral eosinophilia and hyper IgE immunoglobulinemia (120). Immunological mechanisms have been implicated in AD pathogenesis (121), but until recently the possible role of epidermal keratinocytes in AD initiation and maintenance is still largely unexplored (122). As the inflammatory reaction observed in RXR$\alpha^{ep-/-(c)}$ and RXR$\alpha^{ep-/-}$ adults mutants had a weak similarity with AD, we recently generated RXR$\alpha\beta^{ep-/-}$ mutants (Table 1), in which both RXRα and RXRβ are selectively abrogated in epidermal keratinocytes of adult mice in order to avoid any possible functional redundancies between RXRα and RXRβ (119). Most interestingly, we found that these mutant mice develop a skin and systemic syndrome similar to that of human AD (Fig. 1D). First, they display the major clinical features of patients suffering from AD (123,124), including eczematous-like lesions, xeroxis, and pruritus. Second, in

these mice, the skin inflammatory cell infiltrate is mostly composed of CD4 + T helper cells and dendritic cells, associated with eosinophils and mast cells, which together are characteristic of skin lesions of AD patients (120). Third, the profile of cytokines and chemokines, whose expression is increased in RXR$\alpha\beta^{ep-/-}$ inflammatory skin is typical of a Th2-type helper cell inflammation (i.e., IL-4, IL-5, IL-13, IL-10, and IL-31 cytokines; CCL17, CCL22, and CCL8 chemokines), known to be characteristic of human AD inflammation (120,125,126). Importantly, the recently identified thymic stromal lymphopoietin (TSLP) cytokine that is found in skin lesions of human AD is also present in RXR$\alpha\beta^{ep-/-}$ inflammatory skin (see subsequently). In addition, as in chronic human AD skin lesions (127), a delayed minor component of Th1-type inflammation is found in RXR$\alpha\beta^{ep-/-}$ inflammatory skin, as indicated by an increase in Th1-type cytokines (i.e., IFN-γ and TNF-β) and chemokines (i.e., CCL10 and CCL20). Finally, most AD patients exhibit a systemic Th2-like immune syndrome similar to that found in RXR$\alpha\beta^{ep-/-}$ mice, including increased serum levels of IgE and blood eosinophilia, possibly related to IL-4, IL-13, and IL-5 increases (128,129).

At least two lines of evidence support the conclusion that the cytokine TSLP plays a key role in the chain of events that generate the Th2-type skin and systemic atopic syndrome. First, among the various cytokines aberrantly expressed in RXR$\alpha\beta^{-/-}$ mutants, TLSP is the earliest to be strongly produced in epidermal keratinocytes, and its up-regulation is tightly correlated with the occurrence of the atopic skin and systemic syndrome (i.e., TSLP is produced in RXR$\alpha\beta^{ep-/-}$ mice, but not in RXR$\alpha\beta^{af2o}$ mice). Second, and most importantly, a transgene-driven expression of TSLP in epidermal keratinocytes generates both AD-like skin and systemic abnormalities closely mimicking those seen in RXR$\alpha\beta^{ep-/-}$ mice, thus unequivocally demonstrating that TSLP produced in keratinocytes is the initiating cytokine at the top of a chain of immunological events that lead to the atopic syndrome in RXR$\alpha\beta^{ep-/-}$ mutants (119). In fact, TSLP may very well play a similar role in AD patients, as human TSLP has been found to be produced by keratinocytes from AD patients, but not from human normal skin or patients with nickel-induced contact dermatitis (i.e., a Th1-like inflammation) (126,130). It remains to be seen how the overproduction of TSLP is triggered in human skin of AD patients, and to what extent a deregulation of nuclear receptor pathways involving RXRs could be implicated in the pathogenesis of AD.

Interestingly, mutant mice expressing RXRα and RXRβ lacking their AF-2 ligand-dependent activation functions (RXR$\alpha\beta^{epaf2o}$ mice; Table 1) do not develop the AD-like dermatitis, clearly indicating the dispensability of AF-2 in the process by which RXRα and RXRβ prevent the appearance of the inflammatory reaction characterized by the induced expression of TSLP. The dispensability of RXR AF-2s additionally suggests that the induced expression of TSLP in RXR$\alpha\beta^{ep-/-}$ could be due to the relief of transcriptional repression events mediated by RXRα/NR and RXRβ/NR heterodimers (119). If one assumes that RXRα and RXRβ are liganded in keratinocytes (see sections "Activation of Transcription Mediated by Heterodimers Between PPAR$\beta(\delta)$ and Transcriptionally Active RXRα" and

"A RAR–Activating Retinoic Acid Cannot be the Physiological RXRα–Activating Ligand in Mouse Epidermal Suprabasal Keratinocytes"), their transcriptional activity must be subordinated to that of their NR partners (4,82,83). In keeping with this proposal, it is worth noting that administration of the panRXR agonist BMS649 does not relieve the putative repression exerted by RXRs on TSLP expression (119). The identity of the NR heterodimeric partner(s) of RXRα and RXRβ in these events has been revealed recently (131), as RXRα(β)/unliganded RARγ and RXRα(β)/unliganded VDR heterodimers are have been shown to normally repress transcription from the *Tslp* promoter (Fig. 1D).

TOPICAL ADMINISTRATION OF RETINOID AGONISTS TRIGGERS PROLIFERATION OF BASAL KERATINOCYTES THROUGH ACTIVATION OF RXR/RARγ HETERODIMERS IN SUPRABASAL KERATINOCYTES

We have shown that RXRα ablation in basal keratinocytes results in hyperproliferation of interfollicular keratinocytes, a feature that likely reflects the involvement of RXRα in heterodimers with a partner other than VDR (56,65; see previous section). Such possible partners known to be expressed in epidermis include for example RARs (30,55), TRs (132) and PPARs (133,134). As RARγ-null and RARα/RARγ-null keratinocytes cultured in vitro have been shown to be refractory to RA-induced growth arrest (135), RARs could have been the partners of RXRα that mediate an antiproliferative effect in resting (i.e., nonstimulated) epidermis. Our inducible conditional somatic mutagenesis strategy has allowed us to generate mice selectively lacking both RARα and RARγ in adult epidermis. As RARβ is not ectopically expressed in the epidermis of these mice, they actually display a "panRAR-null" epidermis fully devoid of RARα, RARβ, and RARγ. Importantly, we found that the absence of all RARs does not alter the basal keratinocyte homeostatic proliferation (55), in contrast to RXRα ablation (56,65). This observation indicates that RAR/RXRα heterodimers are not involved in controlling the self-renewal of epidermis under basal physiological conditions in vivo.

Although RARs are apparently not involved in the control of homeostatic epidermal proliferation under resting conditions, it is well established that topical treatment of skin with retinoids results in a marked increase in keratinocyte proliferation (30,55,136). We have demonstrated that this retinoid-induced proliferation is mediated by RARγ/RXR heterodimers located in suprabasal keratinocytes, and in which the ligand-dependent transcriptional activity of RXR is subordinated to that of its liganded-RAR partner (55). As cell proliferation only takes place in the basal cell layer of the epidermis in vivo, this finding implies that retinoids induce the synthesis of a paracrine signal in suprabasal keratinocytes, which in turn causes proliferation of basal keratinocytes (Fig. 1E). Several growth factors can affect cell proliferation through binding to membrane tyrosine kinase receptors that are located in basal keratinocytes and belong to the ErbB family (137,138). Amongst the ligands for the ErbB isotypes that are expressed in epidermis,

HB-EGF appears to be the only one regulated by RA (139,140). Furthermore, its mRNA is exclusively expressed in suprabasal keratinocytes prior to the onset of basal cell layer proliferation (140), whereas mature HB-EGF proteins are localized in epidermal basal cells only (141). The positive correlation that we found between expression of HB-EGF and RA-induced proliferation (55) supports the possibility that HB-EGF could be "the" paracrine factor that is synthesized in the suprabasal layers and mediates RA-induced hyperplasia (140). However, it should be noted that RA does not increase HB-EGF expression in RAR$\gamma^{sb-/-}$ mice (Table 1), whereas it still exerts some proliferative effect on their epidermis (55). Thus, HB-EGF may not be the only signaling molecule involved in RA-induced proliferation. In keeping with this conclusion, it was recently shown that expression of amphiregulin in human skin (142) and of epigen (143), epiregulin and transforming growth factor alpha in mouse skin (unpublished results) may significantly participate to the RA-induced proliferation of keratinocytes, whereas HB-EGF contributes but is not the master effecter of RA-induced hyperplasia in mice (144, our unpublished results). On the other hand, using conditional spatio-temporally controlled somatic mutagenesis in basal keratinocytes, we have recently demonstrated that ErbB1 receptor (EGFR) is indispensable to the RA-mediated proliferation, indicating involvement of EGFR (unpublished result). Homodimers and EGFR/ErbB2 heterodimers (unpublished results). Therefore, even though dispensable for homeostatic proliferation in resting skin, a retinoid-inducible RARγ/RXRα-mediated pathway that activates the EGFR/ErbB2 pathway through numerous growth factors is functional in mouse suprabasal keratinocytes.

CONCLUSION

Genetic and pharmacological approaches in vivo, mostly through the somatic ablation of genes involved in retinoid signaling and use of receptor-selective agonists or antagonists, are invaluable tools to dissect and identify individual, specific, RA signaling pathways. Using these combined approaches in the mouse, we notably demonstrated that, under physiological conditions in vivo (*i*) none of the retinoid receptors (RARs and RXRs) is indispensable in keratinocytes for epidermis development (see section "None of the Retinoid Receptor RXR/RAR Heterodimers is Indispensable for Epidermis Formation"); (*ii*) selective panRAR antagonists do not affect the homeostatic renewal and the functions of epidermal keratinocytes, which indicates that RA is not required in interfollicular keratinocytes, and therefore that the possible transcriptional function of RARs in these cells is most probably restricted to repression of gene expression; (*iii*) accordingly, RXRα/RARγ-mediated repression events are cell-autonomously mandatory for LG biogenesis in suprabasal keratinocytes (see section "Repressing Activity of RXRα/Unliganded RARγ Heterodimers") and *Tslp* promoter basal activity is silenced by unliganded RXR$\alpha(\beta)$/RARγ heterodimers, preventing thereby the generation of an AD-like phenotype (see section "Ablation of RXRα

and RXRβ in Keratinocytes Generates an Atopic Dermatitis Triggered by Thymic Stromal Lymphopoietin"); (*iv*) PPAR$\beta(\delta)$/RXRα-mediated events involving an RXRα transcriptionally active AF-2 are required for LG biogenesis, which excludes that the RXRα-activating ligand could be 9-*cis*-RA, as this retinoid would also activate RARγ/RXRα heterodimers, and consequently alter LG biogenesis (see section "Activation of Transcription Mediated by Heterodimers Between PPAR$\beta(\delta)$ and Transcriptionally Active RXRα"); (*v*) a topical treatment with RA or a RARγ-selective agonist triggers a retinoid-inducible RXRα/RARγ-mediated activating pathway, which can enhance basal keratinocyte proliferation, through generation of a paracrine signal (see section "Topical Administration of Retinoid Agonists Triggers Proliferation of Basal Keratinocytes Through Activation Of RXRα/RARγ Heterodimers in Suprabasal Keratinocytes").

These unexpected conclusions raise a number of questions. First, as all of the enzymes required for RA synthesis (from vitamin A) and catabolism are present in epidermis (see section "Is the Skin a Target Tissue for Retinoids?"), is there ever any RA present in keratinocytes to trigger the RA-dependent RXRα/RARγ-mediated pathway that leads to cell proliferation? For instance, could RA be synthesized under stress conditions, such as wound healing? In this respect, note that HB-EGF, whose expression is induced by RA-activated RXRα/RARγ heterodimers, has been identified as the predominant growth factor involved in skin wound healing in vivo (145), and vitamin A deficiency causes delayed wound healing, whereas pretreatment with vitamin A or RA improves epidermal regeneration (146). It is therefore possible that the metabolic machinery expressed in keratinocytes produces endogenous retinoids during wound healing, to activate RARγ/RXRα heterodimers, which at the very least may control HB-EGF signaling in suprabasal keratinocytes and favor wound closure. In this context, an important issue that remains to be solved is how basal keratinocyte proliferation is physiologically controlled. Second, what is the identity of the RXR-activating ligand in keratinocytes, as it cannot be 9-*cis* RA? Docosahexaenoic acid, a long-chain polyunsaturated fatty acid that is abundant in brain and binds RXR with a low efficiency, has been found to activate RXR in cell-based assays (91). These observations raise the interesting possibility that RXR could be bound to low affinity ligands to maximally "sensitize" (through synergistic effects) the transcriptional activity of the RXR/NR heterodimers to discrete variations in concentration of cognate ligands of the NR partner (4). For instance low affinity metabolites may sensitize RXR/PPAR, RXR/LXR, and RXR/FXR heterodimers, which are all involved in regulating energy and nutritional homeostasis (54). Finally, our studies using the RA-responsive F9 mouse embryonic carcinoma (EC) cell line clearly supported the conclusion that through binding of cognate ligands and phosphorylation of their activation domains, retinoid receptors are sophisticated transducers, integrating signals belonging to several signaling pathways, at least in vitro (147,148). It remains to be seen whether these phosphorylation events are relevant in vivo, notably in epidermis physiology.

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3

Retinoids and the Skin: From Vitamin A in Human Epidermis to the Pharmacology of Oral Retinoids in Dermatology

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INTRODUCTION

The observation in the 1920s that vitamin A deficiency may induce follicular hyperkeratosis of the skin (so called phrynoderma) was the rationale for later testing high-dose vitamin A therapy in various hyperkeratotic skin disorders, also in the absence of laboratory or ocular signs of vitamin A deficiency. This "antikeratinizing" effect of natural vitamin A was, however, soon recognized to be associated with a significant risk of developing hypervitaminosis A, that is, headache due to increased intracranial pressure, alopecia, hepatotoxicity, bone pains, etc. These problematic side-effects and the discovery in the 1960s of retinoic acid (RA) as a vitamin A metabolite, initiated a search for more effective, synthetic retinoids with a better therapeutic ratio (effect/side-effect) than vitamin A.

For a discussion about how vitamin A and synthetic retinoids work in the skin, some basic knowledge is needed about the tissue distribution and intracellular metabolism of these compounds. Using fluorescence microscopy, the presence of vitamin A was demonstrated in human skin already 50 years ago (1). Some 20 years later, the introduction of high-performance liquid chromatography (HPLC) made it possible to identify and quantify retinol and its derivatives in lipid extracts of skin biopsy specimens with high specificity and sensitivity (2).

HPLC coupled to liquid scintillation counting also makes it possible to study the metabolism of radioactively labeled retinol and RA in tissue cultures of epidermis or isolated skin cells. This has enabled a characterization of the vitamin A metabolism in both keratinocytes and melanocytes, the predominant cell types in epidermis (3–5). As a corollary, the effects for instance of synthetic retinoids and RA metabolism blocking agents (RAMBAs) can now be studied with respect to effects on the vitamin A metabolism in skin cells.

Additionally, HPLC is often used to characterize the pharmacokinetics and tissue distribution of synthetic retinoids, that provide information crucial to the understanding of the pharmacodynamics of retinoids in clinical praxis.

The purpose of this chapter is to summarize present knowledge about the distribution of vitamin A and synthetic retinoids in the skin under normal and pathological situations, to highlight some characteristic features of the epidermal vitamin A metabolism in humans, and to present some bare essentials about the pharmacological properties of the most commonly prescribed oral retinoids in dermatology.

UPTAKE AND METABOLISM OF VITAMIN A IN HUMAN EPIDERMIS

The vitamin A content of normal, sun-protected human epidermis is about 8 nmoles/g protein or 250 to 300 ng retinol equivalents/g wet tissue (6). This is similar to a value of 13 nmoles/g protein observed in human keratinocytes cultured in the presence of 5% serum (3). The question is: how does this amount of vitamin A reach the skin cells under physiological conditions?

Vitamin A is transported in blood either as retinyl esters bound to plasma lipoproteins or as retinol bound specifically to retinol-binding protein (RBP) (see Chapter 1). Retinoids accumulate in the epidermis either via passive diffusion across cell membranes or via a receptor-mediated uptake of retinol from RBP (7,8). A specific cell surface receptor for RBP has been demonstrated also on keratinocytes (9). Another plausible source of epidermal retinol is beta-carotene accumulating in keratinocytes and melanocytes (see Chapter 15), but this metabolic pathway is probably trivial to man under normal conditions.

Figure 1 shows that, once inside the keratinocyte, retinol may enter several alternative metabolic pathways. It is either re-esterified, desaturated to 3,4-didehydroretinol (ddROH), or oxidized to retinaldehyde and RA. RA, the most active metabolite of vitamin A, which cannot be reduced back to retinaldehyde, is then subsequently deactivated by a series of oxidation steps eventually leading to water soluble excretion products (see Chapter 4). The esterification of retinol (and ddROH), which promotes a local storage of vitamin, is probably essential for modulating the RA production in epidermis. The esterifcation is catalyzed by microsomal lecithin:retinol acyl transferase (LRAT), and to a lesser extent by acylCoA:retinol acyltransferase (ARAT) (see Chapter 1 for details about the enzymes). This is similar to the situation in other tissues, except that epidermal ARAT has an acidic pH-optimum, probably representing an adaptation to the low pH, which prevails in normal human stratum corneum (10).

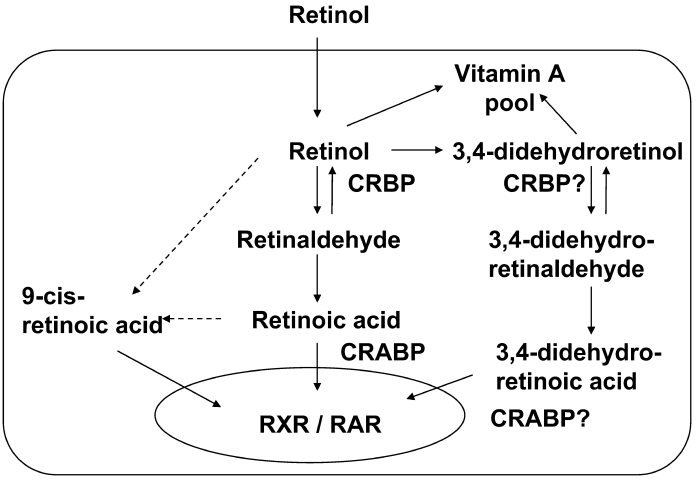
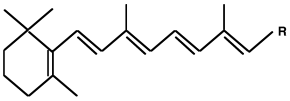


Figure 1 Major metabolic pathways for retinol in human keratinocytes. Dotted lines represent suggested but not proven pathways. *Abbreviations*: CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding proteins; RAR, retinoic acid receptor; RXR, retinoid X receptor. *Source*: Courtesy of H. Törmä.

The biosynthesis of RA in epidermis and the expression of its two binding proteins, cellular retinoic acid binding protein type 1 (CRABP-1) and -2, are similar to that of other tissues, although the latter protein is heavily upregulated in terminally differentiated keratinocytes compared to all other cell types (11).

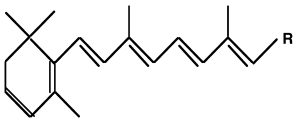
The most conspicuous difference, however, between the retinol metabolism in keratinocytes and other cells is the predominant formation of ddROH, which is then further oxidized to ddRA (3,4). Despite having a 3D structure that is dissimilar to RA, ddRA binds to and transactivates the human RAR receptors in a similar way as RA (12). ddROH, also known as vitamin A2 (Fig. 2), is prevalent in amphibians and certain fishes, but was not know to exist in mammalian tissues until detected in human epidermis some 25 years ago (13). The formation of ddROH is especially evident in hyper-proliferative psoriatic skin and in cultured keratinocytes undergoing terminal differentiation. Another species known to express this unusual metabolic pathway is avian embryos, where ddRA has even been proposed as a morphogenic signal (14). Alas, despite much research efforts the enzymatic basis of the ddROH/ddRA-formation and its putative role in skin biology remain enigmatic.

Analogous to the situation for skin carotenoids, the concentration of natural retinoids is higher in human epidermis than in the underlying dermis (see Chapter 15, Fig. 2); this is especially evident for ddROH and its esters, which are virtually undetectable in blood and other tissues. Within the epidermis there is a mass gradient characterized by increasing concentrations of neutral retinoids in the more differentiated cell layers; in particular the fatty acyl esters accumulate in cells from the outermost layers (15).



All-*trans*-retinoid

Free alcohol	R=CH_2OH
Retinyl esters	R=CH_2-O-acyl
Retinal	R=CHO
Retinoic acid	R=COOH



3,4-didehydro-retinoid

Figure 2 Chemical structures of the two predominating retinoids in human epidermis, vitamin A1 (retinol) and A2 (3,4-didehydroretinol) and their derivatives.

Taken together, all available information about the deposition and metabolism of vitamin A in the skin points in the direction of a homeostatically controlled process, suggesting that vitamin A metabolites play an important role in the maintenance of normal cellular proliferation and differentiation also in human epidermis.

FROM VITAMIN A TO SYNTHETIC RETINOIDS IN DERMATOLOGY

The industry's interest in synthezing RA analogs started already in the 1960s, when chemists at Hoffman-la Roche did most of the pioneering work. It resurfaced in the 1990s, when Ligand and other companies took advantage of the discovery of nuclear retinoid receptors when designing new and highly specific ligands for retinoic acid receptor (RARs) and retinoid X receptor (RXRs), respectively. Three generations of synthetic retinoids can be distinguished, each represented by examples of the structural formulas shown in Figure 3. The first generation encompasses various isomers of all-*trans* RA (13-*cis*, 9-*cis*, etc.); the second generation involves aromatic substitutions of the beta-ionone ring structure (acitretin, motretinid, etc.); and the third generation is represented by the receptor tailored molecules (bexarotene, tazarotene, adaplene, etc.), which structurally look quite distinct from RA, but have in common a polyunsaturated back bone and a COOH-terminus in the tail.

Virtually all retinoid molecules share the following pharmacological and chemical characteristics: (*i*) they are more or less hydrophobic compounds, (*ii*) tightly bound to plasma proteins, (*iii*) chemically unstable (with a few exceptions) in the presence of oxidizing compounds and ultraviolet radiation, and (*iv*) have a comparatively long elimination half-life when administered systemically.

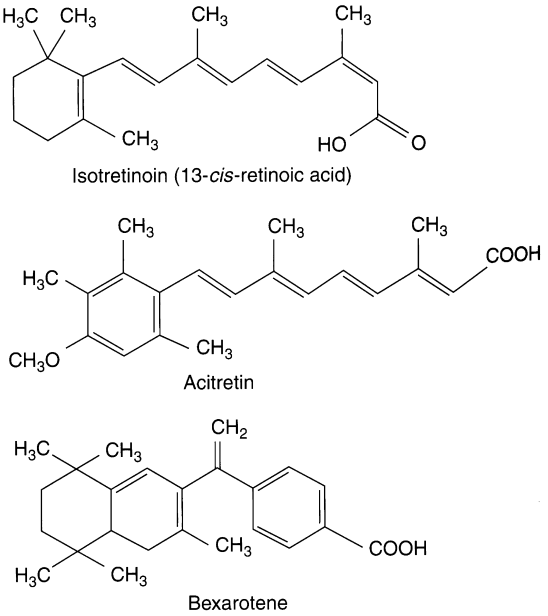


Figure 3 Chemical structures of systemic retinoids approved for use in dermatology.

Table 1 summarizes some pharmacokinetic data for the most commonly prescribed oral retinoids in dermatology. In some countries, high-dose vitamin A therapy is still a viable option when more expensive retinoids cannot be afforded, and at least in Japan, etretinate is still on the market instead of acitretin and is therefore also included in the Table. However, oral alitretinoin (9-*cis* RA), which recently showed promising results in chronic hand dermatitis, and a few other experimental retinoids are not included because they are not readily available on the market.

Isotretinoin

Isotretinoin, which is mainly used to treat acne (see Chapter 6), has a better therapeutic ratio than all-*trans* RA, but does not bind to RAR. Its mechanism of action in reducing sebaceous gland activity is therefore enigmatic.

In contrast to vitamin A that is stored in the liver and has a slow turn-over, isotretinoin is not stored in significant amounts but is more rapidly eliminated via hepatic clearance. Both the unmodified drug and its oxidized metabolites are conjugated to glucuronic acid prior to biliary excretion. The main metabolite, 4-oxo-isotretinoin, is considered a pharmacologically inactive by-product (16). It is the predominant drug metabolite present in plasma during repeated dosing and at steady-state its level is three to four times as high as that of the parent compound. 4-oxo-isotretinoin accumulates slightly during long-term treatment and

Table 1 Some Pharmacologic Properties of Oral Retinoids Used in Dermatology (Data Compiled from the Literature)

	Vitamin A	Isotretinoin	Etretinate	Acitretin	Bexarotene
Chemical configuration					
Ring structure	β-Ionone	β-Ionone	Monoaromatic	Monoaromatic	Polyaromatic
Side-chain	All-*trans*	13-*cis*	All-*trans*	All-*trans*	Not applicable
End group	Alcohol or fatty ester	Acid	Ethyl ester	Acid	Ester (free acid)
Molecular weight	286	300	354	326	338
Therapeutic dosage range (mg/kg per day)	0.2–2	0.5–2	0.5–1.0	0.3–0.75	7–10
Main carrier in plasma	RBP or lipoproteins	Albumin	Lipoproteins	Albumin	Albumin (?)
Estimated oral availability (%)	50–80	21–25	30–70	36–95	No data
Storage sites					
Liver	Yes	No	No	No	No
Fat	(Yes)	No	Yes	No	No data
Terminal elimination half-life	75–250 days	10–20 hr	100–150 days	50–60 hr	<10 hr
Active metabolites	Retinal and retinoic acid	Retinoic acid?	Acitretin/isoacitretin?	Isoacitretin?	Free acid (glucuronides?)
Average tissue levels (ng/gww)[a]					
Epidermis	250	75	150	50	No data
Dermis	200	Not known	175	No data	No data
Subcutis	2000	<30	≥5000	100	No data

[a]Refers to physiological conditions for vitamin A and to long term therapy for other retinoids.
Abbreviation: RBP, retinol-binding protein; ww, wet weight.

clears more slowly ($t_{1/2}$, 17 to 50 hours; mean 25 hour) from the circulation than does the native drug (17). A minor proportion of this metabolite is present in blood in its all-*trans* form, 4-oxo-RA (17,18).

Renal metabolism of isotretinoin is negligible and kidney failure is of no major concern when prescribing the drug. A number of post-transplant kidney recipients with mild renal dysfunction (serum creatinine, 170–250 μmol/L) have taken low-dose isotretinoin (0.2–0.5 mg/kg/day) with good antiacne effect and no clinical signs of retinoid toxicity or deterioration of renal function (19–21). Similarly, a uremic patient undergoing hemodialysis was given the full dose of isotretinoin as acne treatment with no evidence of abnormal drug side-effects (22).

Isotretinoin is metabolized by cytochrome P450 enzymes, which are inducible by ethanol and inhibited by, for example, ketoconazole. Increased blood levels of isotretinoin may therefore be expected when the drug is combined with imidazole fungistatics. Isotretinoin cannot be converted to retinol in vivo, and plasma vitamin A concentrations are not affected by systemic isotretinoin administration (23–25). In contrast, several vitamin A-metabolizing enzymes in the gut, liver, eye, and skin are affected. Thus both intestinal retinal reduction and hepatic retinol esterification are inhibited by isotretinoin in vitro (26). In peripheral tissues, isotretinoin therapy may cause clinically relevant interactions, such as abnormal night vision (27). Also, oral isotretinoin is known to interfere with vitamin A levels in human skin (24). In acne patients given isotretinoin at a daily dosage of 0.5 to 1 mg/kg for three to six months, the epidermal and sebaceous gland contents of vitamin A (retinol including its esters) increased by at least 50% to 100%. In contrast, the cutaneous concentrations of 3,4-dehydroretinol decreased to almost undetectable levels. These effects probably involve metabolic interference of isotretinoin on vitamin A metabolism in the target tissue (42).

Acitretin

Monoaromatic retinoids (acitretin, etretinate, and motretinid) are mainly used to treat psoriasis and other hyperkeratotic diseases. Acitretin binds to RARs with moderate affinity. From both pharmacokinetic and historical viewpoints, etretinate is the prodrug of acitretin and probably lacks biological activity per se (owing to a blocked carboxy terminus) (28). Like isotretinoin and RA, acitretin binds to plasma albumin, whereas etretinate is strongly associated with the lipoproteins (29). The initial hope for acitretin relied on an assumption that the drug would be more rapidly excreted than etretinate after discontinuation of therapy. However, several studies have revealed that acitretin is partly metabolized by esterification to etretinate-like metabolites that are lipid-bound and may persist for variable times after drug discontinuation (30–32). Conversion of acitretin to etretinate seems to be facilitated by a concomitant intake of alcohol (33).

Oxidation of aromatic retinoids requires cytochrome P450 and is thus amenable to enzyme induction, for instance by alcohol and barbiturates. Acitretin appears to be eliminated exclusively by metabolism, with the resultant metabolites

excreted to an equal extent by the hepatic and renal routes. Virtually no unmodified drug is found in the urine or the bile (28).

In pharmacokinetic studies of acitretin, the most conspicuous feature is the rapid appearance of isoacitretin in plasma. The concentration-time curve of the isomer is quite different from that of the unchanged drug, with a half-life about 1.5-fold higher than that of acitretin. Steady-state plasma concentrations ($C_{ss,min}$) of both compounds are reached within 7 to 10 days, which is consistent with the elimination half-life of the two retinoids. During chronic therapy, the predosing level of 13-*cis*-acitretin is usually some three- to five-fold higher than for acitretin. The terminal elimination of acitretin (given as parent drug) is shorter as compared to etretinate; after multiple-dose administration for three months a terminal half-life of 50 to 60 hours has been found (28,34). Occasionally, the half-life is longer (see previously).

Bexarotene

Bexarotene (Targretin, LGD1069) belongs to the rexinoid class of molecules, which bind specifically to RXRs (35). It is used systemically to treat cutaneous T-cell lymphoma (CTCL), but is also available as a topical formulation (1% gel) for psoriasis and hand eczema (see Chapter 10). As far as pharmacokinetics is concerned, bexarotene has not been studied to the same degree as the other retinoids (36). Bexarotene is an esterfied prodrug, the active metabolite of which is the corresponding free acid (37). The tissue levels of the drug do not seem to have been studied in detail.

DISPOSITION OF SYNTHETIC RETINOIDS IN THE SKIN

Steady-state drug concentrations in the skin have been determined for isotretinoin in acne patients and for acitretin (and etretinate) in psoriasis patients (24,30,38,39). Compared with endogenous RA, the therapeutic concentrations of acitretin and isotretinoin in the skin are high; in fact, the epidermal values are usually in the same order as the total vitamin A concentration, that is, 100–500 ng/g wet tissue. No corresponding information is available for bexarotene.

Isotretinoin

During chronic dosing (0.75 mg/kg/day), the isotretinoin levels in epidermis (60–120 ng/g) are generally lower than in the general circulation and comparable to those attained by equipotent doses of acitretin (24,30). The main metabolite of isotretinoin (4-oxoisotretinoin) predominates over the parent drug in epidermis, thus reflecting the situation in plasma. There is no progressive accumulation of the drug in the skin during long-term administration and isotretinoin is not stored or retained in the subcutaneous fat. Analysis of microdissected sebaceous glands from isotretinoin-treated acne patients has shown that the drug is taken up by the target tissue although present at somewhat higher concentrations in the skin compartments surrounding the sebaceous glands (40). The metabolic fate of

isotretinoin in the pilosebaceous apparatus is not known, but the fact that the drug was not detected in surface sebum collected during oral therapy (41) indicates that extensive metabolism or degradation takes place in the sebocytes or in the follicular epithelium. After stopping of isotretinoin therapy, drug concentrations decline rapidly in the sebaceous glands with no detectable retinod three weeks after cessation of the drug (40).

Acitretin (and Etretinate)

The total acitretin concentration in epidermis averages 200 ng/g during chronic dosing (50 mg daily) and does not change with time (31,38); this value includes both acitretin and its ethyl ester, etretinate. Acitretin is found in many tissues during therapy, including keratinocytes (43), and usually constitutes about 30% to 50% of the total drug concentration in epidermis (38). The drug disappears fairly rapidly from the epidermis, a finding consistent with the rapid reversal of mucocutaneous side effects after stopping of treatment. However, etretinate, as a very lipid-soluble metabolite of acitretin, accumulates extensively in other tissues. High-affinity sites for etretinate are fat tissue and the adrenals (44). Drug accumulation in fat explains the very long biological half-life of elimination of etretinate. Thus within a few weeks after starting therapy, the etretinate concentration in subcutaneous fat is almost 100 times higher than in most other tissues. Later on the fat tissues appear to become saturated with etretinate; during chronic therapy the total amount of drug in the fat tissue corresponds to about 50 to 100 mg acitretin (38).

In a preliminary report, Laugier et al. (39) found about 200 and 50 ng/g, respectively, of acitretin and isoacitretin (13-*cis*-acitretin) in full-thickness skin biopsies from psoriasis patients receiving 30 mg of acitretin daily. Considerably lower values (20–50 ng/g) were found in epidermal biopsies by Grönhöj-Larsen et al. (31), who also found that acitretin (or a closely related metabolite) occasionally accumulates in subcutaneous fat although at a much lower rate than etretinate. This storage probably reflects the conversion of acitretin to etretinate during therapy (30). Similar results have been presented by Meyer et al. (32) who also found that the acitretin level in full-thickness human skin fluctuates over time, being maximal 30 ng/g within five hours after an oral dose of 25 mg of the drug.

INTERACTIONS WITH UV-RADIATION AND EFFECTS OF TOPICALLY APPLIED RETINOIDS

Vitamin A and most other retinoids are notoriously sensitive to UV-irradiation and, when present in the superficial layers of the skin, are constantly exposed to other environmental factors as well. Needless-to-say, these interactions may have an impact not only on the tissue levels of retinoids, but also on the mechanism of action of retinoids when treating skin disorders (especially since UV therapy is often combined with retinoids in the treatment of, e.g., psoriasis).

Interestingly, isotretinoin and acitretin behave differently in epidermis when exposed to UV radiation (45); the former retinoid is progressively destroyed, whereas acitretin is isomerized to isoacitretin in a fixed 1:2 ratio without further modification. To what extent these effects can be explained by differences in chemical characteristics or variable skin compartmentalization of the two retinoids (e.g., different protection by cellular binding proteins) is not known.

With topical application of retinoids much higher concentrations are attainable in the superficial layers of the skin (46). RA, isotretinoin and acitretin penetrate more efficiently across the horny layer than etretinate; the dermal concentrations of isotretinoin and acitretin attained after application of 10 μg of retinoid/cm^2 to human skin amount to about 10 μg/g and 1 μg/g, respectively (47,48). The percutaneous absorption pharmacokinetics of acitretin has also been studied in several other species (49). By and large, the systemic availability of topically applied RA, isotretinoin, and acitretin appears negligible (48–50). During topical treatment of light-exposed areas, UV-induced modification of the retinoid molecules is a reality, which may either reduce the therapeutic effect or alter the biological response, that is, via isomerization (48). The interaction between retinoids and UV radiation is probably also significant in melanocytes, which are depleted of vitamin A when irradiated in vitro (51). Biologically, this effect appears to be augmented by a down-regulation of RARs and RXR-α following UV irradiation (52).

CONCLUSIONS

The journey from discovering vitamin A-deficiency as a cause of skin symptoms to today's pharmacotherapy of diverse skin diseases with synthetically tailored, receptor-specific retinoids or RA-metabolism blocking agents is indeed a long and fascinating one, which holds great promise for the future in terms of the development of new and better drugs and an increased understanding of the mechanism of actions of these compounds in the skin. Although side-effects remain a problem, by increasing the specificity of the retinoids and by engineering targeted drug delivery systems these obstacles will hopefully soon be overcome.

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Recent Studies on the Pharmacokinetics and Metabolism of Retinoids in the Skin

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INTRODUCTION

Vitamin A plays a major role for the regular formation of epithelial tissues and its maintenance by the availability and activity of its metabolites—in particular retinoic acid (RA). Therefore, interconversion, storage, uptake, metabolism, and efflux are factors that determine retinoid effects on individual skin cells as well as the body as a whole. The general metabolic pathways of retinoids, which have been reviewed in Chapter 1 and elsewhere (1–3). The metabolic pathways include inter alia: (*i*) retinyl ester storage in the liver; (*ii*) transport from the liver to the target organ after binding to plasma retinol-binding protein; (*iii*) conversion of retinol to retinal and RA respectively by alcohol dehydrogenase (ADH) and retinaldehyde dehydrogenase (RALDH); (*iv*) binding to the cellular retinol binding protein (CRBP) or cellular retinoic acid binding protein (CRABP) as well as to retinoic receptors (RARα, β, and γ) or retinoic X receptors (RXRα, β, and γ) (2).

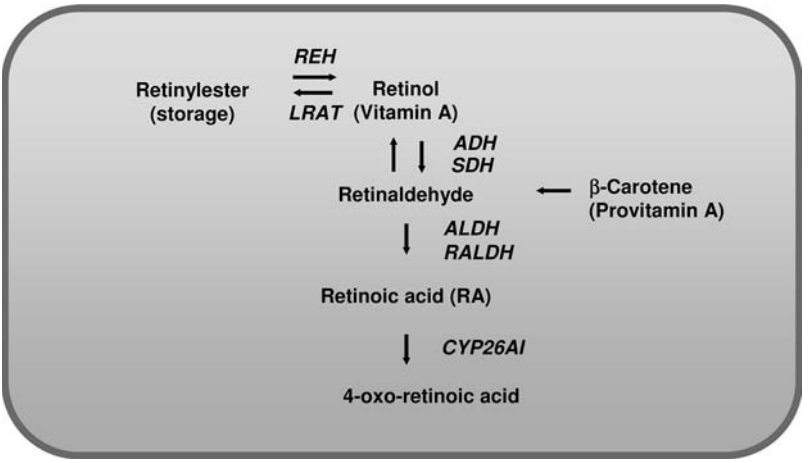

Figure 1 Intracellular pathways of retinoid metabolism. Retinylesters and β-carotene are ingested and converted to retinylesters for storage, mainly in the stellate cells of the liver. Demand for retinol results in the release of retinol–retinol binding protein complexes, which are taken up by human skin. The storage of retinol in human skin occurs through esterification mediated by lecitin: retinol acyltransferase. Hydrolysis of retinylester to retinol is regulated by specific retinyl ester hydrolase. The retinoid acid is derived from retinol precursor by conversion to retinaldehyde by ADH or short-chain dehydrogenase/reductase and subsequently to retinoic acid by ALDH activity.

This chapter will focus on more recent findings of: (*i*) specific aspects of membrane-associated transport of retinoids, (*ii*) their metabolism by inducible enzymes in skin cells, (*iii*) and the effect of RAs and their metabolites on different skin cell types.

ACTIVE TRANSPORT OF RETINOIDS IN SKIN CELLS

For many years, the skin was thought to be a passive structural barrier between the body and the environment. It is now clear that (*i*) human epidermal keratinocytes express various transport-associated and detoxifying metabolic enzymes and (*ii*) these cells are capable of active uptake, biotransformation and antitransport of different small molecular weight compounds including xenobiotics such as drugs, solvents, and carcinogens or vitamins such as retinoids.

Efflux-transport associated proteins, such as P-glycoprotein multidrug resistance protein 1 (MDR1) and multidrug resistance-associated proteins (MRPs) are overexpressed in drug-resistant cell lines and various human tumors, including malignant melanoma (4,5). Several studies have shown that human skin cells express these transport proteins and regulate the distribution of specific substrates (6,7).

Reserve-transcriptase polymerase chain reaction (RT-PCR) RT-PCR analysis of normal human epidermal keratinocytes (NHEK) revealed constitutive expression

of MRP1 as well as MRP3-6 but was negative for MDR1 and MRP2. Expression of MDR1 mRNA was weakly detectable following treatment with the corticosteroid dexamethasone. In addition, expression of the relevant proteins was confirmed by immunoblots showing constitutive MRP1, 3, and 5 as well as MDR1 after induction with dexamethasone. Immunohistological analysis of normal human skin showed positive staining for MRP1 in the plasma membrane of epidermal keratinocytes (6). Functional activity of these MRPs in NHEK and dermal fibroblasts was verified in vitro using the calcein acetoxymethyl (AM) ester assay (8). It is also known that mutations in the MRP6 gene are linked to pseudoxanthoma elasticum (PXE), a connective tissue disorder affecting elastic structures in the body including the skin, although their role in the pathogenesis of PXE remains undefined (9).

Recent studies have focused on identifying specific substrates of the MRP-transporters expressed in skin cells. Active transport of retinoids mediated by membrane-associated transport proteins was demonstrated in NHEK. Treatment of cells with the cytokine IL-6 and sIL-6R increased expression of MRPs and active efflux transport of all-trans [20-methyl-^{3}H] RA in NHEK (10). Specific MRP-inhibitors, such as indomethacin, can inhibit this transport (Heise et al., unpublished data). MDR1 does not affect efflux transport of all-trans RA (11) and is not constitutively expressed in NHEK or dermal fibroblasts (6).

The role of influx-transport proteins, such as the organic anion transporting polypeptides (OATPs), which are also constitutively expressed in human skin cells is currently unclear (12). Studies in rat retina showed that N-retinyl-N-retinylidene ethanolamine, an unusual cationic, amphiphilic retinoid seems to be transported by OATP2, which is localized at the interface between the pigment epithelium and the photoreceptor outer segments (13). Active transporter mediated influx of retinoids in skin cells is yet to be proven.

EXPRESSION OF RETINOID-METABOLIZING ENZYMES IN HUMAN SKIN

All-*trans* RA is thought to be the major "natural ligand" of all three nuclear RA receptors. Cellular levels of all-*trans* RA are stringently regulated through a balance of uptake, biosynthesis, catabolism, and efflux-transport (10,14). In human skin, the primary metabolite of all-*trans* RA is 4-hydroxy all-*trans* RA, which is further metabolized to 4-oxo all-*trans* RA. However, the metabolic pathways for RA transformation are only partially understood and the specific cytochrome P450 (CYP) enzyme responsible for the conversion of RA to 4-oxo-RA in human skin has not been defined. Smith et al. (15) recently identified CYP2S1 (16) in human skin cells and showed its ability to metabolize all-*trans* RA. Up to a two-fold change in CYP2S1 mRNA expression was observed following topical application of all-*trans* RA to human skin. Duell et al. (17) showed a 4.5 fold increase in conversion of all-*trans* RA to 4-oxo all-*trans* RA in skin treated with all-trans RA. In addition, incubation of all-*trans* RA with heterologously expressed

CYP2S1 (15) yielded 4-hydroxy-RA and 5,6-epoxy-RA but not 4-oxo RA. These data indicate that CYP2S1 is a CYP enzyme responsible for RA-metabolism in human skin but that other metabolic pathways must exist as well.

In 1997, the first mammalian RA-inducible retinoid acid-metabolizing cytochrome P450 (CYP26AI) was cloned (18,19). White et al. (18) showed that CYP26AI generates several hydroxylated forms of RA, including 4-OH-RA, 4-oxo-RA and 18-OH RA in COS-1 cells transfected with a human CYP26AI expressing vector. They also showed that MCF10A cells in which no inducible expression of CYP26AI was detectable, did not generate 4-oxo or 4-OH RA in either control or RA-treated cells. In contrast, MCF7 cells with highly inducible CYP26AI expression produce these metabolites following treatment with all-*trans* RA (18).

Since NHEK can metabolize RA it is tempting to speculate that CYP26AI activity is responsible. Recent analysis of monolayer cultures of NHEK and dermal fibroblasts by quantitative real-time PCR and RT-PCR revealed no basal expression of CYP26AI mRNA, whereas specific transcripts were detectable following addition of 10^{-6} M all-*trans* RA (20). Immunofluorescence and Western blot analysis showed a weak expression of CYP26AI in NHEK, which was increased by treatment with all-*trans* RA, forming an autoregulatory feedback loop to control RA levels. In striking contrast to monolayer cultures in which CYP26AI was only weakly detectable, strong constitutive expression was seen in vivo and in 3D organotypic culture where it was found to be mainly expressed in basal epidermal keratinocytes, as well as eccrine sweat glands and sebaceous glands (Fig. 2). These studies verified the capacity of human skin to metabolize

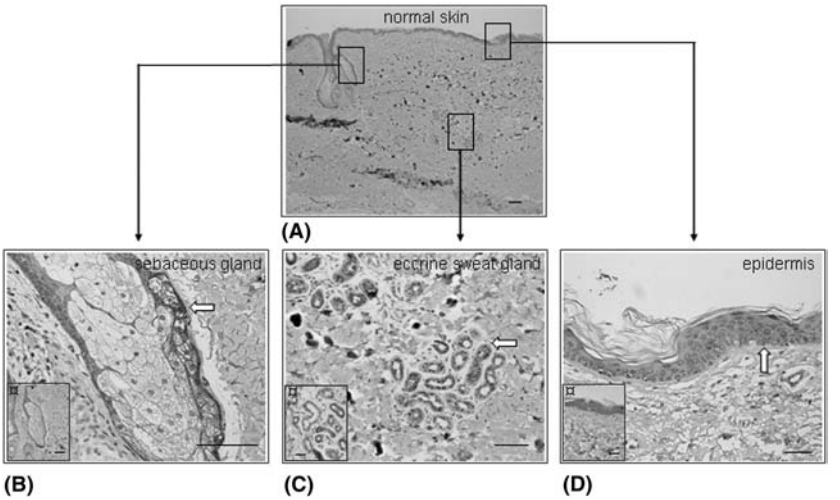

Figure 2 (*See color insert*) Immunohistochemical analysis of the expression of the retinoid acid metabolizing CYP26AI in normal human skin. (**A**) Overview, (**B**) staining of the sebaceous gland, (**C**) the eccrine sweat gland, and (**D**) the epidermis. *Note*: ¤ indicates an isotypic control; ⇒ indicates expression of CYP26AI, bar = 200 μm. *Source*: From Ref. 20.

RA, although substantial differences exist in CYP expression between normal skin and 3D skin models compared to monolayer cultures.

EFFECT OF RETINOID-METABOLITES ON SKIN CELLS

RA exerts a variety of effects on gene transcription that results in the regulation of growth, differentiation and inflammation in normal and neoplastic skin cells. The therapeutic efficacy of RA and other retinoids appears to relate to these interactions. As described previously, human skin cells have the capacity to metabolize RA isomers (15,17,20). Since the effect of metabolic transformation of RA on its efficacy and toxicity in human skin cells was unknown the functional activity of 4-oxo- RA metabolites in NHEK and dermal fibroblasts was analyzed. Although the 4-oxo-metabolites of RA are generally thought to be inert catabolic end-products, studies by Baron et al. (21) indicate that these substances display strong and isomer-specific transcriptional regulatory activity in both NHEKs and dermal fibroblasts. Microarray and proteomic analyses identified a number of novel genes/gene products that are influenced by RA treatment of NHEKs or fibroblasts, including genes for enzymes catalyzing biotransformation of retinoids, corticosteroids and antioxidants and structural and transport proteins known to be essential for homeostasis. Interestingly, all of the 4-oxo-RA metabolites showed transcriptional regulatory activity including 4-oxo-13-*cis*-RA, which has generally been thought to be an inactive metabolite of 13-*cis*-RA. 4-oxo-13-*cis*- and 4-oxo-all-*trans*-RA had similar effects on the expression of genes related to extracellular matrix and cellular metabolism, whereas 4-oxo-9-*cis*-RA differed in this regard. Each of the 4-oxo-metabolites showed distinctive gene expression profiles, suggesting that the relative stoichiometry of the different 4-oxo metabolites in the nucleus may account for the different therapeutic actions observed for the three RA isomers.

CONCLUSION

The effect of retinoids on skin cells is influenced by numerous factors including interconversion, storage, uptake, metabolism, and efflux transport. Developing knowledge regarding these mechanisms has helped enhance our understanding of retinoid pharmacology, but many unanswered questions remain. For example, the pathways responsible for the metabolism of all-*trans* RA in skin cells are still not completely understood. It seems likely that variations in the expression of transporters and metabolizing enzymes may be responsible for the individual variability in reaction of patients to oral or topically applied retinoids, but more clinical and in vitro studies are needed to clarify these points.

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Anti-aging Effects of Retinoids and Mechanisms of Action

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INTRODUCTION

Over the past 100 years, there has been a radical shift in the public's attitude to sun exposure. Until Coco Chanel introduced the suntan as an essential fashion accessory approximately 80 years ago suntans were not desirable. Since then, almost as a direct consequence, lifelong sun exposure in individuals has increased exponentially. The accessibility of vacations in the sun, commercial tanning parlors and the perception of the suntan as being healthy have compounded this. The direct sequelae of increased sun exposure have been photoaging and, more significantly, photocarcinogenesis. Indeed, the incidence of melanoma and nonmelanoma skin cancers has been described as epidemic by some authors (1). Thus, preventive and medical strategies to target the effects of chronic sun exposure are essential. Preventive strategies are beginning to take effect particularly in Australia. Retinoid therapy is one of the most well studied medical strategies.

The term "retinoids" defines natural or synthetic compounds with vitamin A-like biological properties. Retinoids are essential for mammalian embryogenesis, development, vision, immunity, and epithelial cell differentiation. Abnormal

dark-vision adaptation, dry skin, dry hair, broken fingernails, and decreased resistance to infections are among the first signs of retinoid deficiency in adult humans. Retinoids were first used in dermatology in the 1950s for the treatment of acne and other skin disorders. In 1986, Kligman et al. (2) first reported the results of a clinical study showing that topical tretinoin (all-*trans* retinoic acid) partially reversed the cutaneous damage associated with photoaging. Since then, a large number of clinical studies have demonstrated the beneficial effects of retinoids in the treatment of clinical signs of skin aging (see below).

In this chapter, we detail the clinical characteristics and molecular mechanisms of skin aging, and we describe the clinical effects of topical retinoids in the treatment of skin aging. We also provide an up-to-date overview of our current understanding of the mechanism of action of retinoids in the treatment of human skin aging, and discuss the side effects of retinoid therapy.

SKIN AGING

Organization of Human Skin

Human skin is separated into three compartments: superficial epidermis, dermis, and subcutaneous fat. The epidermis provides a tight barrier between environment and internal organs, and is mainly composed of keratinocytes, melanocytes, and Langerhans cells. The dermis provides physical and metabolic support to the skin. It comprises mainly fibroblasts, blood vessels, and appendages, embedded in collagenous extracellular matrix. Subcutaneous tissue is composed primarily of fat-producing adipocytes, and located beneath the dermis.

Collagens represent about 90% of the total proteins of the skin. Type I collagen is the most abundant protein of the dermal extracellular matrix, and is secreted by dermal fibroblasts into the extracellular space as soluble precursor called procollagen. The levels of procollagen molecules are a reliable measure of ongoing collagen production.

Enzymatic cleavage of procollagen molecules gives rise to insoluble collagens, which are incorporated into fibrils to form collagen fibers. The formation of mature collagen fibers (or fibrillogenesis) involves enzymatic and nonenzymatic cross-linking, and remains only partially understood (3). Type I collagen is necessary for the maintenance of skin's structure and function. Alterations of collagen fiber structure, organization, and/or number are believed to be determining factors in formation of wrinkles in skin.

Clinical and Histological Features of Skin Aging

Due to its physical exposure to the environment, skin is particularly susceptible not only to damage from xenobiotics (drugs, chemicals, atmospheric pollution, and cigarette smoke) but also to ultraviolet (UV) irradiation from the sun. On areas of the body that are often not covered by clothing (i.e., face, nape of the neck, hands, and forearms), photoaging and chronological aging are superimposed, leading to more pronounced alterations of the appearance of the skin.

Comparison of sun-exposed and sun-protected skin areas within the same individual reveals that sun exposure is primarily responsible for "aged" appearance of the skin. Photoaging is a cumulative process that greatly depends on the degree of skin pigmentation and sun exposure. Darkly pigmented skin is more resistant to photoaging than fair skin, mainly due to higher concentration of melanin, which absorbs UV radiation and thereby acts as a natural sunscreen (4–6).

The clinical signs of photoaging are diverse and include fine and coarse wrinkles, mottled hyper-pigmentation, actinic lentigines, freckles, roughness, telangiectasia, and sallowness (Fig. 1). Actinic lentigines are even-colored and pigmented macules often (erroneously) called "liver" or "age spots." These are not related to natural aging, but are a direct consequence of cumulative sun exposure seen only at sun-exposed sites.

Photoaging is also accompanied by passive vascular dilatation and increased vessel tortuosity leading to telangiectasia. These vascular alterations may explain the "rosy cheeked" appearance of photoaged skin (particularly in people of celtic heritage), and the appearance of Bateman's or senile purpura at sites of chronic sun exposure.

Histological examination of photoaged skin reveals irregular epidermal thickness, thickening of the stratum corneum, and significant increase in the

Figure 1 Photoaged skin is characterized clinically by the presence of wrinkles and actinic lentigines.

number of melanosomes in basal keratinocytes (causing the appearance of pigmentation). In addition, there are focal irregularities in cell and nuclear size and shape. Actinic lengitines are characterized by hyperkeratosis and increased number of basally-located melanocytes. These epidermal changes overlie the dermal changes, mainly alteration of the organization of extracellular matrix and accumulation of amorphous elastin-containing material that resides beneath the epidermal dermal junction (described as "solar elastosis"). In contrast, naturally aged skin is characterized by thinning of the epidermis, and general atrophy of the dermis due to a combination of decreased collagen synthetic activity and lack of mechanical tension (7). As a result, naturally aged skin is characterized by a loss of collagen and elastin content and disorganization of the fibrillar network (7–9). Alterations of the organization of collagen and elastin are typically more pronounced in photoaged skin, compared to sun-protected aged skin (10).

Biochemically, naturally aged and photoaged skin are characterized by sustained down-regulation of collagen production (Fig. 2) (11,12) and sustained

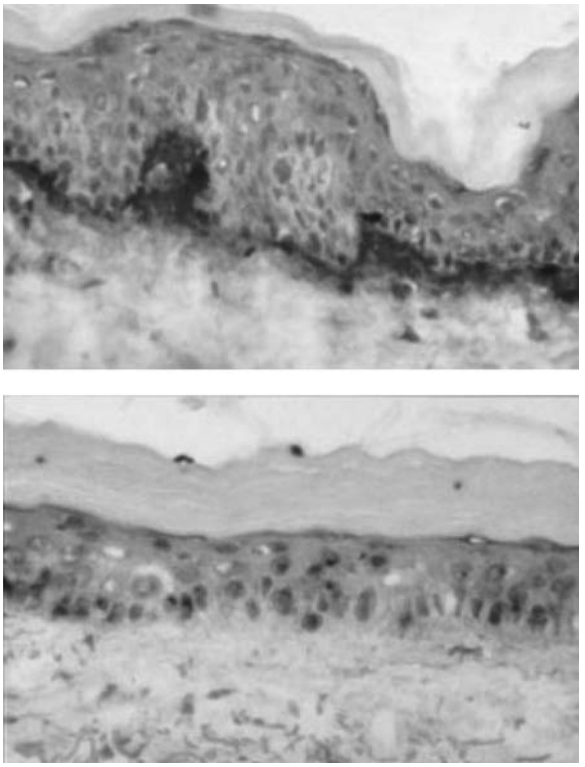

Figure 2 (*See color insert*) Photomicrograph of sun-protected (*upper panel*) and sun-exposed (*lower panel*) skin stained for procollagen I in the papillary dermis of sun-exposed skin. *Source*: Courtesy of Massachusetts Medical Society.

upregulation of collagen-degrading enzyme activities, primarily those of matrix metalloproteinases (MMPs) (13,14). Imbalance between dermal extracellular matrix degradation and production results in alterations of skin structure and function, and is the principal cause of reduced youthful appearance of skin with advancing age.

Molecular Mechanism of Skin Aging

Reactive Oxygen Species Initiate Damage to the Extracellular Matrix

The aging process is associated with oxidative stress due to enhanced production of reactive oxygen species (ROS) and decreased oxidative defenses (15,16). As a result, accumulation of ROS alters the structure and properties of nucleic acids (17) and proteins (18), which leads to skin damage. In sun exposed areas, chronic oxidative stress that occurs over a lifetime is superimposed with transient generation of ROS, which occurs after UV irradiation of human skin (19,20).

The sequence of events that are involved in the alterations of skin structure after UV irradiation exposure is partially characterized (Fig. 3). First, UV irradiation is absorbed by chromophores in epidermal and dermal cells. The absorbed energy is dissipated by photochemical reactions that produce multiple ROS, including superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO′). ROS are activators of several receptor tyrosine kinases and strong reversible inhibitors of protein tyrosine phosphatases (PTPs) (21). Inhibition of PTPs' active-site cysteine residue by UV irradiation raises the phosphorylated (i.e., activated) state of many cellular tyrosine kinases, including cytokine and growth factor cell surface receptors, such as epidermal growth factor (EGF) receptor (22–25), tumor necrosis factor (TNF)-α receptor (26), platelet activating factor (PAF) receptor (26), insulin receptor (27), interleukin (IL)-1 receptor (28), and platelet derived growth factor (PDGF) receptor (29). Indeed, in vitro down-regulation of receptors for EGF, IL-1, and TNF-α results in 85% reduction of UV irradiation-activated signal transduction (28). Through activation of cytokine and growth factor cell surface receptors, UV irradiation stimulates acute cellular responses, equivalent to the synergic effect observed when cells are stimulated simultaneously with several growth factors (28).

The variety of molecular changes that result from stimulation of cell surface receptors includes activation of members of the mitogen-activated protein kinase (MAPK) family: Erk, c-Jun NH_2-terminal kinase (JNK), and p38 (30). JNKs and p38 are strongly activated by UV irradiation of human skin, and induce the phosphorylation of the transcription factors c-Jun and activating transcription factor (ATF)-2, which in turn stimulate c-Jun transcription (31). Elevated c-Jun associates with constitutive c-Fos to form an active activator protein 1 (AP-1) complex. In human skin in vivo, AP-1 is induced throughout the epidermis and in dermal cells one hour after UV irradiation and remains elevated for at least 24 hours (23).

AP-1 is a critical regulator of several members of the MMP family. The MMPs that contain an AP-1 site in their promoter include MMP-1 (interstitial

collagenase or collagenase 1, which initiates degradation of types I and III fibrillar collagens), MMP-9 (gelatinase B, which further degrades collagen fragments generated by collagenases), and MMP-3 (stromelysin 1, which degrades type IV collagen of the basement membrane and activates proMMP-1). Induction of MMP-1, MMP-3, and MMP-9 transcripts occurs within eight hours following UV irradiation of human skin in vivo, and is accompanied by increased enzyme activities in keratinocytes and fibroblasts (23). As a result, mature fibrillar collagen is degraded within 24 hours after UV irradiation in human skin in vivo (32). Importantly, AP-1-driven MMPs can degrade other extracellular matrix proteins including laminins, proteoglycans, and elastin (33). Therefore, elevated MMP activity following UV irradiation causes alterations of several constituents of the dermal extracellular matrix. Interestingly, topical application of antioxidant such as *N*-acetyl cysteine (NAC) 24 hours prior to UV irradiation reduces activation of c-Jun and consequent induction of MMPs (19). This observation further supports the important role of ROS in UV irradiation-mediated MMP induction (Fig. 3).

In addition to causing collagen and other extracellular matrix protein breakdown, UV irradiation reduces deposition of new type I collagen by transiently decreasing procollagen production (32), and impairing organization of collagen fibrils (34). Decreased collagen production occurs by two main mechanisms. First, AP-1 negatively regulates transcription of type I collagen genes (35,36), and second, UV irradiation prevents the action of transforming growth factor (TGF)-β.

TGF-β and its downstream target connective tissue growth factor (CTGF) are two major profibrotic cytokines (37,38). The effects of TGF-β are diverse and include stimulation of synthesis of collagen and fibronectin and other extracellular proteins, and inhibition of MMP-1 and MMP-3 (38). The binding of TGF-β to its receptors (TβRI, II, and III) initiates downstream signal transduction, which involves activation of Smad-2, and -3. Activated Smad-2 or -3 binds to Smad-4, and the complex translocates into the nucleus, where it acts to stimulate transcription of target genes, including TGF-β1 and CTGF. TGF-β signaling is controlled by inhibitory Smad-7 that interferes with activation of Smad-2 and -3 and terminates TGF-β signaling (38). UV irradiation inhibits expression of TβRII, and upregulates Smad-7 (39), thereby inhibiting expression of CTGF (40). Inhibition of TGF-β signaling in the dermis contributes to down-regulation of collagen production after UV irradiation. Procollagen production in human skin in vivo is decreased within eight hours following UV exposure, and lasts for 24 to 48 hours (32).

Altogether, UV irradiation leads to a transient and localized alteration of the extracellular matrix. The driving force for photoaging is photochemical generation of ROS. The oxidative theory of natural aging posits that ROS generated by normal metabolism damages cellular constituents, triggers destructive signaling pathways (41). Thus, natural aging and photoaging share key molecular features including increased ROS levels (16,42), increased JNK and

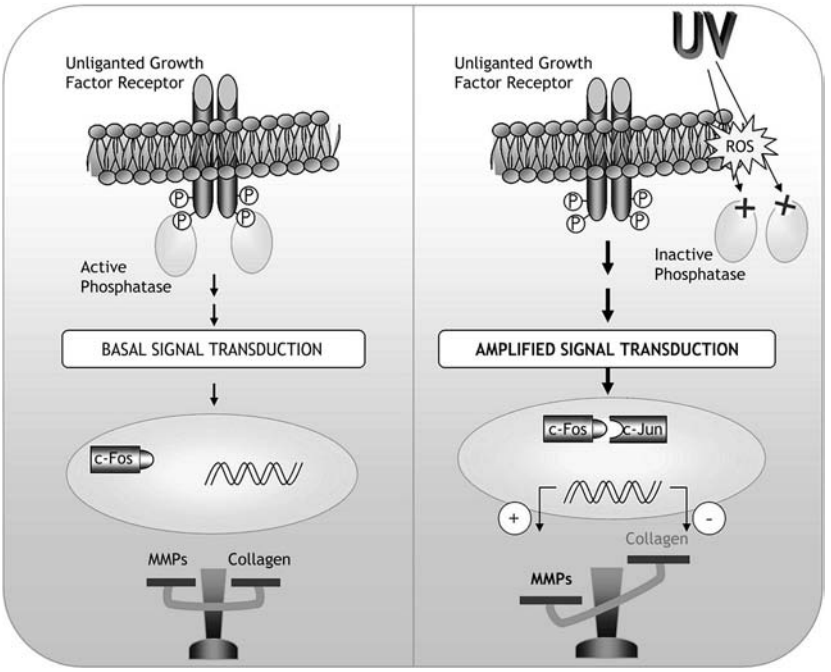

Figure 3 Increased oxidative stress caused by UV irradiation alters skin cell secretion pattern. In non-UV irradiated skin (*left*), active phosphatases regulate the activity of cytokine and growth factor receptors to keep signal transduction pathways at basal level. In response to UV irradiation (*right*), increased reactive oxygen species (ROS) inactivate phosphatases and thereby prolongs the active state of various cytokine and growth factor receptors. Activated receptors amplify downstream signal transduction cascades that enhance transcription factor AP-1 activity. AP-1 stimulates transcription of collagen degrading enzymes [matrix metalloproteinases (MMPs)] and represses collagen transcription. Imbalance between degradation and production of collagen leads to impairment of skin structure accompanied by decreased rebuilding capacity of the skin.

AP-1 activities (43), and increased production of MMP-1, MMP-3, and MMP-9 (14,43,44).

Overall, increased ROS production associated with photo- and natural aging results in an imbalance between degradation and synthesis of collagen, which contributes to local damage and loss of dermal collagen.

Extracellular Matrix Alterations Promote the Phenotype of Aged and Photoaged Skin

Biochemical examination of naturally aged and photoaged skin reveals a sustained upregulation of MMP levels, as well as a sustained down-regulation of procollagen formation rate. The degree of reduced collagen production correlates with the severity of clinical photodamage (12) and the age of individuals (9,44).

Recent studies have increased our understanding of how extracellular matrix alterations promote the phenotype of naturally aged and photoaged human skin. UV irradiation or age-dependent increases of ROS initiates fragmentation of existing collagen. The fragmented collagen is not fully repaired or replaced with newly synthesized collagen. Over a lifetime and with repeated sun exposure, imperfectly repaired collagen and associated deficits in the structure and function of the skin connective tissue accumulate. Accumulation of fragmented collagen fibrils has been demonstrated in photoaged and naturally aged skin by electron microscopy and biochemical analyses (7,45). Increased collagen fragmentation alters the organization and mechanical properties of the dermal extracellular matrix. Normally, dermal fibroblasts bind to intact collagen fibrils, and through cell surface collagen receptors (integrins), which are coupled to cytoskeletal elements, exert contractile forces on the extracellular matrix. Resistive forces exerted by the extracellular matrix create a state of dynamic tension within the fibroblasts and the extracellular matrix. Maintenance of optimal balance between collagen production and collagen degradation by fibroblasts is dependent on adequate mechanical tension (46–48). The weakened mechanical resistance of fragmented extracellular matrix is not adequate to maintain collagen homeostasis and shifts the balance to net increased degradation and reduced synthesis (49). Thus, damaged collagen that accumulates during photo- and natural aging promotes further net collagen fragmentation and further reduction of new collagen production by dermal fibroblasts.

RETINOID TREATMENT OF SKIN AGING

Given the previous scenario, one can easily grasp the importance of stimulating new collagen production for improvement of naturally aged and photoaged skin. Topical retinoic acid (RA) has been demonstrated to stimulate collagen synthesis and improve the appearance of aged and photoaged human skin.

Retinoid Treatment of Photoaged Skin

Topical drugs that are used for the treatment of skin aging are primarily applied on sun-exposed areas, especially the face. Indeed, the majority of clinical studies that have been conducted with retinoids were done on photoaged skin. Fewer reports have demonstrated beneficial effect of retinoids on sun-protected, naturally aged human skin.

Among the very large number of existing natural and synthetic retinoids, few are used in the treatment of skin photoaging. Among them, topical all–*trans* retinoic acid (tRA, tretinoin) has been used in numerous clinical trials and is still widely prescribed by clinicians. However, topical tretinoin therapy causes side effects, which include local irritation and scaling (see below). These side effects can be moderately severe and are a leading deterrent to usage. In a search for alternate compounds with fewer side effects, other topical retinoids have also been studied. These are 13-*cis*-RA (isotretinoin), 9-*cis*-RA (alitretinoin), retinaldehyde

(RAL), retinol (ROL), and the synthetic retinoids, tazarotene and adapalene. Each of these compounds has been proven to be variably effective in treating clinical signs of skin aging; however, each also produces side effects similar to tRA. Retinoid side effects are dose dependent and are mediated by the actions of retinoid receptors (50,51). Therefore, without adjunct intervention, therapeutic effects are expected to be accompanied by unwanted side effects (see below).

Tretinoin

A chance, clinical observation directed researchers to investigate the role of topical retinoids in the management of photoaging. Kligman et al. observed improvements in fine periorbital wrinkling in women using topical tretinoin for the treatment of acne, suggesting that retinoids could repair one of the key features of photoaged skin (2). This hypothesis was first tested on an animal model. Irradiation of a hairless mouse with UVB for 10 weeks produces wrinkles and histologic features of solar elastosis similar to those observed in photoaged human skin (52). Treatment of photoaged mouse skin with topical tretinoin over the course of 10 weeks produced a "repair zone" of new collagen in the papillary dermis—this correlated with the concomitant clinical effacement of wrinkles. This observation was followed up by an open, uncontrolled trial of tretinoin 0.05% cream applied once daily to photoaged human skin. Face and forearms were treated from 3 to 10 months in each volunteer. As observed in the animal model, there was notable clinical improvement in wrinkling associated with deposition of reticulin fibers and angiogenesis in the papillary dermis (2). Double-blind, vehicle-controlled trials followed soon after. In the first of these trials (53), 0.1% tretinoin cream was applied once daily for four months to photoaged facial and forearm skin. There was significant improvement in fine, and coarse facial wrinkling (Fig. 4), sallowness, tactile roughness, and actinic lentigines. However, 92% of patients in the study, reported a "retinoid dermatitis" as a side-effect of treatment, evident clinically by erythema, scaling, and pruritus. The presence of "retinoid dermatitis" has been used as "evidence" that the improvement seen in photoaged skin treated with retinoids is due to this inflammatory "irritant" reaction, and not a retinoid specific event. In an attempt to address this debate the efficacy and irritancy of two concentrations of tretinoin cream, 0.1% and 0.025%, were compared (54). Once daily usage for 48 weeks produced no significant difference, between the two concentrations of tretinoin in overall improvement in photoaged skin but the higher concentration (0.1%) was significantly more irritating. The implication of these findings is that irritancy and efficacy of retinoids can be separated, however low-grade irritancy cannot be excluded entirely as a mechanism of action.

Two multicenter studies (55,56) confirmed the effectiveness of tretinoin as a treatment for photoaged skin. These studies recruited a total of 547 subjects, of whom 393 used tretinoin cream once daily in concentrations ranging from 0.001% to 0.05%. The investigators concluded that six months of once daily applications of a 0.05% tretinoin emollient cream formulation is effective in improving the clinical features of chronic sun exposure. Histologic examination of tretinoin

Figure 4 Clinical improvement in fine wrinkling following 48 weeks' treatment with topical 0.05% tretinoin.

treated skin from this study revealed clinical improvements to be accompanied by epidermal changes, including compaction of the stratum corneum, spongiosis (intercellular edema, which causes widening of the interkeratinocyte spaces), a thickened granular cell layer, increased basal keratinocyte mitotic index and deposition of mucin (57).

In the aforementioned studies, clinical outcome was assessed on subjective criteria and although blinded, still open to observer's bias. To circumvent such potential for bias an objective measure—optical profilometry—was developed by Grove et al. (58). This involves silicone molding of the "crow's feet" area of the face, before and after treatment with tretinoin cream. The ridges and furrows on

the replica are measured by a high-resolution camera coupled with computer-based software. Employment of the objective technique of optical profilometry reaffirmed the clinical findings of previous studies.

The marked improvements in photoaged skin produced by topical tretinoin are from relatively short-term studies. Are these clinical improvements maintained once treatment is stopped, or do they regress? To address this question, the multicenter trials (55,56) were prolonged: subjects were randomized to either stop tretinoin or to continue for a further 24 weeks of treatment (59). Results showed that applying tretinoin emollient cream 0.05% thrice weekly maintained improvement, but stopping therapy resulted in some clinical reversal of beneficial effects. Most clinicians would advocate long-term treatment with tretinoin to at least maintain and perhaps to enhance the clinical gains made in the short term, for example six months. Few vehicle-controlled clinical studies of tretinoin for the treatment of photoaging persist for longer than a year. This issue was addressed partly in a study by Kang et al. (60) who performed a two-year vehicle-controlled trial of once daily applications of 0.05% tretinoin emollient cream or vehicle in 204 subjects. Significant improvement in the clinical features of facial photoaging, particularly fine wrinkles, occurred as early as four months of tretinoin treatment; this improvement was maintained for the two-year treatment period.

Hyperpigmentation, particularly in the form of actinic lentigines, is the other cosmetically troubling feature of photoaged skin. In a 10 month vehicle-controlled double-blind trial of once daily 0.1% tretinoin significant clinical lightening in actinic lentigines and mottled hyper-pigmentation of the hands, forearms, and face occurred in the tretinoin-treated group (Fig. 5) (61). This particular study also addressed the question of whether actinic lentigines recurred after

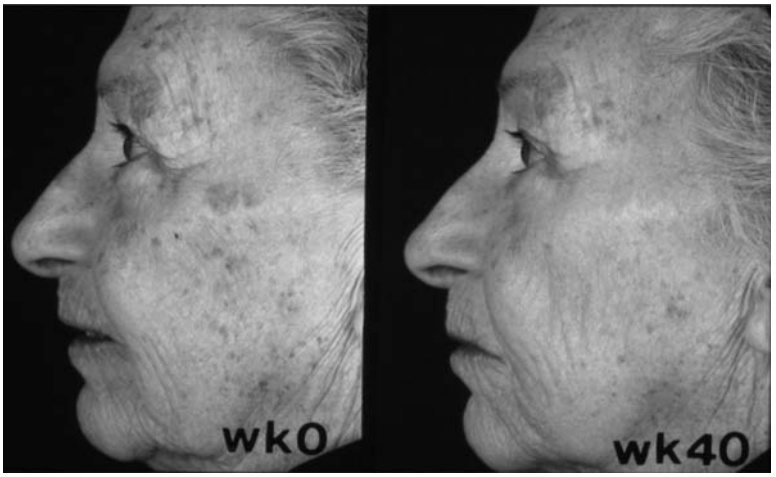

Figure 5 (*See color insert*) Significant lightening of facial actinic lentigines following 10 months' treatment with 0.1% tretinoin cream. *Source*: Courtesy of Massachusetts Medical Society.

withdrawal of treatment. Subjects were followed up six months after stopping tretinoin; those actinic lentigines that had disappeared on tretinoin treatment did not return although further sun exposure could result in new lesions. Although actinic lentigines and sun-induced hyperpigmentation are the major features of photoaging in caucasians, they are seen as an especially prevalent and cosmetically troubling problem in Far East Asia. Thus there is significant interest in the efficacy of topical tretinoin in treatment of sun-induced hyperpigmentation in this population. As a result, the antiphotoaging effects of tretinoin cream have been studied in Far East Asian subjects (62–64). It appears that clinical lightening and/or disappearance of actinic lentigines occurs with tretinoin treatment (5). The first significant improvements are noted to occur at four to eight weeks of treatment and are maintained at three-year follow-up.

Histologically, retinoid therapy is associated with compaction of stratum corneum, thickening of epidermis, increased distribution of melanin, and increased formation of type I collagen, glycosaminoglycans, and anchoring fibrils (65). On the other hand, retinoids have no proven effect on solar elastosis (collagen VII) (65). When used for at least six months, tretinoin reduces melanocytic and keratinocytic atypia, and increases the thickness of papillary dermal collagen, which likely contributes to tretinoin-mediated enfacement of wrinkles (66).

Isotretinoin

Isotretinoin is an effective treatment for facial wrinkling, seemingly with less irritancy than tretinoin (64). These data are supported by other studies (67,68) including a six-month multi-center, double-blind study of a 0.05% formulation of isotretinoin containing an SPF 15 sunscreen (69). There have been no direct comparative studies of topical isotretinoin with tretinoin, but it is widely believed that tretinoin is probably more effective for the treatment of photoaging. Creidi et al. (70) reported that topical RAL improved wrinkling, although retinyl propionate cream was ineffective (71).

Tazarotene and Adapalene

Third generation synthetic retinoids have been developed and licensed for the treatment of acne and psoriasis and, unsurprisingly perhaps, their effectiveness in the treatment of photoaged skin has been investigated also. Tazarotene, developed initially for the treatment of plaque psoriasis, selectively targets retinoic acid receptors (RARs)-β and -γ (72). Several studies have examined the efficacy and safety of 0.1% tazarotene cream in the treatment of photaging. A pilot study (73) demonstrated that objective clinical and histologic improvements in both wrinkles and pigmentary mottling of forearm skin following daily applications of 0.1% tazarotene cream over 12 weeks. Subsequent longer-term multicenter trials confirmed these data (74,75). A six-month dose-response study (74), demonstrated that a 0.1% formulation of tazarotene was superior to lower concentrations of the retinoid in clinical improvement of fine wrinkles and mottled hyperpigmentation of facial skin. The study showed also that 0.1% tazarotene cream was comparable

in efficacy to 0.05% tretinoin emollient cream. A second six month duration (75) double-blind, vehicle-controlled study of once daily 0.1% tazarotene confirmed efficacy, which when extended to 12 months in an open fashion, revealed continued improvement in wrinkling, hyperpigmentation, surface roughness, and global severity of photoaging.

Adapalene, licensed for the treatment of acne, is effective for the treatment of photoaging. A vehicle-controlled trial of once daily applications of adapalene gel (either 0.1% or 0.3%) for four weeks, followed by twice daily applications, if tolerated, for up to nine months produced significant clinical lightening of actinic lentigines (76).

Retinoids for the Treatment of Naturally Aged Skin

The role of topical retinoids in the treatment of naturally aged skin is an emerging area. Photoaging and natural aging appear to share a number of molecular and biochemical features, particularly in subjects over 70 years of age (77). Naturally aged, sun-protected skin is characterized by reduced levels of collagens I and III and increased levels of matrix metalloproteinases (77). Varani et al. (78) used a modified skin organ culture model to examine the effects of tretinoin on adult skin, both sun-protected and photoaged, and on neonatal foreskin. Adult skin from both sites, (sun-protected and photoaged) responded equally well to tretinoin as regards epidermal keratinocyte proliferation and dermal production of collagen. By contrast, neonatal skin was relatively unresponsive. These data imply that retinoids are able to repair naturally aged, as well as photoaged skin. Further in vivo studies have corroborated these findings (44). Kligman et al. (79), in a six-month study, compared topical applications of tretinoin to vehicle on sun protected upper, inner thighs of elderly women. There was a histologic "rejuvenation" of aged skin with tretinoin. More recently, long-term use of topical tretinoin in subjects aged over 80 years has been shown to produce significant improvement in the clinical appearance of aged skin (80). Interestingly, naturally aged skin is characterized by an increase in the levels of RAR-γ in the epidermis, and transfecting a keratinocyte line with the RAR-γ transcript produces features of senescence, including enhanced matrix metalloproteinase production (81). Collectively, these observations imply that naturally aged skin may be "retinoid deficient" relative to younger skin.

CELLULAR MECHANISMS OF ACTION OF RETINOIDS

Vitamin A (all-*trans* retinol, tROL) and other naturally occurring retinoids (e.g., beta-carotene) are supplied by the diet, absorbed in the gut, and transported in the blood as ROL coupled to retinol-binding protein (82). ROL is stored as retinyl esters (REs), primarily in the liver. Esterification is reversible, which allows release of ROL from the liver into the circulation for uptake by peripheral tissues. In peripheral cells, ROL may be esterified for storage or converted to active metabolites.

In target cells, RA is the most biologically active form of retinoids. All-*trans*-, 9-*cis*-, and 13-*cis*-RA are naturally occurring isomers of RA. When needed, REs are converted into ROL, which is then transformed into RA in a two-step process: the first step is reversible and gives rise to RAL, whereas the second dehydrogenation into RA is irreversible. Retinoids are stabilized in the cytoplasm by interaction with carrier proteins. Cellular ROL binding proteins (CRBP-I and -II) are specific for ROL and RAL (83,84), and cellular RA binding proteins (CRABP-I and -II) interact primarily with RA (85). Unlike CRABP-I, CRBP, and CRABP-II are induced by RA (86). CRAPB-II binds tRA with a stronger affinity than 9-*cis*-RA (87).

As opposed to CRABP-I that has a storage role in the cytoplasm, CRABP-II transports RA into the nucleus by a ligand-activated nuclear localization signal (NLS) "switch" mechanism (88). Binding of RA to CRABP-II in the cytosol induces a structural rearrangement in the protein and brings together three basic amino acids in CRABP-II's tertiary structure. These three residues form a sequence that is identical to a classical NLS. Exposure of the NLS upon RA binding is responsible for the recognition of CRABP-II by nuclear importins, and the translocation of the RA-CRABP-II complex into the nucleus (Fig. 6) (88). CRABP-I does not translocate into the nucleus when bound to RA (89).

RA exerts its action in the nucleus via RARs and retinoid X receptors (RXRs), which are members of the superfamily of nuclear receptors. These are nuclear receptor ligand-activated transcription factors. Each type of retinoid receptors has three isoforms named α, β, and γ, which are encoded by distinct genes whose expression is tissue specific. Adult human skin expresses predominantly RAR-γ and RXR-α (90,91). RXRs specifically bind 9-*cis*-RA, whereas RARs bind tRA and 9-*cis*-RA (92). 13-*cis*-RA does not bind either RARs or RXRs with appreciable affinity. Upon binding of RA, RAR, which is heterodimerized with RXR, becomes activated. Activated RAR regulates transcription of numerous genes by (*i*) binding to retinoic acid response elements (RAREs), which are located in the promoter of responsive gene (transactivation) (e.g., CRABP-II), or (*ii*) modulating the activity of other promoters (e.g., AP-1) via protein–protein interactions (transrepression) (93).

RA is oxidized into inactive 4-oxo-RA before elimination. This oxidation is catalyzed by the RA-4-hydroxylases (CYP26A1 isoforms). RA-4-hydroxylase gene contains a RARE in its promoter, which provides a mechanism of autoregulation to limit RA levels within cells (94).

MECHANISMS OF ACTION OF RETINOIDS IN THE TREATMENT OF SKIN AGING

Improvement of clinical signs of skin aging (reduction of wrinkles) is attributed to the capacity of retinoids to improve dermal functions, especially increasing extracellular matrix protein production by dermal fibroblasts. Retinoids also act to

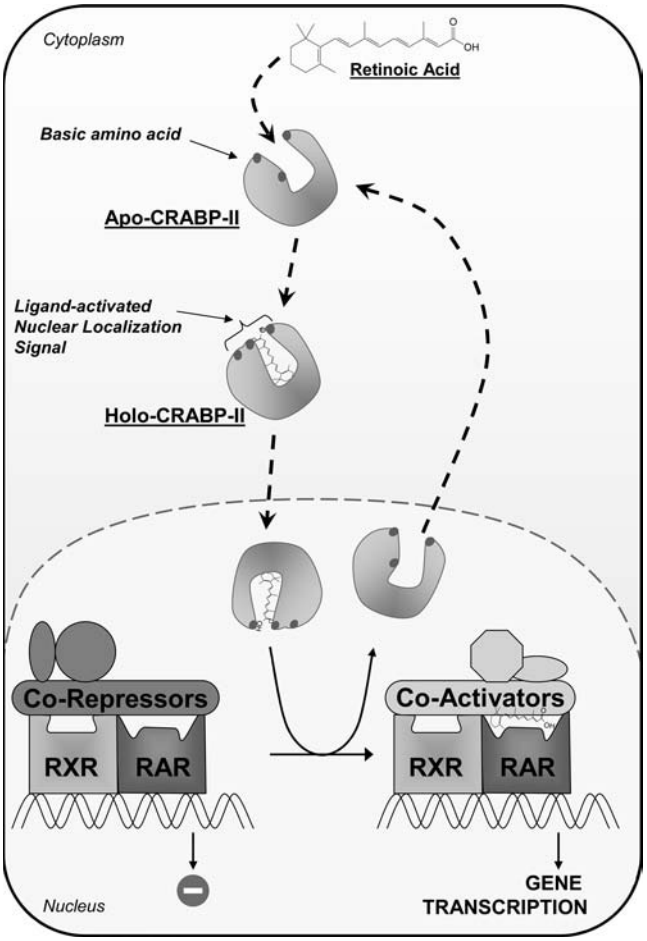

Figure 6 Cellular retinoic acid binding proteins (CRABP)-II-mediated tRA translocation into the nucleus. Apo-CRABP-II is located in the cytoplasm, where it binds tRA. tRA binding induces a conformational change that exposes a nuclear localization signal in the tertiary structure of holo-CRABP-II, allowing its translocation into the nucleus. In the nucleus, holo-CRABP-II delivers RA to retinoic acid receptors (RAR). tRA binding to RAR causes the release of corepressor complex and the recruitment of coactivators, which results in activation of target gene transcription.

stimulate growth of epidermal keratinocytes, and smooth skin pigmentation. In the early stages of retinoid therapy (i.e., within the first few weeks), effects on epidermal growth primarily contribute to initial signs of retinoid-induced improvement of skin appearance. With more prolonged retinoid therapy, stimulation of keratinocyte growth produces scaling and reddening. Scaling is the major side effect and primary deterrent to topical retinoid therapy.

Mechanism of Action of Retinoids in the Dermis

The detailed molecular mechanisms by which retinoids stimulate dermal extracellular matrix protein production remain elusive, largely because of lack of a suitable cell-based in vitro model. For instance, in vivo data demonstrate that retinoids increase synthesis of new collagen by dermal fibroblasts. However, human dermal fibroblasts in culture produce a large amount of collagen that cannot be further increased by retinoid treatment (Rittié L, Fisher GJ, unpublished observations). Whether this lack of stimulation is due to culture-associated loss of retinoid responsiveness of dermal fibroblasts (50), or lack of a necessary additional mediator(s) of retinoid action (e.g., coactivators, extracellular matrix component) remains to be determined.

Topical treatment of sun-protected aged skin with 1% ROL for seven days increases the number of fibroblasts in the skin, increases collagen production, and decreases MMP-1 and MMP-9 production in human skin in vivo (44). Interestingly, treating aged skin with 1% ROL for seven days prior to skin biopsy enhances fibroblasts outgrowth of skin explants placed in culture (44). This observation suggests that retinoids increase fibroblast activity and/or fibroblast migratory capacity in human skin in vivo. Detailed mechanisms for such stimulation remain to be elucidated.

Evidence from studies in mouse skin and nonskin cell types suggests that TGF-β may be an important mediator of retinoid action in skin. TGF-β is secreted in a biologically latent form that must be activated in order to bind to its receptors and exert its activity (95). RA increases TGF-β in mouse skin (96,97) and enhances the production of plasminogen activator, activator of latent TGF-β, in various cell types (98–100). Furthermore, RA upregulates the levels of TβRII transcripts and, to a lesser extent TβRI transcripts, in bovine endothelial cells in vitro, resulting in enhancement of TGF-β activity in these cells (101). Whether retinoids enhance TGF-β activation and/or signaling in human skin remains to be determined.

In vitro fragmentation of collagen gels with exogenous MMP-1 reduces collagen production by skin fibroblasts cultured in the gel, in a similar manner as collagen fragmentation down-regulates collagen production in naturally aged and photoaged skin (45,102,103). Interestingly, in the in vitro model, procollagen synthesis can be restored to the level of untreated gels by further treatment of the fragmented collagen gel by gelatinases, suggesting that collagen fragments exert an inhibitory influence on procollagen production. However, retinoids down-regulate gelatinase activity (MMP-9, 92-kDa gelatinase) in human skin, in vivo (44,104). While induction of other collagen fragment-degrading enzymes by retinoids remains a possibility, it has yet to be described in human skin.

Finally, retinoid pretreatment decreases UV-induced AP-1 activity in human skin in vivo (32). Recently, Ramirez et al. showed that tRA decreases AP-1 activation by decreasing c-Jun phosphorylation in an ovarian carcinoma cell line (105). This mechanism does not involve inhibition of the kinase activity of JNK, but rather induction by RA of JNK phosphatase activity (i.e., inactivation of JNK via dephosphorylation). Since increased AP-1 expression is observed in aged

and photoaged skin and AP-1 is a pivotal mediator of elevated MMPs and reduced procollagen expression, it is conceivable that tRA acts on AP-1 activity to restore the balance between production and degradation of extracellular matrix proteins in human skin. Such a mechanism remains to be demonstrated in human skin in vivo.

Mechanism of Action of Retinoids in the Epidermis

Topical retinoid therapy is often associated with reddening and scaling at the site of application. These side effects, often referred to as "retinoid dermatitis," are observed in up to 92% of treated patients (53) and are the major deterrent to topical retinoid therapy. Scaling is proportional to the dose and time of treatment, and is due to increased proliferation of keratinocytes in response to retinoids (106). The numerous proliferating keratinocytes in the lower layers of the epidermis give rise to increased number of postmitotic cells, which desquamate at the surface of the skin (Fig. 7). Animal studies have shown that stimulation of keratinocyte proliferation and consequent scaling are mediated by ligand activation of retinoid receptors in keratinocytes (50,107).

The magnitude of the side effects can be mitigated in several ways. First, decreasing the dose of retinoid applied to the skin reduces reddening and scaling. However, this approach is limited by the fact that molecular mechanisms of efficacy and side effect both involve activation of retinoid receptors. Therefore, decreasing the concentration of tRA below 0.025% to minimize epidermal growth also tends to reduce therapeutic benefit (55). Many over-the-counter retinoid-containing preparations contain concentrations of retinoid that are too low to cause either side effect or therapeutic effect.

Another approach to limit the concentration of RA in the skin is through topical application of metabolic precursors of RA (e.g., ROL, ROL-ester, or RAL). These precursors are enzymatically converted to RA within skin cells. Since this conversion is tightly regulated, the amount of RA produced from its precursors is relatively low, compared to direct topical application of RA. Indeed, ROL and, to a lesser extent, RAL, have been shown to cause less side effects than RA (108), while still providing therapeutic benefit for the clinical signs of aging. However, for reasons described earlier, neither ROL nor RAL, at effective concentrations, are completely without side effects.

Recent insights into the molecular basis of retinoid-induced epidermal hyperplasia offer the possibility of effectively limiting retinoid side effects. In human epidermis in vivo, retinoids increase secretion of heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (AR), two ligands that bind to and activate EGFR (51,109) (Fig. 7). The mechanism of this induction appears to be indirect because neither HB-EGF nor AR genes contain RARE in their promoters. Importantly, it has been shown in skin organ culture in vitro that blocking AR and HB-EGF with specific antibodies, or direct pharmacological inhibition of EGFR tyrosine kinase activity, prevents retinoid-induced epidermal hyperplasia (51,110). This observation has been extended to human skin in vivo using genistein,

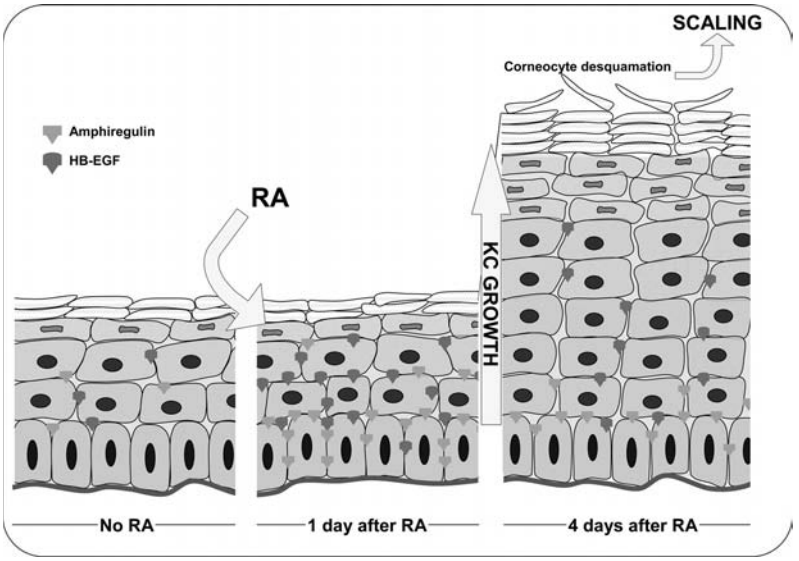

Figure 7 Retinoic acid (RA)-induced epidermal hyperplasia and scaling. In untreated epidermis (*left panel*), keratinocytes in the bottom (basal) layer are capable of cell division. Basal keratinocytes migrate to upper (suprabasal) layers, and differentiate. The last step of differentiation gives rise to corneocytes that desquamate at the surface of the skin. Topical retinoid (RA) treatment (*middle panel*) increases amphiregulin and heparin-binding EGF-like growth factor (HB-EGF) levels. Amphiregulin is induced primarily in basal keratinocytes, whereas HB-EGF is induced in both basal and suprabasal layers. These two ligands activate epidermal growth factor receptor, which leads to increased keratinocyte proliferation. Stimulation of keratinocyte proliferation by retinoids results in increased number of suprabasal cells (*right panel*). These suprabasal cells migrate to the skin surface where they desquamate, thereby causing scaling.

a naturally occurring EGFR inhibitor, that is present in high concentration in soy (19). When applied simultaneously with tRA or tROL, genistein significantly reduced retinoid-induced epidermal hyperplasia in human skin in vivo (51). These data provide proof of concept that inhibition of retinoid-induced EGFR activation has the potential to mitigate unwanted side effects of RA therapy.

CONCLUSIONS

Both sun-induced premature skin aging and natural skin aging are associated with epidermal and dermal alterations, including fragmentation and loss of dermal collagen, which impairs the skin's structure and functions. Topical vitamin A derivatives are among the few drugs with proven effectiveness in treating the clinical signs of aging. More research is needed to clearly understand the mechanisms by which retinoids act to stimulate production of extracellular matrix proteins, and thereby improve the appearance of aged skin.

The use of topical retinoids is limited by their side effects of skin reddening and scaling. However, the magnitude of these side effects can be mitigated by reducing dose and frequency of applications, by using metabolic RA precursors, or by preventing the action of retinoids in the epidermis by compounds, such as genistein, which inhibit EGFR activation. Ultimately, better understanding of retinoid mechanisms of action may lead to the design of new drugs that can specifically activate dermal repair without causing unwanted epidermal effects.

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6

Retinoid Therapy of Acne and Sebocyte-Related Disorders

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INTRODUCTION

Retinoids that include the natural and synthetic derivatives/metabolites of vitamin A as well as its functional analogs [i.e., chemicals that bind to retinoic acid receptors (RARs)], have been shown to exert multiple and profound effects on diverse biological systems (see Chapters 1 and 4). The effects of synthetic retinoids on epithelial cell proliferation, differentiation and keratinization, cellular cohesiveness, immunomodulation, dermal matrix repair and regeneration, and on the functioning of the sebaceous gland, have lent these molecules wide applicability in the systemic and topical treatment of many cutaneous disorders. In particular, retinoids have found great usefulness in the treatment of acne.

Acne vulgaris is the most common dermatologic entity, believed to affect 85% to 95% of the world's population at some point in their lives (1). Most commonly, adolescents are affected, as the disease is induced as a result of the dramatic increase in androgen production that occurs with adrenarche. However, in increasing numbers, patients—particularly women—assert that the disease appears to erupt in the third, fourth, or fifth decades of life, with the absence of an adolescent onset.

Acne vulgaris is a chronic inflammatory disease affecting the pilosebaceous unit, and has multifactorial pathogenetic mechanisms, which account for the various

clinical presentations of lesion morphology and severity. The pathogenesis is now understood to involve four main factors:

1. an increase in sebum production stimulated by androgen;
2. altered follicular epithelial differentiation leading to intrafollicular hyperproliferation in the setting of abnormal intrafollicular desquamation. This results in the formation of the precursor lesion of acne, the microcomedo;
3. proliferation of *Propionibacterium acnes*, a gram-positive, anerobic diphtheroid, within the follicle; and
4. inflammation, as a result of:
   a. the ability of *P. acnes* to induce the innate immune response through activation of toll-like receptors (TLRs), thereby causing the elaboration of interleukin (IL)-1α, IL-8, tumor necrosis factor (TNF)-α and other proinflammatory cytokines (1,2);
   b. the rupture of the microcomedo (3); or
   c. the proinflammatory properties of sebaceous lipids themselves (4); accounting for the papules and pustules seen clinically in inflammatory acne.

Topical retinoids, have become, arguably, the cornerstones of acne therapy, as they target the precursor of all other acne lesions, the microcomedo, by correcting the abnormal intrafollicular hyperkeratosis and desquamation that results in microcomedo formation. Besides their comedolytic activity, topical retinoids have been shown to have direct and indirect anti-inflammatory properties as well. Topical retinoids are most often utilized in combination with topical and/or systemic antibiotics, but have been found to be efficacious as monotherapy in mild to moderate acne, including all but the most severe inflammatory acne, as well as in maintenance therapy (5).

TOPICAL RETINOIDS IN THE TREATMENT OF ACNE

Tretinoin

The prototypical, first-generation topical retinoid molecule, tretinoin (all-*trans*-retinoic acid), was introduced over 30 years ago as the first retinoid for the topical treatment of acne (6) and was approved as such by the Food and Drug Administration (FDA) in 1971. All-*trans* retinoic acid is a lipophilic, naturally-occurring vitamin A metabolite with a molecular weight of 300.44 (7). Although all-*trans* retinoic acid is the physiologic ligand of nuclear RARs, topical application of current preparations results in supra-physiologic concentrations in the epidermis.

Mode of Action

Its primary mode of action, as with all other topical retinoids, is to normalize the altered proliferation and differentiation/keratinization of the follicular epithelium, thereby disrupting the primary lesion, the comedo, as well as preventing formation

of the precursor lesion, the microcomedo (8,9), thus halting the progression to inflammatory lesions. As explained previously, this is thought to be achieved through activation of gene transcription and subsequent protein synthesis, although it has not been established whether the clinical effects of topically-applied tretinoin are mediated by RARs, due to topical irritant effects, or both. However, it is known that tretinoin, having a side chain with several alternating single and double bonds, making it flexible and able to adapt to a number of conformations, binds with high affinity and activates all three RARs, RAR-α, -β, and -γ(highest affinity is for RAR-γ, which controls epithelial differentiation). Despite this structural flexibility, tretinoin binds negligibly to retinoid X receptors (RXRs), except at very high concentrations (10). Although tretinoin neither binds to nor activates RXRs, heterodimerization of an RAR with an RXR is required for DNA binding and gene transcription to occur in keratinocytes.

In addition to its potent comedolytic activity, tretinoin has more recently been documented to have anti-inflammatory effects. All-*trans* retinoic acid has been shown to down-regulate TLR2 expression and function in human monocytes in vitro, as well as to down-regulate monocyte cytokine induction by *P. acnes* (11). All-*trans* retinoic acid has also been shown to significantly inhibit cyclooxygenase-2 (an enzyme responsible for the metabolism of arachidonic acid into the inflammatory mediators, prostaglandins) activity and production of TNF-α (a proinflammatory cytokine produced by monocytes) in vitro (12).

Topical tretinoin has been found to decrease pigmentation, and is useful in reducing postinflammatory hyperpigmentation in black patients, a very common complication of acne vulgaris in this population (13).

As mentioned previously, topical retinoids are often utilized in conjunction with topical antibacterial agents to enhance efficacy. Combination products are marketed, containing tretinoin and erythromycin base, indicated for treatment of mild to moderate comedonal and inflammatory acne [e.g., Stievamycin® Stiefel Laboratories, Inc., Canada, (Table 1); and Antibio-Aberel®, Janssen Cilag, France]. These tretinoin/erythromycin combination products are not currently available in the United States. A combination product, containing tretinoin and clindamycin phosphate, approved in Europe for the treatment of mild to moderate acne vulgaris, is currently under clinical investigation in the United States (14).

Effects on Sebaceous Gland Activity

It is generally accepted that acne vulgaris and seborrhea go hand-in-hand (15). Despite the fact that it is the sebum impacted within the pilosebaceous follicle, and not sebum on the skin surface, which is involved in the pathogenesis of the disease, patients are often disturbed by the surface shine. Topical tretinoin does not appear to affect sebaceous gland activity (16). However, the vehicle may play a role in abating the shiny appearance of seborrhea, as tretinoin 0.1% gel in a vehicle with a microsphere delivery system (Retin-A Micro®, Table 1) has been reported to be effective at reducing facial shine, when compared to a traditional tretinoin 0.025% cream (17).

Table 1 Tretinoin Preparations for Topical Acne Treatment[a]

Brand name	Manufacturer	Availability	Comments
Avita® (U.S.A.)	Mylan Bertek Pharmaceuticals Inc.	0.025% cream (20 g, 45 g) 0.025% gel (45 g)	Cream and gel vehicles contain polyolprepolymer-2
Retin-A® (U.S.A., Canada)	Ortho-McNeil Pharmaceutical	0.025% cream (20 g, 45 g) 0.05% cream (20 g, 45 g) 0.1% cream (20 g, 45 g) 0.01% gel (15 g, 45 g)	
Retin-A Micro® (U.S.A.)	Ortho Dermatological	0.04% gel microsphere (20 g, 45 g) 0.1% gel microsphere (20 g, 45 g)	Gel vehicle has microsphere controlled-release delivery system
Retisol-A® (Canada)	Stiefel Laboratories, Inc.	0.01% cream (25 g) 0.025% cream (25 g) 0.05% cream (25 g) 0.1% cream (25 g)	Cream contains SPF 15 sunscreens: 7.5% Parsol MCX and 2% Parsol 1789
Stieva-A® (Canada)	Stiefel Laboratories, Inc.	0.01% cream (25 g) 0.025% cream (25 g) 0.05% cream (25 g) 0.01% gel (25 g) 0.025% cream (25 g) 0.05% cream (25 g) 0.025% solution (50 mL)	
Stieva-A Forte® (Canada)	Stiefel Laboratories, Inc.	0.1% cream (25 g)	
Stievamycin® (Canada)	Stiefel Laboratories, Inc.	0.01% gel (25 g) 0.025% gel (25 g)	Also contains erythromycin USP 4.0%
Stievamycin Forte® (Canada)	Steifel Laboratories, Inc.	0.05% gel (25 g)	Also contains erythromycin USP 4.0%
Tretin-X® (U.S.A.)	Triax Pharmaceuticals, LLC	0.025% cream (35 g) 0.05% cream (35 g) 0.1% cream (35 g) 0.01% gel (35 g) 0.025% gel (35 g)	

[a]Brand names and commercial availability data listed for North America only.

Adverse Effects

Irritation and the retinoid dermatitis. The most common adverse effect encountered with topical application of tretinoin is a transient irritant dermatitis, usually of mild to moderate severity, consisting of erythema, desquamation, burning, and itching. These symptoms constitute what is known as "retinoid dermatitis" or

the "retinoid skin reaction" and usually are experienced early in the course of treatment (the first few months), with the tendency to remit with continued use, possibly through the poorly-understood phenomenon of "hardening."

The severity of the irritation experienced is affected by many variables, including: the frequency and amount of each topical application; the barrier integrity of the skin to which it is applied; concomitant use of topical therapies and emollients; the concentration of tretinoin; and the vehicle of the preparation. The tendency is to believe that if a little is good, a lot is better, but this is not applicable, as it were, in the treatment of acne with topical retinoids. The "retinoid dermatitis" that results from overzealous application of tretinoin preparations limits compliance and, thus, efficacy, and the recommendation is for application of a thin layer to the entire face.

Tretinoin is optimally applied once daily, usually at night. However, those with sensitive skin may not tolerate daily applications at the initiation of therapy. In those individuals, it may be preferable to begin applications at a lower frequency (e.g., every other day or twice weekly) and increase the frequency of application every three to four weeks as tolerability increases.

As noted previously, an intact skin barrier is important to the tolerability of topically-applied tretinoin. Epidermal absorption and the resultant irritant effects are enhanced in skin with impaired barrier function. For this reason, it is prudent to avoid application to dermatitic or sunburned skin. In addition, wet skin has greater absorptive capacity than does dry skin; therefore, it is advisable to wait 20 to 30 minutes after washing the face to apply tretinoin-containing products (17).

Tretinoin is marketed for topical acne therapy in concentrations of 0.01%, 0.025%, 0.04%, 0.05%, and 0.1% and in solution, cream, and gel formulations. The lower the concentration, the lower the efficacy and the lower the risk of irritation and, in general, creams are better tolerated than gels, and solutions are the least well-tolerated. However, one gel formulation of topical tretinoin utilizing a vehicle with "microsponge" technology (round, microscopic particles of synthetic polymer called "microspheres" that deposit tretinoin in a gradual, controlled-release manner) was found to be less irritating to individuals with sensitive skin than a traditional tretinoin cream of equal concentration (17). In addition, cream and gel vehicles that incorporate polyolprepolymer-2 (Avita® 0.025% gel and cream, Table 1), a liquid polymer that retains tretinoin on the skin surface, also with slow, controlled-release, have also been found to have equivalent efficacy with a significantly lower irritant potential than traditional tretinoin gel of equal concentration (18). Judicious use of noncomedogenic emollients can also mitigate much of the irritant effect of topically-applied tretinoin.

Some patients experience an apparent flare of acne during the initial few weeks of topical retinoid therapy, thought to represent the extrusion of deeper rooted microcomedones and comedones. This exacerbation usually resolves spontaneously with persistent use and continued patient compliance should be encouraged.

Instability and photosensitivity. Tretinoin is known to be susceptible to photodegradation by sunlight; accordingly, the recommendation is for night-time application. However, the vehicle may play an important role in enhancing photostability. Tretinoin 0.1% gel microsphere was found to be significantly more photostable (i.e., degraded at a much slower rate) under conditions of simulated solar ultraviolet irradiation than was tretinoin 0.025% gel without the microsphere delivery system (19).

In addition to the sensitivity of the molecule itself to light, treatment with topical tretinoin has a reputation for heightening photosensitivity in individuals who use it. Some believe that this is due to the thinning of the stratum corneum, which results from topical retinoid therapy, while others have suggested that the apparent "photosensitivity" is really postinflammatory hyperpigmentation subsequent to the commonly-experienced "retinoid dermatitis" (20). Nevertheless, the recommendation is: to use at night, rather than during the day; to wear *SPF 15* or higher sunscreen when anticipating any sun exposure and; to avoid using other photosensitizing medications concomitantly (7).

The molecule all-*trans* retinoic acid is not only photolabile, it is also very susceptible to oxidative degradation. The molecule's side chain exhibits great structural flexibility, which allows it to conform to and bind all subtypes of RARs, but also exposes it to instability in the presence of light and oxygen. Of particular clinical relevance is benzoyl peroxide, a topical antimicrobial agent, which is often used in combination with other topical therapies in the management of acne, and a potent oxidative agent. Tretinoin is rapidly oxidized in the presence of benzoyl peroxide to inactive metabolites (21) so, in a daily topical regimen that includes both medications, it is advisable to substantially separate the application times for each.

Concerns of potential teratogenicity. Oral tretinoin that is used to treat acute promyelocytic leukemia, is a potent teratogen in animals. Consequently, there is much concern about the potential for teratogenicity of topical tretinoin in humans. The absorption of tretinoin applied to the skin has been found to be negligible in humans (22), and less than that, which occurs naturally, but there have been sporadic case-reports of congenital defects (23,24) associated with its use in early pregnancy. Causation, however, is not proven and, in our opinion, is improbable. It is very unlikely that the plasma concentration of retinoids is meaningfully affected by any contribution made from topically-applied tretinoin, but there is, at present, no formal agreement on what constitutes the safe use of topical retinoids during pregnancy. The FDA has deemed topical tretinoin Pregnancy Category C, and it is not recommended during lactation, due to equivocal animal studies using supra-pharmacologic doses of topical tretinoin (7). Although some authors believe topical tretinoin to be perfectly safe for use in pregnant women, it is probably wise to use tretinoin in pregnancy, only if absolutely essential. This issue remains controversial.

Adapalene

In the early 1990s, there were several reports of a new molecule, CD271, a synthetic naphthoic acid derivative with retinoid activity both in vitro and in vivo (25,26).

In 1993, adapalene, as this new molecule was eventually called, showed itself to be both a safe and effective topical treatment for acne in a pharmaceutical-sponsored study (27). In 1996, the FDA approved adapalene for the topical treatment of acne. Adapalene is found to be efficacious both as monotherapy and in combination with topical antimicrobials for the treatment of mild to moderate comedonal acne, as well as in maintenance therapy (28). Adapalene has also been found useful in the treatment of postinflammatory hyperpigmentation that results from acne, a particular concern in black patients (29).

Mode of Action

Since adapalene was the first topical retinoid developed after tretinoin, the comparisons are inevitable. Similar to tretinoin, the basis for the efficacy of adapalene in the topical management of acne is its comedolytic activity (30). Moreover, like tretinoin, adapalene has significant antiinflammatory properties (31), although the mechanisms of inflammatory mediation may differ for the two retinoids (25,32). Adapalene has no effect on sebaceous gland activity (33).

In contrast to tretinoin, however, the ability of adapalene to bind to nuclear RARs and activate gene transcription appears to be independent of CRABPs in the cytoplasm of keratinocytes, as adapalene is incapable of binding to CRABP. Despite this difference, adapalene seems to exert similar control as all-*trans* retinoic acid over keratinocyte differentiation and epidermal morphology (34).

The synthetic, second-generation topical retinoid also differs from the prototype in its receptor-selectivity. The adapalene molecule was designed to have more conformational rigidity than that of tretinoin. As a result, adapalene demonstrates relative selectivity for RAR-β and RAR-γ, unlike the flexible, receptor-nonselective molecule of tretinoin, which binds to all RARs. Since RAR-β is not expressed on human keratinocytes, adapalene is believed to attribute its cutaneous effects and specific side effect profile to its ability to bind to RAR-γ. Adapalene does not bind to RXRs (33) but, like with tretinoin, RXRs act as silent partners in the RAR/RXR heterodimers that is required for activation of gene transcription.

Adverse Effects

Irritation and the retinoid dermatitis. Adapalene has a slight propensity towards cutaneous irritation. Nevertheless, when evaluated alongside tretinoin with regard to tolerability, adapalene 0.1% gel compares favorably in many studies (35–37), with at least the same efficacy as tretinoin 0.025% gel (38). Much like tretinoin, the slight irritation occurs during the first few weeks of treatment and abates with continued therapy, and usually is not sufficiently severe to be an obstacle to compliance. Moreover, adapalene retains it reputation of low irritancy, even when applied to wet skin immediately after washing (39).

Adapalene is currently commercially available in a 0.1% concentration as a cream, gel, solution, and pledgets (Differin®, Galderma Laboratories, Texas, U.S.A.). However, in a recent study comparing adapalene 0.3% gel to adapalene 0.1% gel, it was concluded that the higher concentration demonstrated superior efficacy, as

well as increased potential for irritation. Hence, the irritancy potential along with the efficacy may be dose-dependent. This new, more concentrated gel formulation of adapalene is currently in the final phase of clinical investigation (40).

Instability and photosensitivity. The conformational stability of the adapalene molecule renders it stable in the presence of light and oxygen. Also, recent studies suggest that adapalene does not enhance photosensitivity (41).

Concerns of potential teratogenicity. Adapalene, being even more lipophilic than tretinoin, accumulates in the pilosebaceous follicle after topical application, with miniscule absorption into the bloodstream. Despite this, reports have surfaced of congenital defects associated with its use in early pregnancy (42). Similarly to topical tretinoin, the U.S. FDA has labeled adapalene Pregnancy Category C. The benefits of treatment during pregnancy must significantly outweigh the potential risks to the fetus.

Tazarotene

In the mid 1990s, a novel acetylenic retinoid designated AGN 190168 was developed. This molecule was ground-breaking as it was the first topical retinoid found to be valuable in the treatment of mild to moderate plaque psoriasis (43). This molecule was also found to be efficacious topically for acne and, in 1997, the molecule, named tazarotene, was approved by the US FDA as topical therapy for mild to moderate inflammatory acne.

Tazarotene has demonstrated superior efficacy over other retinoid molecules available to date for topical acne therapy (44,45), has shown promise in treating the postinflammatory hyperpigmentation that results from acne in dark-skinned patients (46), and is efficacious in maintenance therapy.

Metabolism and Mode of Action

Following topical administration, tazarotene is rapidly hydrolyzed by esterases to its active free acid metabolite, tazarotenic acid (47).

Like other retinoids, tazarotenic acid exerts its effects through binding and activating gene transcription via nuclear RARs. Similar to the other third-generation topical retinoid, adapalene, tazarotene is receptor-selective—it exhibits high affinity binding to RAR-β and -γ, with no binding to RAR-α, or the RXRs (47)—which, at least theoretically, limits undesirable side-effects.

Three novel genes are upregulated by tazarotene: tazarotene-induced gene (TIG)-1, TIG-2, and TIG-3; which are speculated to mediate its antiproliferative/comedolytic effects (48). Tazarotene also demonstrates significant antiinflammatory properties (31,49).

Adverse Effects

Irritation and the retinoid dermatitis. The adverse effect most commonly seen with the topical use of tazarotene is local skin irritation of mild to moderate

severity, with symptoms of erythema, itching, burning, and desquamation (50). With continued usage, these effects diminish (51).

Tazarotene is commercially available for the treatment of acne as 0.05% cream and gel, and 0.1% cream and gel (Tazorac®, Allergan Inc., U.S.A. and Canada; Zorac®, Allergan Inc., Europe, Africa, and the Middle East). As expected, the creams are better tolerated than the gels in each concentration, and tolerability is dose-dependent (52). Studies show the irritation experienced with topical administration of tazarotene to be subjectively and objectively greater than that from adapalene, but tazarotene is better tolerated than tretinoin preparations (50–55). The emollient effects of some concurrent topical acne medications may decrease irritation and increase compliance as well as efficacy, however (56).

Innovative application strategies are employed to increase tolerability of tazarotene and compliance with therapy while maintaining efficacy, namely: alternate day applications at the initiation of therapy; and short-contact therapy (57,58).

Instability and photosensitivity. Tazarotene and its active metabolite, tazarotenic acid, are both structurally more rigid molecules than the tretinoin molecule. This structural rigidity confers on tazarotene photostability (44,59). Because tazarotene can be photosensitizing (59), however, (it was shown to significantly decrease the minimal erythema dose of ultraviolet-B light in psoriatics, and significantly reduced the ultraviolet-A exposure required to induce immediate pigment darkening) it is prudent to advise nocturnal application and the judicious use of sunscreens on treated skin.

Tazarotene has proven to be a chemically stable topical retinoid, unlike tretinoin, and appears to mix well with other topical medications without being degraded or affecting the stability of other molecules (60).

Concerns of potential teratogenicity. After topical administration, tazarotene is minimally absorbed into the bloodstream (47). However, the systemic levels of its active metabolite, tazarotenic acid are measurable but small, below 1 microgram/liter after topical application of tazarotene 0.1% gel to the entire face of acne patients and individuals with normal skin (61–63). Tazarotenic acid is further metabolized to its sulfoxide and other polar metabolites with no significant accumulation in adipose tissue as it and its metabolites are hydrophilic (64). Tazarotenic acid has a relatively short terminal half-life and is eliminated by the kidneys and gastrointestinal system in about 18 hours (61). In addition, no drug-related metabolic, hematologic, or ophthalmologic consequences have been seen with topical administration of tazarotene (65). In animal studies, topical doses were not found to be teratogenic, mutagenic nor carcinogenic, nor did they affect fertility (47).

Oral tazarotene that has been evaluated in clinical trials as a treatment for psoriasis, also undergoes rapid and extensive hydrolysis by esterases to tazarotenic acid, which is by seven days post oral administration completely excreted through the urine and feces, along with its inactive, polar metabolites, with no accumulation in adipose tissues (66).

However, the FDA has labeled topical tazarotene Pregnancy Category X. It is contraindicated in pregnancy.

Topical Isotretinoin

The molecule 13-*cis* retinoic acid, or isotretinoin, will be discussed at length in a later section, as it is not approved by the FDA for the topical treatment of acne. It is approved in the United States for the oral treatment of severe acne and in Europe as a topical treatment for mild to moderate acne (e.g., Isotrex® 0.05% isotretinoin gel, Stiefel Laboratories, Inc., Canada; Isotrexin® 0.05% isotretinoin/2.0% erythromycin gel, Stiefel Laboratories, Inc., Canada).

Topical isotretinoin appears to be similar to topical tretinoin with regard to comedolytic activity, efficacy against inflammatory lesions, lack of influence on sebaceous gland activity (67–69) and potential to induce photosensitivity (70), but differs from tretinoin in being somewhat more photostable (71) and is less irritating (72).

Topically-administered isotretinoin is minimally absorbed (73) and animal studies show that 60 times the human therapeutic dose topically applied resulted in no fetal harm (70).

Topical Retinaldehyde

Retinaldehyde, or retinal, is a precursor of retinoic acid, and thus acts physiologically as a retinoid. It has been demonstrated to be comedolytic in the rhino mouse model (74). Topical retinaldehyde is marketed as a cosmeceutical in both the United States and Europe for use in "blemish-prone" skin (e.g., Diacnéal® 0.1% retinaldehyde/6% glycolic acid cream, Eau Thermale Avène, France) and is found to decrease postinflammatory hyperpigmentation as well, with greater tolerability than tretinoin preparations (75–77). Retinaldehyde is also found to differ from other topical retinoids in demonstrating antibacterial activity against various gram-positive bacteria, including *P. acnes* (78).

Future of Topical Retinoids?

The search continues for the development of the perfect, receptor-selective retinoid molecule with maximum topical therapeutic potential, stability and tolerability, and minimal side effects. In November 2005, a report of a new retinoid, designated ER36009, showed that in the rhino mouse model this new RAR-γ-selective molecule had 96 times the activity of tretinoin in the utricle reduction assay, and shows promise as a potent, new comedolytic agent (79).

ORAL RETINOIDS IN THE TREATMENT OF ACNE

Isotretinoin

Oral isotretinoin, first synthesized in 1955, and approved by the FDA in 1982 for severe, recalcitrant, nodulocystic acne, has revolutionized acne therapy. Isotretinoin,

or 13-*cis* retinoic acid, was the first treatment to induce prolonged remissions in severe, nodular (cystic) acne refractory to other treatment, and acne conglobata (80), and was the only oral therapy indicated solely for acne. It remains, over 20 years later, the treatment of choice for these conditions. Its use is usually reserved for patients who have failed to improve with conventional therapy, which should include topical retinoids and systemic antibiotics, or who have significant physical or emotional scarring, because of the potential serious toxic effects of systemic isotretinoin.

Mode of Action

Oral isotretinoin is the only acne therapy available that targets all four factors implicated in the pathogenesis of acne. Unlike its isomer tretinoin, isotretinoin barely binds to CRABPs or RARs (81).

Comedolytic/keratinolytic effects. As with topical retinoids, 13-*cis* retinoic acid administered orally is potently comedolytic and keratinolytic (87). Ultrastructurally, disintegration of desmosomes accounts for the lack of cohesion between intrafollicular corneocytes (82).

Anti-inflammatory effects. Isotretinoin has powerful antiinflammatory and immunomodulatory effects (83). The molecule significantly inhibits monocyte and neutrophil chemotactic responses in vivo (84,85).

Effects on sebaceous glands. In contrast to available topical retinoids, including topical isotretinoin, systemic isotretinoin is a strong sebostatic agent (86). The size of the sebaceous lobules, as well as the amount of sebum secreted is dramatically decreased (82,87,88) and skin surface lipid composition is thus altered (89). Isotretinoin decreases proliferation of basal sebocytes, possibly through cell cycle arrest and induction of apoptosis via RAR-independent mechanisms (90). 13-*cis* retinoic acid also inhibits sebocyte differentiation (81). The effect on sebum production is achieved through reduction in skin androgen receptor levels (91)—there are minimal effects on circulating hormone levels (92), although it was recently postulated that isotretinoin inhibits the conversion of 3-alpha-androstanediol and androsterone into dihydrotestosterone and androstenedione by the enzyme retinol dehydrogenase-4 (93). The sebosuppressive effect is slowly reversed on discontinuation of therapy.

Isotretinoin is the only systemic retinoid found to have these profound effects on sebum production, and this sebostatic effect is felt to account for its efficacy in severe acne, as the other oral retinoids are ineffective in treating acne (94).

Effects on *Propionibacterium acnes* proliferation. *P. acnes* is among the resident flora of adult human skin and is found in high densities in skin sites rich in active sebaceous glands (95) because it uses sebum triglycerides to satisfy some of its nutritional requirements. Through the activity of bacterial lipase, triglycerides in sebum are broken down into glycerol and free fatty acids. The decreased sebum production that results from systemic isotretinoin administration causes

reduced substrate (triglycerides) for *P. acnes*, and the populations within the follicle decrease (82,96).

Side Effects

Mucocutaneous toxicity. The most common side effect of systemic isotretinoin, cheilitis, is so common that it is often used to monitor compliance. Desquamation of the skin, xerosis, xerostomia, conjunctivitis, eye irritation, contact lens intolerance, dry nose, epistaxis, photosensitivity, and pruritus are also commonly experienced (97,98) and usually do not limit compliance.

Isotretinoin is usually given in doses of 0.5–2.0 mg/kg/day, with 1 mg/kg/day considered optimum dosing in terms of balancing benefits with risks. Treatment is usually continued for 16 to 20 weeks with a cumulative dosage goal of 120–150 mg/kg, which has been found to reduce the potential for relapse and prolong the remission period after discontinuation of the drug. The aforementioned mucocutaneous side effects tend to be dose-dependent. Doses as low as 0.1 mg/kg/day have been shown to be effective, but relapse rates are high at this dose and the period for remission is shorter than at higher doses (96)—(this study of low-dose isotretinoin did not continue long enough, however, to achieve the maximal cumulative dose of 120 mg/kg).

Isotretinoin therapy also, less commonly, can result in paronychia, excessive granulation tissue, particularly periungually (99,100), eruptive pyogenic granulomas (101) and, rarely, keloid formation following minor trauma, laser surgery, or dermabrasion (102).

Teratogenicity. After ingestion, approximately 25% of isotretinoin is absorbed into the bloodstream, where it is highly bound to plasma proteins, and measurable serum levels are attained after 30 minutes. Bioavailability can be increased 1.5 to 2 times by ingestion with food. Maximal concentrations are reached two to four hours after ingestion. About 10% to 30% of isotretinoin is metabolized as its isomer, tretinoin. Isotretinoin undergoes hepatic metabolism and its major metabolites are 4-oxo-isotretinoin and 4-hydroxy-isotretinoin. The parent drug and its metabolites are excreted in the feces and urine. Isotretinoin has an elimination half-life of less than one day, although 4-oxo-isotretinoin, a teratogenic metabolite, has a half-life of several days (103). There is no progressive accumulation in the serum, skin or adipose tissue, and after four weeks, there is no evidence of isotretinoin, its isomer, tretinoin, or its metabolites in the serum or skin (104).

Isotretinoin crosses the placenta and is a potent teratogen in humans (105,106). Adverse events relating to use during pregnancy include spontaneous abortion and birth defects, such as microcephaly, hydrocephalus, anomalies of the great vessels, microtia/anotia (absence of the external ears), cleft palate, micrognathia, thymic aplasia, microphthalmos, orbital hypertelorism, and retinal/optic nerve abnormalities (107,108). Isotretinoin is among the most potent human teratogens on the market; accordingly, it is classified Pregnancy Category X by the FDA. Two forms of contraception should be used in women of childbearing

age from one month before initiation of treatment to three months postdiscontinuation and monthly pregnancy testing is required in this population.

On March 1, 2006, the FDA restricted prescription access to isotretinoin by approving the implementation of a risk management program known as "iPledge," which seeks to prevent fetal exposure to isotretinoin. Only physicians who are registered with the iPledge program can prescribe isotretinoin, and only patients who comply with all the program's requirements, (including being registered with the iPledge program, signing an informed consent form, obtaining counseling on the risks of use and the various requirements for safe use, and compliance with necessary pregnancy testing in women of child-bearing age) can get the medication (109). Manufacturers, wholesalers, and pharmacists must also be registered with the program and pregnancy testing must be done with a certified lab.

Effects on plasma lipids and liver function. Isotretinoin can induce hypertriglyceridemia, increases in low-density lipoprotein (LDL) cholesterol and decreases in high-density lipoprotein (HDL) cholesterol. The short-term of treatment, however, is not considered to pose a risk for cardiovascular disease. These lipid disturbances return to normal within two months after therapy is discontinued (110,111) and treatment of excessive hyperlipidemia usually allows for continuation of isotretinoin therapy.

Isotretinoin is also known to cause a reversible chemical hepatitis, with dose-dependent increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (111).

Baseline fasting serum lipids and liver function tests, then two weeks after initiation of therapy are recommended. If triglycerides are found to be elevated, monthly fasting serum lipids should be checked until serum triglycerides stabilize; if the hepatic enzymes are found to be elevated, monthly checks are recommended and should be repeated at the end of therapy. Systemic isotretinoin is relatively contraindicated in those with significant hepatic dysfunction or uncontrolled hyperlipidemia.

Other gastrointestinal side effects. Acute pancreatitis can occur secondary to an isotretinoin-induced severe hyperlipidemia (112), which requires discontinuation of the isotretinoin. In addition, there are reports of inflammatory bowel disease associated with isotretinoin treatment (113). The onset of inflammatory bowel disease may occur months after discontinuation of systemic isotretinoin therapy, and a causal relationship has not been firmly established. Nevertheless, caution is advised in patients with known inflammatory bowel disease. A. R. Shalita has treated several patients with stabilized inflammatory bowel disease without difficulty and without recurrence of the bowel disease.

Effects on musculoskeletal system. Isotretinoin has several effects on the skeletal system, some resulting from its effects on vitamin D metabolism (114). In contrast to the other side effects of systemic retinoids (with the exception of teratogenicity), the skeletal side effects are generally not reversible.

Isotretinoin can induce the formation of often asymptomatic skeletal hyperostoses, including changes mimicking the diffuse idiopathic hyperostosis (DISH) syndrome (115). This effect appears to be dose-related, with a higher incidence at higher doses (116) and is a result of chronic, systemic administration of isotretinoin (115), particularly at high doses.

High doses and chronic use of oral retinoids, including isotretinoin, are also known to cause premature epiphyseal closure in children, resulting in short stature (115).

Mild, transient arthralgias and myalgias may occur with isotretinoin therapy, but these usually do not require discontinuation of therapy (117,118). There are also sporadic reports of arthritis responding to dose reduction (119) and Achilles tendonitis (120).

Effects on vision and central nervous system. In addition to the xerophthalmia commonly experienced, and meibomian gland atrophy and corneal opacities reported with isotretinoin use, photophobia and decreased dark adaptation/night blindness can also occur (121,122). The loss of dark adaptation may be permanent (122).

Isotretinoin administration is known, rarely, to precipitate pseudotumor cerebri, resulting in severe headaches (123). This side effect is reversible with discontinuation of isotretinoin in conjunction with the administration of a systemic corticosteroid.

Although a causal relationship has not been established, reports link suicidal depression to isotretinoin use (124,125) even in individuals with no prior history of depression or suicide attempts (126). In general, nonetheless, that successful treatment of severe acne with systemic isotretinoin can have an immensely positive impact on psychological outlook. There is no currently known pharmacologic mechanism to account for psychiatric symptomatology associated with isotretinoin and this issue remains controversial. Careful monitoring during therapy can identify those patients with this relatively rare but serious side effect, and timely psychiatric referral and dose-reduction or discontinuation of isotretinoin can prevent adverse sequelae.

SUMMARY

Despite toxicities that limit their use, topical and systemic retinoids are indispensable in the treatment of acne, from the very mild, comedonal form to the severe, recalcitrant nodular variant. Timely intervention in acne, regardless of clinical severity, may prevent irreversible sequelae, such as scarring, and has profound effects on the psyche. Knowledge of potential side effects and close surveillance, particularly with systemic retinoids, is essential for appropriate and successful management of acne with these agents and can result in significant improvement in quality of life. With the increased understanding of the mechanisms through, which these molecules exert their effects, and how structure relates to function and stability, come the possibility of developing newer, better retinoids with limited side effects and optimized clinical effects.

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7

Retinoids and Retinoic Acid Metabolism Blocking Agents in Psoriasis

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INTRODUCTION

It is well known that psoriasis is a retinoid-sensitive disease. For decades, topical as well as oral retinoids have been widely used as antipsoriatic treatments. In general, topical treatment remains the most frequently used therapy for patients with mild to moderate psoriasis, whereas systemic retinoids contribute to the therapeutic management of patients with more severe and recalcitrant types of psoriasis.

The aim of this chapter is to give a brief overview of the history of retinoids in psoriasis and to focus on the clinically relevant data of topical tazarotene and oral acitretin, two retinoids that are largely contributing to the therapeutic management of patients with psoriasis. Oral tazarotene will be discussed briefly. Finally, the characteristics and future perspectives of the rexinoids (bexarotene) and the retinoic acid metabolism blocking agents (RAMBAs) in psoriasis will be highlighted.

HISTORY AND DISEASE CHARACTERISTICS

Psoriasis is a chronic, multi-factorial and complex skin disorder affecting 1% to 3% of the world's population. It is characterized by well-defined red patches covered by silvery, flaky scales ranging from a few spots on the extensor surfaces and the scalp to large areas all over the body. The disease can evolve with periodic flare-ups. There are several variations of psoriasis but the most common type is chronic plaque psoriasis. The cause of psoriasis is unknown and although psoriasis manifests itself as a skin disorder, it is not excluded that the disease is caused by impaired or defective cell mediated immunity (T-cell driven). It has been known for quite a long time that psoriasis runs in some families. Analysis of concordance rates amongst monozygotic and dizygotic twins is another source of evidence that genetic factors are important for psoriasis. Psoriasis is associated with HLA-B13, HLA-B17, HLA-B37, and HLA-Bw16. Further studies revealed that these latter associations were secondary to an association with HLA-Cw6.

Based upon analyses of family pedigrees, a polygenic inheritance provides the best model for the complex genetics of psoriasis. Genome-wide linkage scans have demonstrated several genetic loci for psoriasis; these studies have recently been reviewed by Rahman and Elder (1).

Table 1 summarizes gene loci linked to psoriasis. *PSORS1* has been mapped to chromosome 6p21.3 by independent groups and is considered to be a major gene involved in psoriasis. It was found that about 50% of psoriatic patients had involvement of *PSORS1*. In fact, the critical region appears to be a 300 kb interval around the centromere end of class I major histocompatibility complex (MHC). Although HLA-Cw6 is clearly associated with psoriasis, it is unlikely that HLA-C itself is the susceptibility gene.

Histological examination of the psoriatic lesions reveals the following abnormalities: epidermal hyperproliferation, premature keratinization, and immune cell infiltration (2). Up to now no preventive therapy exists for psoriasis, symptomatic therapy is the only therapy available.

The history of the development of retinoids in dermatology goes back decades and comprises several generations, which have been extensively described

Table 1 Gene Loci in Psoriasis

Gene locus	Position on chromosome
PSORS1	6p21.3
PSORS2	17q25
PSORS3	4q32-35
PSORS4	1q21
PSORS5	3q21
PSORS6	19p13-q13
PSORS7	1p35-34
PSORS8	16q12-13
PSORS9	4q31-34
PSORASI	16q12

in the first chapter of this book (3,4). Briefly, already in the beginning of the last century, it was known that vitamin A (retinol) plays a major role in the proliferation and differentiation of epithelial structures. Vitamin A deficiency in humans results in dry skin with follicular hyperkeratosis. At that time, an essential role of vitamin A was also attributed in diseases like Darier's disease and ichthyosis.

Vitamin A was investigated at nonphysiological doses in acne and psoriasis. Megadoses of vitamin A (2–4 million IU) resulted only in a slight improvement of psoriasis, but the symptoms of vitamin A intoxication, such as intracranial hypertension with nervous disturbances, dryness of the mucous membranes and skin, peeling of the skin were unacceptable (5). Hundreds of derivatives of vitamin A were investigated and tretinoin [all-*trans*-retinoic acid (all-*trans*-RA)] as well as isotretinoin (13-*cis*-retinoic acid) proved to be therapeutic substances in the topical treatment of acne (6). However, tretinoin and isotretinoin had no anti-psoriatic potential following topical application. We had to wait until 1997 before tazarotene, the first topical retinoid to be active in psoriasis, became available.

Because the efficacy of orally administrated vitamin A and all-*trans*-RA was insufficient, the search for synthetic analogs with a better efficacy and safety profile resulted in the synthesis of the mono-aromatic retinoids like etretinate and later of its free active carboxylic acid metabolite acitretin. Etretinate and acitretin are highly and equally effective systemic treatments for psoriasis and adverse events appear to be similar in severity and incidence. Today acitretin is the preferred systemic retinoid for the treatment of psoriasis because of its better pharmacokinetic profile. A major problem with systemic retinoids is their teratogenicity and a separation of this phenomenon from their therapeutic effects has still not been achieved. The search for new ligands offering at least the same efficacy and a better risk/benefit ratio than the existing retinoids has resulted in the development of specific retinoid X receptors (RXR) ligands or rexinoids like bexarotene, which are effective but still not devoid of serious side effects.

Another new approach is to increase the endogenous retinoic acid levels in the skin by blocking the catabolism of intracellular all-*trans*-RA with a RAMBA. RAMBAs such as liarozole and its successor Rambazole™ (R115866) have been proven efficacious in the treatment of keratinization disorders like psoriasis, ichthyosis, and acne. Currently, Rambazole is intensively investigated for the treatment of psoriasis (Phase 2b).

TOPICAL RETINOIDS: TAZAROTENE

Tazarotene is the first topical retinoid being approved for the treatment of psoriasis, despite the fact that several topical retinoids have been available for decades for retinoid sensitive diseases.

Mechanism of Action

Tazarotene is a synthetic acetylenic retinoid. It is in fact, an ethyl ester prodrug that is rapidly hydrolyzed to tazarotenic acid, its active and more water-soluble

form. Having a more selective affinity for the retinoic acid receptors (RARs) than tretinoin, tazarotenic acid shows transactivation through RAR-γ and RAR-β and less through RAR-α (7,8). It does not bind RXR. The mechanism of action of tazarotene in psoriasis is not defined, but the drug has been shown to have both antiproliferative and anti-inflammatory activities (9–11).

Pharmacokinetics

Percutaneous penetration of topical tazarotene is limited (12). The systemic absorption after a single application in psoriatic patients is less than 1% of the applied dose, and 2.6% and 5.3% after once daily applications during two weeks of tazarotene 0.05% or 0.1% gel, respectively. After 12 weeks treatment with 0.05% and 0.1% tazarotene the systemic absorption was 1.8% and 3.9%, respectively (13). In phase III trials with either the gel or the cream, low tazarotene plasma concentrations (≤0.15 ng/mL) were observed in 1% to 3% of the psoriatics after 12 weeks of treatment (12–15). Low but detectable (>0.05 ng/mL) plasma levels of tazarotenic acid were observed in 47% to 69% of these patients.

The mean half-life (t½) of topical tazarotenic acid is similar in normal and psoriatic patients and is approximately 18 hours. Tazarotenic acid has a high affinity for plasma proteins (>99%) and has a relatively small volume of distribution (1.97 L/kg). Tazarotene is mainly eliminated via urinary and fecal routes as sulfoxides and sulfones. CYP2C8 and flavine-containing mono-oxygenases mediate oxidation of tazarotenic acid to its sulfoxide, which is the primary metabolite in urine (13).

Indications and Contraindications

Topical tazarotene has been approved for the treatment of chronic plaque psoriasis in several countries and is available as a 0.05% and 0.1% cream or gel formulation (16–19). In the United States, tazarotene cream is indicated for plaque psoriasis and the gel for stable plaque psoriasis with up to 20% body surface involvement. In Europe, tazarotene gel is indicated for mild-to-moderate plaque psoriasis involving up to 10% body surface area.

Tazarotene is applied once daily in the evening and it is recommended to start with the 0.05% formulations and to increase the concentration if necessary and tolerated.

In view of its teratogenic potential, tazarotene is contraindicated in pregnant women and in women who are not taking adequate birth control. Tazarotene is also contraindicated in patients being exposed to substantial sunlight and are not using adequate sun protection and in patients using photosensitizers or having photodermatitis.

Efficacy

Tazarotene as Monotherapy

Tazarotene gel (0.05% and 0.1%) applied once daily for 12 weeks has been shown to be efficacious as monotherapy for mild to moderate plaque psoriasis in two large

vehicle-controlled clinical trials (14). With 0.1% tazarotene, already at week one, a statistically significant improvement compared to the vehicle could be observed whereas for the 0.05% formulation four weeks of treatment were needed.

Also the cream formulation proved beneficial in the treatment of psoriasis in two vehicle-controlled studies (15). Both the 0.05% and 0.1% cream formulations applied once daily for 12 weeks followed by a 12 weeks treatment, free follow-up were significantly more effective than the vehicle cream. The 0.1% tazarotene cream was generally more effective than the 0.05% cream, although slightly less well tolerated. During the 12 weeks post-treatment phase, both concentrations of tazarotene cream were significantly better than vehicle in maintaining therapeutic effect.

In one study 0.1% and 0.05% tazarotene gel formulations applied once daily for 12 weeks followed by a 12 week treatment free follow-up were compared to a potent corticosteroid, fluocinonide 0.05% cream twice daily (20). At the end of the 12 weeks treatment course, both tazarotene gel formulations had a similar efficacy as the potent corticosteroid fluocinonide 0.05% cream, but the corticosteroid showed an earlier onset of efficacy. Compared to tazarotene 0.05%, fluocinonide cream was significantly better from week two to eight and compared to tazarotene 0.1% at week four. Fluocinonide 0.05% cream should be the preferred treatment when a fast clearing is mandatory. On the other hand, tazarotene treatment has a slower relapse as compared to fluocinonide.

Comparative studies between tazarotene 0.1% gel once daily and calcipotriol 0.005% ointment twice daily revealed a superior efficacy of calcipotriol during the first eight weeks, but an equal efficacy after 12 weeks of treatment (21). Local irritation was noted only in the tazarotene treated lesions.

Combination Therapy with Corticosteroids

Whilst tazarotene is effective on its own, it is frequently used in the clinic in combination with corticosteroids or phototherapy in order to avoid retinoid dermatitis, which develops in a significant proportion of patients treated with tazarotene as a monotherapy.

The combination of tazarotene with various topical corticosteroids has been compared to tazarotene monotherapy (22–24), corticosteroid monotherapy (25) and calcipotriol (26). In all trials, the topical corticosteroid and tazarotene 0.1% gel were applied once daily, except in one study (24), where they were applied on alternate days.

Table 2 summarizes the response rates obtained in two large scale studies comparing a 12 week treatment of plaque type psoriasis with tazarotene 0.1% gel monotherapy versus the combination with a corticosteroid (22,23). The combinations of 0.1% tazarotene gel with mid- to high-potency corticosteroids show a higher efficacy than tazarotene monotherapy after 12 weeks treatment (23). The results from the low potency corticosteroid group did not statistically differ from the vehicle group.

It has also been shown that the median time to initial treatment success was substantially improved by combining tazarotene 0.1% gel with a corticosteroid

Table 2 Response Rates to Tazarotene 0.1% Gel in Combination with Topical Corticosteroids (Responses Evaluated After 12 Weeks of Treatment)

Reference	Treatment	Potency of corticosteroid	No. of patients reaching at least 50% overall improvement from baseline (%)
21	Tazarotene	N.A.	35
21	Tazarotene + fluocinonide 0.05% ointment	High	48
21	Tazarotene + mometasone furoate 0.1% ointment	High	66
21	Tazarotene + diflorasone diacetate 0.05% ointment	High	55
21	Tazarotene + diflorasone diacetate 0.05% cream	Mid-high	44
21	Tazarotene + betamethasone dipropionate 0.05% cream	Mid-high	78
21	Tazarotene + fluticasone propionate 0.005% ointment	Mid-high	57
22	Tazarotene + placebo cream	N.A.	80
22	Tazarotene + fluocinolone acetonide 0.01% cream	Low	79
22	Tazarotene + mometasone furoate 0.1% cream	Mid	91
22	Tazarotene + fluocinonide 0.05% cream	High	95

Abbreviation: N.A., not applicable.

(23). It took at least four weeks for tazarotene monotherapy + placebo to reach 50% improvement of psohasis area and severcity index (PASI) versus three weeks for the combination of tazarotene with fluocinolone acetonide 0.01% cream (high-potency) and only two weeks for the combination with mometasone furoate 0.1% cream (mid-potency). The best performing steroid was betamethasone dipropionate 0.05% cream, but the best strategy based on efficacy and tolerability in both studies was the concomitant use of tazarotene 0.1% gel with mometasone furoate

0.1% cream. Mometasone furoate 0.1% cream causes less local and systemic side effects than betamethasone dipropionate 0.05% cream.

The efficacy of the combination of tazarotene 0.1% gel once daily + calcipotriol 0.005% ointment twice daily compared to a clobetasol ointment treatment twice daily, in a two-week open label, left-right comparison study was not statistically significant different (27). There seems to be no chemical incompatibility between calcipotriol ointment and tazarotene gel.

Combination with Phototherapy

Combination therapy of tazarotene and phototherapy with ultraviolet B (UVB) proved to be rather effective, although we have to be aware of the enhancement of phototoxicity of tazarotene by simultaneous treatment.

Several studies have indicated that tazarotene in combination with phototherapy is well tolerated and superior to monotherapy in terms of antipsoriatic efficacy as well as in onset of action (28–31). In a clinical broad band UVB study, the combination of tazarotene gel 0.1% with UVB clearly shortened the time to reach 50% improvement (25 days vs. 53) and lowered the median cumulative UVB dose needed from 1394.5–390 mJ/cm^2_compared to UVB monotherapy (31).

In another study, topical tazarotene plus narrow-band UVB (311 nm) resulted in a 64% reduction of PASI assessed after four weeks as compared to a 48% reduction by UVB monotherapy (30).

One study comparing the effect of narrow-band UVB irradiation in combination with topical tazarotene versus the combination with calcipotriol and a second study comparing psoralen plus ultraviolet A (PUVA) in combination with tazarotene or tacalcitol ointment both failed to reveal significant differences between the respective regimens (32,33).

Side Effects

Local irritation is the most common side effect of topical retinoids. The most common side effects associated with topical tazarotene as a monotherapy are desquamation, burning/stinging, dry skin, erythema, and pruritus (14,15,20). The incidence of side effects was higher in patients using the 0.1% formulations as compared to the 0.05% formulations. When compared to calcipotriol monotherapy, the frequency of the previously mentioned adverse events was higher using the tazarotene gel, whilst the efficacy is the same. No significant phototoxicity was associated with the combination of topical tazarotene and UVB (30,31).

Treatment Strategies

In general, topical treatment remains to be the most frequently used approach in treating patients with mild to moderate psoriasis. Topical tazarotene and topical vitamin D3 analogs have become a first line treatment the last decade. Dithranol and tar treatment are prescribed for patients not responding to other topicals and

UVB treatment and for those patients who are not or not yet eligible for a systemic treatment. High-potency topical corticosteroids as a monotherapy are used in those patients having strong irritation to vitamin D3 analogs or tazarotene. Combining topical tazarotene with a topical corticosteroid reduces both the irritancy to tazarotene monotherapy as well as the well-known side effects of corticosteroids, such as tachyphylaxis, striae, skin atrophy, and adrenal gland suppression. The combination also increases the rate of improvement and the ultimate treatment efficacy as compared to tazarotene monotherapy. As compared to corticosteroid monotherapy, the combination lengthens the remission.

Reduction of the application time to a few minutes by washing-off a topical treatment has been shown to enhance efficacy and reduce irritancy. Short contact application has become a popular approach in dithranol treatment. It also has been shown for topical tazarotene that reduction of the application to a few minutes reduces irritancy substantially without compromising efficacy too much (34).

SYSTEMIC RETINOIDS: ACITRETIN

Today, acitretin, a second-generation systemic retinoid, is the preferred systemic retinoid in the treatment of psoriasis for more than 10 years (35,36). Acitretin can be used as a monotherapy, but is frequently used in combination with other antipsoriatic drugs.

Mechanism of Action

Acitretin, which is the pharmacologically active metabolite of etretinate, has been shown to activate all three RARs, without evidence that it actually binds to these receptors (37). Acitretin has been shown to reduce scaling and to interfere with various aspects of cutaneous inflammation, without evidence for immune suppression.

Pharmacokinetics

The absorption of acitretin is very variable amongst individuals and is optimal when ingested with food. The mean elimination half-life of acitretin is about two days (38,39), whereas the mean elimination half-life of etretinate, the ethyl ester of acitretin, is 120 days, but can still be detected in the subcutis as long as 18 months after treatment (40). Acitretin was developed because it was thought to be cleared from the body more rapidly than etretinate. However, re-esterification of acitretin to etretinate in the human body has found to be possible, especially if the patient has a substantial alcohol intake (41,42). Therefore, in view of the teratogenic effect, women of childbearing potential who have been treated with acitretin have to continue contraception for two years in Europe and for three years in the United States after stopping the therapy.

Drug interactions with acitretin may be relevant, in particular with drugs interfering with cytochrome P450 metabolism, such as cyclosporine and with drugs competing for plasma protein binding such as phenytoin.

Table 3 Indications and Contraindications for Acitretin in the Treatment of Psoriasis

Indications
Severe psoriasis that cannot be managed by topical treatments or phototherapy, including day-care and in-patient dithranol treatment
Erythrodermic or pustular psoriasis
Contraindications
Moderate to severe liver dysfunction
Severe kidney dysfunction
Pregnancy and lactation
Women of childbearing potential who cannot guarantee adequate contraception during and up to 3 yr following discontinuation of acitretin
Hyperlipidemia, especially hypertriglyceridemia, which cannot be controlled
Concomitant medications which interfere with retinoids
Concomitant hepatotoxic drugs
Diabetes mellitus which cannot be controlled
Alcohol abuse

Indications and Contraindications

Table 3 summarizes the indications and contraindications of the use of acitretin in psoriasis. The recommended initial and ongoing evaluations are outlined in Tables 4 and 5, respectively.

Efficacy

Acitretin as Monotherapy

For palmo-plantar and generalized pustular psoriasis (von Zumbusch) and for erythrodermic psoriasis retinoids can be considered to be first-line therapies. Lesions respond more rapidly in monotherapy than with most other therapies. Initial dosages of 1 mg/kg/day for pustular psoriasis, 0.5 mg/kg/day for chronic plaque psoriasis, and 0.25 mg/kg/day for erythrodermic psoriasis are recommended. Since correct dosing is the determinant for optimal efficacy of acitretin,

Table 4 Preacitretin Screening

History to exclude contraindications
Liver function tests (AST, ALT, γGT, alkaline phosphatase, and bilirubin)
Serum triglycerides and cholesterol
Glucose
Serum creatinine
Pregnancy test
Spinal X-ray (initially performed during the first 3 mo of therapy if long-term treatment is anticipated)

Abbreviations: ALT, alanindamino transferase; AST, aspertate amino transferase; γGT, γ glulamyl transferase.

Table 5 Evaluation During Acitretin Treatment

Monitor mucocutaneous side effects
Serum cholesterol/triglycerides and liver enzymes (every 2 wk for the first 6 wk, then every 6–12 wk)
Serum creatinine (elderly patients or patients with mild to moderate renal dysfunction)
Monitor for development of hyperostoses by history and by X-ray of spine (once every 2 yr)
Pregnancy test (before treatment commences)

the dosage sometimes needs to be adjusted. In general, mild cheilitis is indicative for a sufficient bioavailability of acitretin.

In generalized pustular psoriasis, acitretin has been shown to be effective in 84% of the patients (Fig. 1) (43). The lack of immune suppression of acitretin makes this therapy also suitable for psoriasis associated with human immunodeficiency virus infection (44).

The plaque-type psoriasis responds variably and the use of acitretin as a monotherapy should be limited to severe forms not manageable by topical treatments or phototherapy, including day care and in-patient dithranol treatment. The clinical response is dose-dependent; different dosages of 10–75 mg/day have been evaluated (45–52). Starting with high doses of acitretin (0.5–1 mg/kg/day) may result in an initial worsening and the sudden appearance of side effects on the skin (cheilitis, dry skin, and pruritus) jeopardizing the compliance of the patient.

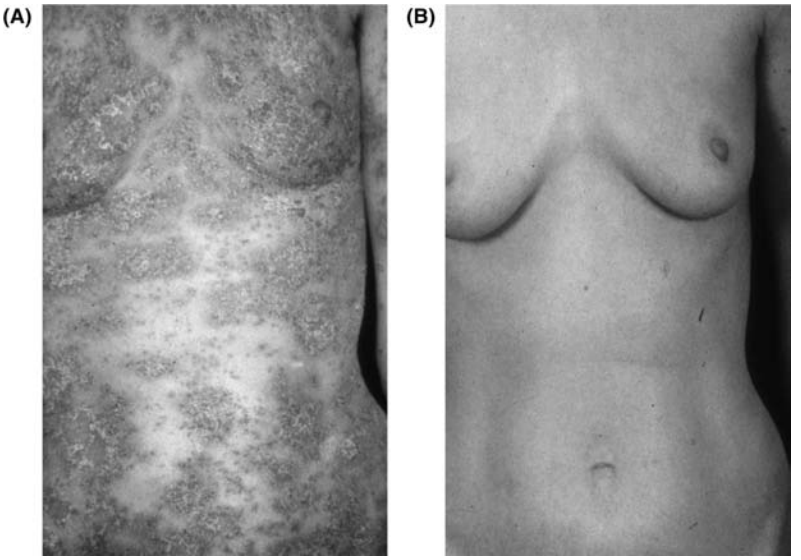

Figure 1 (*See color insert*) Clinical response to acitretin (1 mg/kg/day) in a patient with generalized pustular psoriasis (**A**) before treatment and (**B**) after four weeks treatment.

Starting with a low dosage (10 mg/day) and a progressive increase of the dosage seems to be more acceptable for the patient and can avoid the worsening of the existing lesions.

In a comparative study versus etretinate, at least 75% improvement in the PASI was observed in 23.1% of the patients treated with 50 mg acitretin after eight weeks of treatment and at least 50% improvement of PASI was reached by 54% of the patients (53). In a second study, the mean PASI improvement after 12 weeks was between 70% and 75% (54).

Acitretin has been shown to be an effective maintenance therapy in patients with chronic plaque psoriasis. Of all patients, 75% and 88% reached PASI-50 after six months and 12 months treatment respectively (45,55,56).

Treatment with acitretin during several months might improve nail psoriasis considerably (57). Although present in 80% to 90% of all psoriasis patients (58) and negatively affecting their quality of life (59), no specific placebo-controlled studies are currently available in nail psoriasis.

It is generally agreed that acitretin is not effective in psoriatic arthritis. Studies with acitretin between 25 mg and 60 mg daily have been shown to considerably reduce the risk of developing new cutaneous malignancies in patients who have received high dose PUVA treatment in the past or have been treated with cyclosporine (60–63).

Combination with Phototherapy

A consensus conference on the treatment of acitretin in combination with UVB or PUVA revealed that the combination enhances efficacy and limits the treatment frequency, duration and the cumulative doses compared to the UVB or PUVA monotherapy. The combination also permits lower acitretin doses, which imply an increased safety and compliance (64,65).

Broadband UVB in combination with acitretin has been shown to be highly effective as compared to monotherapies (66–69). A study comparing UVB treatment combined with placebo to UVB treatment combined with acitretin (50 mg/day) resulted in a 74% improvement in PASI in patients treated with UVB plus acitretin 50 mg/day versus 35% with UVB and placebo and 42% with acitretin monotherapy (67).

The preferred schedule when using the combination is pretreatment during two weeks with low-dose acitretin followed by the combination. In case of insufficient response to UVB monotherapy, acitretin 25 mg may be added which requires a 50% reduction of UVB dose.

The combination of 25 mg acitretin daily with narrowband UVB three times a week has been shown to be highly effective in patients with difficult-to-treat psoriasis (70). An improvement of at least 75% of the severity score was reached in 72.5% of the patients, only 12.5% had less than 50% improvement.

Also the combination of acitretin and PUVA (71–73) or Bath PUVA (74) has been shown to be more effective than the monotherapies. In general, 14 days prior to PUVA treatment patients have to take acitretin 25 mg/day. As with UVB,

adding acitretin to PUVA monotherapy requires a reduction of the initial UVA dose by 50%.

Combination Therapy with Topical Vitamin D Analogs

Compared to each of the monotherapies, an improved efficacy has been observed for the combination of acitretin with topical calcipotriol (50 μg/g) (75).

Combination Therapy with Biologicals

Today no studies have been performed comparing the efficacy of the combination of acitretin and a biological with both monotherapies. The combination of acitretin 25 mg/day with etanercept 25 mg twice weekly has been shown to be safe and effective in difficult-to-treat psoriasis (76). Also, several case studies have been reported showing that the combination of acitretin with a biological may be rather effective (77–79). A case of Acrodermatitis Continua Hallopeau responding successfully to the combination of etanercept and acitretin has been reported as well as a beneficial long-term improvement in a patient who had unsuccessfully treated with acitretin for 43 months, but who improved substantially by a 12 week combination course of alefacept and acitretin (80,81).

Contraindicated Combinations and Combinations for Restrictive Use

Cyclosporine inactivation by the cytochrome P450 system can be inhibited by acitretin meaning that the combination of acitretin and cyclosporine carries the risk of accumulation of cyclosporine. The effect of etretinate on the cytochrome P450-mediated metabolism of cyclosporine has been studied in vitro and indicates that there is no metabolic interaction; it is likely that the two drugs are metabolized by different P450 isoenzymes (82). The combination of methotrexate and acitretin has been used in those patients in whom all treatments have failed (83). Although this combination can be very effective, due to severe hepatotoxicity, this combination is no longer advisable (84).

Strategies of Combination Treatment

Psoriasis patients deserve long-term control of their disease with optimal benefit/risk ratio. The existing therapies, although providing excellent short-term control, may produce acute or chronic toxicities limiting their long-term use. Combination therapy can offer a solution. Cather and Menter (85) defined three objectives for a combination therapy: the first objective would be to achieve a similar efficacy and a lower toxicity with a lower dose; the combinations calcipotriol plus acitretin and UVB/PUVA plus acitretin are well established approaches to reach this goal. The second objective is to achieve fast clearing and subsequently maintain the improvement by sequential therapy. Powerful clearing therapies such as cyclosporine, methotrexate, photo(chemo)therapy, infliximab, or the traditional topical treatment with dithranol may be followed by acitretin maintenance resulting in a long-term safe control of the disease. In particular, in patients with previous exposure to potentially carcinogenic

treatments this approach is useful. The third objective is to avoid cumulative toxicity by rotational treatment. Patients with difficult-to-treat psoriasis who require a continuous treatment should use rotate treatments in order to reduce toxicity and still maintaining improvement of their psoriasis.

Side Effects

Mucocutaneous side effects of acitretin comprise cheilitis, dryness of the eyes, nasal and oral mucosa, epistaxis, xerosis, brittle nails, hair loss, and burning or sticky skin. In fact, some degree of cheilitis is an indication for sufficient bioavailability and therefore is, to some extent, a parameter to individualize the optimal dosage. Another side effect frequently seen during the first four weeks of treatment is the initial aggravation of psoriasis with an increase of the body surface area involved. A more seldom mucocutaneous side effect is "retinoid dermatitis," which may mimic "unstable psoriasis." Periungual pyogenic granulomas may occur during long-term use of acitretin (45,86).

In 25% to 50% of patients treated with acitretin hyperlipidemia has been reported and it is, in most patients, reversible when reducing the dosage or when treatment is stopped (87). Highly increased levels of triglycerides imply increased risk for pancreatitis and chronic increases may increase the risk for cardiovascular comorbidities. In patients treated with acitretin, one should be aware that diabetes mellitus, obesity, increased alcohol intake are risk factors for development of hyperlipidemia. In patients with moderate to severe psoriasis increases in serum lipids and dislipidemias have been reported without retinoid treatment (88–91). Therefore, figures on occurrence of hyperlipidemia in psoriasis should be corrected for the overall predisposition under psoriatics to develop hyperlipidemia. Lifestyles changes to prevent hyperlipidemia should be stimulated in psoriatics and in particular those who are treated with retinoids. Elevation of transaminases is seen in 13% to 16% of acitretin treated patients (86). A liver biopsy study did not reveal a significant risk for liver toxicity (92). Severe increases of liver test functions are rare, but may indicate toxic hepatitis induced by acitretin (93).

Strict contraception in women of childbearing potential is required during and two to three years following acitretin treatment. Teratogenic complications following acitretin treatment is discussed elsewhere in this book (94–96).

Less frequently, patients receiving acitretin may experience arthralgia, myalgia, or paraesthesia as well as possible worsening of pre-existing bone disorders during long-term therapy (97–99). Pseudotumor-like symptoms and signs have been recorded sporadically. The combination of retinoids with tetracyclins may increase the risk of this side effect. Symptoms and signs of intracranial hypertension are severe headache, nausea and vomiting, visual disturbances, and papilloedema (95). Although the relationship to acitretin has not been convincingly shown, depression and suicide have been suggested to occur during acitretin treatment (95). Decreased color vision and impaired night vision might occur during acitretin treatment.

SYSTEMIC RETINOIDS: TAZAROTENE

Oral tazarotene (4.5 mg/day) has been shown to be efficacious in moderate to severe psoriasis in two double-blind placebo-controlled trials and in a long-term open label trial (100).

Results of the phase III trials, reported at the Winter American Academy of Dermatology in 2003, demonstrated effectiveness of oral tazarotene versus placebo.

Additional data of the open label study, reported at the 62nd annual meeting of the American Academy of Dermatology 2004, also showed efficacy of oral tazarotene in long-term treatment of psoriasis (52 weeks, once daily). A moderate to complete clearing (at least 50% global improvement) was achieved by 56% of the patients by week 16 and 68% by week 24. A marked improvement (at least 75% global improvement) was achieved by 44% of patients by week 36. During long-term treatment, oral tazarotene was associated with adverse events, such as alopecia, hypertriglyceridemia, hepatotoxicity, ocular dryness, desquamation, and pruritus.

Pharmacokinetic data of oral tazarotene indicate that the drug poses little risk for deep tissue storage and accumulation in humans suggesting a shorter post-treatment waiting period for women who wish to conceive after discontinuation of oral tazarotene treatment.

However, although efficacious in psoriasis, oral tazarotene has not yet been approved by the FDA due to lack of safety data (100).

SYSTEMIC REXINOIDS: BEXAROTENE

Innovations in retinoid treatment in psoriasis are to be expected from RXR-selective ligands or rexinoids. Bexarotene (Targretin®, LGD1069) a novel synthetic specific RXR-ligand has been developed for the treatment of cutaneous T-cell lymphoma (CTCL) where it was shown to inhibit T-cell accumulation, which is also an important feature of psoriasis (101,102,103). Thus, as it might provide a new approach in the treatment of psoriasis, bexarotene is currently under investigation for this indication.

Mechanism of Action

Bexarotene selectively binds and activates RXR receptors: RXRα, RXRβ, and RXRγ(104) and not to RARs. RXR receptors function as heterodimers with other nuclear receptors [e.g., RAR, vitamin D receptor (VDR), thyroid receptor (TR), and peroxisome proliferator activator receptor (PPAR)] who play an important role in cell function and physiology, meaning that the biological activities of bexarotene could be broader than that of compounds that activate RARs.

Bexarotene has been shown to inhibit the growth of tumor cell lines of hematopoietic and squamous cell origin and to induce apoptosis (105–110). In vivo bexarotene induces tumor regression in some animals (105), while in other

models, tumor induction is prevented (107). As such, bexarotene has been developed for the treatment of CTCL (101).

In clinical studies, bexarotene (300 mg/m^2/day) proved to have an activity in patients with CTCL, even in patients in a late stage of the disease and who were not responsive to other systemic therapy, resulting in a regression or even temporary complete clinical response. The exact mechanism of action of bexarotene in the treatment of CTCL is unknown.

Pharmacokinetics

Oral bexarotene is absorbed with a T_{max} of about two hours and its T½ is about seven hours. Studies in patients with advanced malignancies show approximate single dose linearity within the therapeutic range and low accumulation with multiple doses.

Bexarotene is highly bound (>99%) to plasma proteins. In vitro studies suggest that cytochrome P450 3A4 is the major cytochrome P450 responsible for formation of the oxidative metabolites, which may be glucuronidated. The oxidative metabolites are active in in vitro assays of retinoid receptor activation. Neither bexarotene nor its metabolites are excreted in urine in appreciable amounts. Bexarotene is thought to be eliminated primarily through the hepatobiliary system (103,111).

Efficacy

Benign hyperproliferative and retinoid sensitive disorders, such as psoriasis are good candidates for studies with bexarotene. In a first study by Smit J.V. (112), it was shown that systemic treatment with bexarotene, up to 3.0 mg/kg/day, decreases epidermal proliferative and inflammation parameters (112). In a phase II dose finding study (0.5, 1, 2, and 3 mg/kg/day for 12 weeks), no significant dose-dependent difference between the four doses groups for PASI (modified PASI), PEL (plaque elevation), and physician's global assessment (PGA) was demonstrated (113). There was a statistical improvement of psoriasis (≥50%) with mean response rates of 22% for in PASI, 52% for PEL and 36% for PSA. Based on these data, doses higher than 3.0 mg/kg/day and lower than 0.5 mg/kg/day should be investigated in a placebo-controlled design. If higher doses are needed, side effects known from the treatment of patients with CTCL may be a limiting factor for bexarotene in the treatment of psoriasis or the drug could also be evaluated in combination with other antipsoriatic therapy.

Side Effects

The FDA approved oral bexarotene for the treatment of cutaneous manifestations of CTCL in patients who are refractory to at least one prior systemic therapy. In the high doses used to treat CTCL, nearly all patients suffer adverse effects, which can include hyperlipidemia (risk of pancreatitis), hypothyroidism, and

hematological reactions (leukopenia, anemia) (114). The many side effects limit the use of bexarotene as a monotherapy for CTCL.

Adverse events in the psoriasis trial were comparable to the CTCL trial with a lower frequency and severity (112). No serious adverse events related to the drug occurred. However, hyperlipidemia (56%) and a decrease in free T4 serum levels (54%) were most frequently reported adverse events related to the drug. Typical retinoid-related adverse events were pruritus, in only 14% of the patients and cheilitis in 10%.

Although the clinical data suggest an antipsoriatic effect of bexarotene, the side effects, especially hypertriglyceridemia and hypothyroidism, may require intervention and may restrict the use of higher doses in psoriasis patients. Further studies are needed to ascertain the relevance of bexarotene to the treatment of psoriasis (114).

RETINOIC ACID METABOLISM BLOCKING AGENTS

A new class of molecules named RAMBAs sometimes referred to as retinoic acid 4-hydroxylase inhibitors have proven to be of therapeutic benefit in keratinization disorders like psoriasis, ichthyosis, and acne. In this section, the mechanism of action of RAMBAs and their history will be presented followed by an illustration of the proof of principle in psoriasis with liarozole, the first RAMBA ever been in clinical development for indications like psoriasis and ichthyosis. Today, Rambazole (R115866) representing a new generation of highly potent and selective RAMBAs is in clinical development for the treatment of psoriasis. Specific information on its pharmacology and pharmacokinetics will be provided. Furthermore, results of a phase 2a study in psoriasis will be highlighted, suggesting that Rambazole has a promising profile for treating psoriasis.

Mechanism of Action

All-*trans*-RA, a minor metabolite of retinol and β-carotene, is believed to be the most active naturally occurring retinoid. It is significantly more active (100 to1000 fold) than retinol in experimental systems. One of the pathways of all-*trans*-RA inactivation occurs by hydroxylation at the C4 position of the cyclohexenyl ring to form 4-hydroxy-all-*trans*-RA, which is converted by a reductase into 4-keto-all-*trans*-RA that is in turn further transformed into more polar metabolites. Both the 4-hydroxylation process of all-*trans*-RA and the further oxidation of 4-keto-all-*trans*-RA to more polar metabolites involve microsomal cytochrome P450 (CYP) dependent enzymes (115,116). RAMBAs are nonretinoid drugs that block the the CYP dependent 4-hydroxylation, which results in an increase of the intracellular all-*trans*-RA concentration (Fig. 2) (117,118).

Unlike existing therapies where massive amounts of exogenous retinoids, natural or synthetic are administered to the body, a RAMBA modulates the body's own production of all-*trans*-RA to achieve the same therapeutic effects.

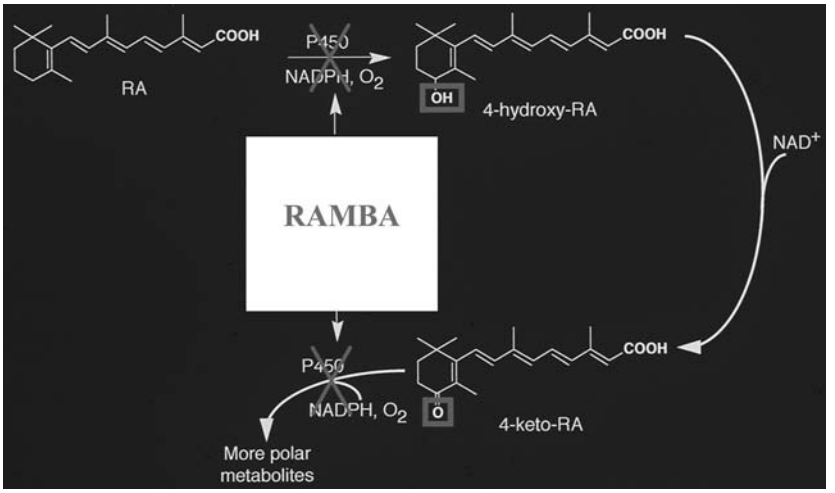

Figure 2 Mechanism of action of retinoic acid metabolism blocking agent (RAMBA).

By inhibiting its intracellular catabolism, endogenous all-*trans*-RA accumulates in the skin to therapeutic levels. Once treatment is stopped, a RAMBA is quickly eliminated from the body returning the metabolism of retinoic acid back to normal and declining the elevated all-*trans*-RA levels back to physiological levels. The rapid clearance of the RAMBAs is important as it decreases the potential for side effects in contrast to the available synthetic retinoids, which may stay in some organs for a long time, and which can cause retinoid related chronic toxicity and birth defects for months after discontinuation of therapy.

History of RAMBAs

The first azole shown to display RAMBA properties was the antifungal ketoconazole. Besides its antifungal activity, ketoconazole was shown to inhibit in an apparently competitive manner the CYP mediated metabolism of all-*trans*-RA by hamster liver microsomes (119). The search for more selective CYP inhibitors of all-*trans*-RA metabolism led to the identification of liarozole. Liarozole is an imidazole derivative that is lacking antifungal activity, but shares with ketoconazole its inhibitory effects on epidermal all-*trans*-RA metabolism (IC_{50} = 2 μM) and on the CYP mediated 17-hydroxylase-17,20-lyase in testes microsomes. Like ketoconazole, administration of liarozole to rats enhances endogenous plasma concentrations of endogenous RA and reduces the elimination rate of intravenously injected RA from plasma (120,121). Liarozole also inhibits several other cytochrome P450-dependent steroid biosynthesis reactions—mainly the conversion of androgens to estrogens (aromatase) and of 11-deoxycorticosterone to corticosterone (11-hydroxylase) (122,123).

Developed in the 1990s as a nonhormonal agent in the treatment of prostate cancer and various other cancers, liarozole was considered to be of potential benefit in the treatment of skin disorders like psoriasis and ichthyosis based on to the fact that the reported adverse events in the cancer trials were mainly cutaneous reactions showing a striking similarity with vitamin A related symptoms. Currently, liarozole is in development as an orphan drug for ichthyosis (excluding autosomal dominant ichthyosis vulgaris) (124–126). In this chapter, only its effects in psoriasis will be reviewed. Whereas, the first RAMBAs where nonselective CYP inhibitors, a thorough screening of hundreds of molecules against different CYP-isozymes yielded a very selective and highly active retinoic acid 4-hydroxylase inhibitor namely Rambazole™.

Liarozole: Proof of Principle in Psoriasis

A total of six clinical trials assessing the efficacy and safety of oral liarozole in psoriasis have been conducted (127–131). All trials were double-blind, placebo-controlled, randomized, parallel group trials, except for one open pilot trial and one active-controlled trial with acitretin at daily doses of 25 mg. All trials initially consisted of a treatment period of 12 weeks and a follow-up of 8 to 12 weeks, except for the open study in which no follow-up period was foreseen. Liarozole was administered in daily doses ranging from 50 to 300 mg.

A pooled analysis of the efficacy data of the six trials included in total 828 subjects of which 501 had been randomized to liarozole treatment (127). Significant mean decreases in PASI score compared to baseline and improvement in disease conditions were observed for all groups receiving liarozole. Nevertheless, large differences in response and improvement percentages between the groups receiving a liarozole dose lower than 150 mg and higher than 150 mg were observed. Both PASI score evaluations and global clinical evaluations pointed out that relative increases in response were largest when increasing the liarozole dose from 75 to 150 mg. Upon further increasing the liarozole dose, no further significant changes in percentage of responders were noticed. All doses of liarozole were found to be well tolerated, although the occurrence of the most recurrent adverse events (e.g., skin disorders, pruritus, dry mouth) was notably higher in subjects receiving a liarozole dose higher than 150 mg. However, the incidence of such adverse events in the 150 mg liarozole group did not surpass that of the acitretin group. A liarozole dose of 150 mg was therefore found to be optimal for the treatment of psoriasis.

Rambazole: A New Generation RAMBA

Rambazole (R115866) a substituted benzyl-1,2,4,-triazole derivative, has been identified as a third generation of RAMBAs with improved potency and selectivity (132). The investigational drug is a stable, enantiomerically pure base, which has been identified both in vitro and in vivo as a compound with highly specific and selective CYP inhibiting properties against hydroxylases involved in the catabolism of all-*trans* RA. Regarding its potency, Rambazole is an inhibitor of

all-*trans* RA catabolism in the nanomolar range (IC_{50} = 4 nM, human CYP26 transfected yeast microsomes) and is about three orders of magnitude more powerful than liarozole (IC_{50} = 3 μM). As for its selectivity Rambazole shows, in comparison to its retinoid catabolism inhibiting activity, only trivial inhibitory effects (IC_{50} = 1.2 – 2.6 μM) on the CYP-dependent biosynthesis of steroids (testosterone and estradiol). Furthermore, consistent with its ability to enhance endogenous RA content in tissues and plasma, Rambazole was found to generate retinoid-mimetic biological activities in various retinoid sensitive animal models of keratinization (132). In these studies, no major effects other than its primary pharmacodynamic effect were evidenced. By virtue of this property and the modulating effects of RA on epithelial growth and differentiation, Rambazole is currently in clinical investigation for both oral and topical treatment of psoriasis and acne.

Pharmacokinetics

In vitro studies using isolated hepatocytes and subcellular liver fractions from mouse, rat, dog, and human indicated that Rambazole was metabolized by all of the species. The potential of Rambazole to interact with specific cytochrome CYP substrates was studied in human liver microsomes. The lowest IC_{50} values were observed for two substrates metabolized via CYP3A4; being 1.3 μM and 2.6 μM. These concentrations, however, are considerably higher than those observed following administration of Rambazole to humans (133).

The pharmacokinetics of Rambazole after single and repeated oral dosing was studied in healthy male subjects. The oral absorption after a single dose (0.6–20 mg) was fast with peak plasma concentrations reached within one hour after dosing. Rambazole was eliminated with a mean dominant half-life of one to two hours and a mean terminal half-life of six to seven hours. The single-dose pharmacokinetics of Rambazole appeared to be dose-proportional. After repeated b.i.d. dosing for eight days (0.5-2-4 mg), steady-state was attained after one day. At the end of treatment on day 8, mean dominant and terminal half-lives were on the order of 2 hours and 15 hours, respectively. Steady-state plasma concentrations increased proportionally with the dose between 0.5 mg and 2 mg b.i.d., and more than dose-proportionally between 2 mg and 4 mg b.i.d. A dose-related increase in plasma RA was observed. On day 8 of the b.i.d. dosing of Rambazole, the exposure of RA in terms of C_{max} was lower than on day 1, whereas in terms of $AUC_{0\text{-}12h}$ the exposure remained fairly similar (133).

Tissue distribution data are not available from humans, but were collected following single oral administration of Rambazole at 2.5 mg/kg to male rats. Rambazole was extensively distributed throughout the body with a volume of distribution at steady-state of 5.2 L/kg. As in plasma, maximum concentrations of Rambazole were observed at one hour (the first sampling time) postdose in all tissues investigated except for the skin (three hours postdose). Maximum tissue concentrations of Rambazole in the skin (43.1 ng/g) were similar to those in plasma (40.1 ng/mL). Concentrations of Rambazole in tissues declined at a rate similar to those in plasma, except in adrenals where the rate of decline was slightly slower (133).

Indications and Contraindications

Today, no clinical data are available on drug-interactions and contraindications with Rambazole. Even though Rambazole belongs to the class of azole derivatives, the drug's main mode of action in skin is based on the inhibition of the CYP-mediated metabolic inactivation of the body's own retinoic acid. Hence drug interactions and other forms of interactions as well as contraindications and precautions related with retinoids should be taken into consideration (e.g., prevention of pregnancy, monitoring of lipid levels, avoiding comedication inhibiting or inducing CYP 3A4) (133).

Efficacy

The efficacy and safety of oral Rambazole in moderate to severe plaque-type psoriasis was assessed in an open label, phase 2a trial where 19 subjects were treated once daily with 1 mg Rambazole for eight weeks (134). Efficacy analysis performed at the end of the treatment phase and after a two-week treatment free follow-up period showed a time-dependent improvement of PASI. At end of treatment, five subjects (38%) showed an improvement in PASI of at least 50% compared to eight subjects (62%) at follow-up meaning that the optimal clinical efficacy has not yet been reached with an eight-week treatment schedule. In all treated subjects, throughout the whole treatment period, plasma levels of RA increased 0.5–1.5 ng/mL, 2 to 4.5 hours after intake of 1 mg Rambazole and returned to approximately baseline levels within 24 hours. Figure 3 shows the

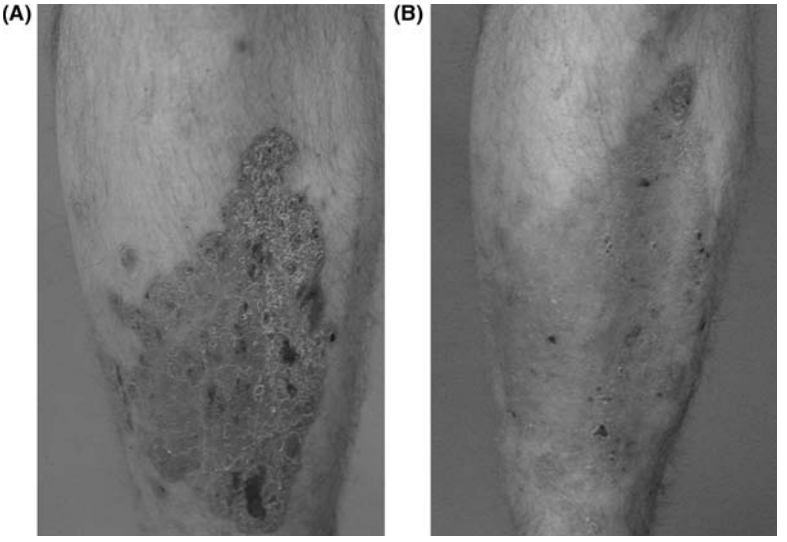

Figure 3 (*See color insert*) Clinical response to Rambazole™ (1 mg/ day) in a patient with moderate to severe plaque type psoriasis (**A**) before treatment and (**B**) after 10 weeks (eight weeks treatment + two weeks treatment-free follow-up).

leg of a patient before treatment with Rambazole and at the end of the two weeks follow-up.

Side Effects

Adverse experiences reported in association with the use of Rambazole are expected to be fewer and less severe than those reported for retinoids. The most common observed symptoms in the clinical studies performed until now are those associated with increased levels of RA, that is, pruritis, xerosis, and cheilitis. Additionally, epistaxis and cases of increased blood triglycerides have been reported (133,134).

CONCLUSIONS

Several retinoids have been developed and used successfully over the years for the treatment of psoriasis. However, today, acitretin (Neotigason®, Soriatane®) is the only oral, and tazarotene (Zorac®, Tazorac®) the only topical retinoid, approved for psoriasis in most European countries and in the United States. Oral tazarotene has completed phase III studies, but at the time of writing, its manufacturer has received a nonapprovable letter from the FDA. Despite the fact that many new therapies, like the biologicals, have been developed, retinoids as monotherapy or in combination with other treatment modalities remain a useful therapy for the treatment of psoriasis. The search goes on and synthetic retinoids continue to evolve and improve in terms of specificity and decreased toxicity. Rexinoids and new approaches like RAMBAs are currently intensively investigated with promising results.

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8

Retinoid Treatment of the Disorders of Cornification

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INTRODUCTION

In the 1970s and 1980s dermatology was exploring and developing systemic retinoid therapy as a treatment for a wide variety of disorders of cornification (DOC). This direction was not based on a detailed understanding either of the molecular mechanisms of retinoid action nor an understanding of the pathophysiologic mechanisms causing the clinical disorders. At the time this was developing, there was no knowledge of the nuclear retinoid receptors and X-linked ichthyosis due to steroid sulfatase deficiency was the only main DOC whose pathophysiology was even barely understood. Today our understanding of the genes involved in a wide spectrum of DOC's has exploded, revealing that these disorders are caused by a wide spectrum of diverse mechanistic abnormalities that include abnormal keratin intermediate filament structural proteins, connexins , transglutaminase, filaggrin, lipozygenases, transport proteins, etc. It is amazing that disorders with such a broad spectrum of diverse etiologies could respond to the same few systemic retinoid drugs. At the same time, progress has been made in understanding of molecular mechanisms underlying retinoid action. Despite this enormous progress in understanding both pharmacology and pathophysiology, this knowledge has not translated into the development of more compounds for treatment of the DOC's.

DISORDERS OF CORNIFICATION

The cutaneous DOC's are a heterogeneous group of diseases that are characterized by abnormal cornification (keratinization). "Cornification" is a descriptive term for the process that keratinocytes undergo during their maturation (differentiation). As individual keratinocytes mature, they move from the basal cell layer towards the outer surface of the epidermis, finally arriving in the stratum corneum. Within the stratum corneum, these keratinocytes, now corneocytes, function as part of the epidermal barrier. As they move through the stratum corneum, they undergo profound changes, which in the normal situation, enable them to be shed into the environment in a regular and organized fashion, leaving a smooth epidermal surface with a functioning barrier. The characteristic feature linking these DOC's is that the epidermis is abnormal. Several different epidermal abnormalities may coexist. There may be abnormal quality or quantity of scale produced, abnormal quality or quantity (thickness) of stratum corneum, abnormal keratinocyte kinetics, or other abnormality in the process of epidermal maturation (cornification).

Retinoids have diverse biologic effects. They can affect cell growth, differentiation, morphogenesis, immune function, cellular cohesiveness, and they can inhibit tumor promotion and malignant cell growth. The clinical result of these retinoid effects is dependent on several factors including the specific retinoid molecule, the retinoid concentration and the specific cell or tissue being exposed. It is interesting to speculate how retinoids could improve a broad range of DOC's that have vastly different causes. In many tissues, retinoids have the general effect of altering differentiation. In many DOC's with different etiologies, there is the formation of a stratum corneum which is abnormal in quality in addition to being thickly hyperkeratotic. Retinoids have a keratolytic/shedding effect that can help remove the excessive hyperkeratotic horn leading to a more normal thickness and improved function. In some disorders, such as Darier disease, there is dyskeratosis associated with the hyperkeratosis, and the thick horn can become colonized with large numbers of organisms, which then stimulate inflammation. The inflammation may lead to further barrier impairment and more hyperkeratosis. In this situation, removal of hyperkeratotic horn can lead to improvement in skin integrity.

In 1925, Wolbach and Howe identified epithelial changes in vitamin A deficient animals and thereby related low levels of vitamin A to dyskeratotic skin conditions (1,2). This was followed by the clinical use of systemic vitamin A for the treatment of disorders, such as Darier disease and pityriasis rubra pilaris, in which follicular keratoses resembled the lesions of vitamin A deficiency (3,4). The beneficial effects of vitamin A in these disorders was followed by the use of systemic vitamin A for other diseases of the epidermis and epidermal appendages.

Since the doses necessary to improve skin conditions are close to the doses associated with hypervitaminosis A, the development of synthetic retinoids was an attractive approach to improve the balance between retinoid efficacy and toxicity.

All-*trans* retinoic acid (tretinoin), a natural retinoid metabolite, was used clinically in the treatment of pityriasis rubra pilaris, ichthyosis, psoriasis, acne, and actinic keratoses (5–7). However, early systemic use of tretinoin was limited by its toxicity (8).

In the mid 1970s, isotretinoin was shown to be effective for a variety of previously treatment resistant DOC's including ichthyoses, Darier disease and pityriasis rubra pilaris (9,10). Since then numerous reports have documented benefit of isotretinoin, etretinate and then acitretin in these and other DOC's (11–23). Much of the clinical experience in the treatment of the DOC's with retinoids was based on treatment with the aromatic retinoid etretinate. At the time of this writing, the drug etretinate is no longer available in the United States and Europe, but is available in Japan and a few other countries. Etretinate is the ethyl ester form of the drug, that when ingested is metabolized to acitretin, the acid form. However, while the efficacy and toxicities of these two aromatic retinoids are similar, they are not identical. Acitretin treatment of the DOC's is similar to the many earlier reports describing the effects of etretinate, and this body of clinical experience with etretinate is often the best information we have to make clinical predictions in the treatment of the DOC's today.

MANAGEMENT OF THE DISORDERS OF CORNIFICATION

In addition to issues related to cosmetic appearance, patients with DOC's can have medical issues related to abnormal skin function. These can include pruritus, thickening of the skin with painful cracking and fissuring, decreased range of motion at joints, decreased tactile sensitivity of the fingers, hypohidrosis with heat intolerance, and for some disorders increased tendency for skin infection. These symptoms can vary between patients and even within the same patient over time. Because of this, it is important to adjust therapy to the individual patient's current needs.

Hydration, Lubrication, and Antimicrobials

The skin in many DOC's is characterized by abnormal barrier function and increased transepidermal water loss. In some conditions, the skin may be prone to poor moisture retention, easy irritation, and enhanced penetration of drugs. Three important mechanisms are involved in the action of most agents used in the treatment of DOC's: hydration, lubrication, and keratolysis. For DOC's with increased tendancy for skin infections, antimicrobials are another group of widely used agents. For most DOC's, the first line therapy includes hydration and lubrication, which can improve barrier function and facilitate desquamation.

Keratolytics

For DOC's with increased scaling and those with a thickened stratum corneum, keratolytic agents are used to decrease keratinocyte adhesion, to promote

desquamation of the stratum corneum and to increase water binding thereby enhancing hydration. There are a wide variety of topical keratolyitcs including the α-hydroxy acids (lactic acid, glycolic acid, etc.), salicylic acid, urea, propylene glycol, and topical retinoids. Prescription Lac-Hydrin is often reported to be particularly effective. Topical retinoids including tretinoin adapalene and tazarotene are available in a variety of concentrations and vehicles (24). When these agents fail to achieve a sufficient improvement, systemic retinoid therapy may be a useful addition to basic topical therapy.

CONSIDERATIONS IN STARTING SYSTEMIC RETINOID THERAPY

There are several issues to consider in the decision to start systemic retinoids. These include the choice of retinoid drug, the specific disease, and the age and child bearing potential of the patient.

Choice of Retinoid

While the spectrum of efficacy and toxicity of different retinoids are similar and overlap, they are not identical. In different clinical situations, there may be advantages of one retinoid over another. For the DOC, both isotretinoin and the aromatic retinoids (etretinate, acitretin) have been found to be efficacious (18,25,26). This is in contrast to the treatment of acne, where isotretinoin is highly effective, but the aromatic retinoids (etretinate, acitretin) are ineffective. Both retinoids have been found to be effective in Darier disease, lamellar ichthyosis (LI), congenital ichthyosiform erythroderma (CIE) and pitgriasis nubra pilaris (PRP). Additional experience with these retinoids has indicated that for different DOC's, there are differences in the degree of disease responsiveness. *The choice of retinoid drug may be influenced by the specific disease to be treated.* The aromatic retinoids (etretinate, acitretin) are superior in the treatment of many of the DOC's including psoriasis, erythrokeratodermia variabilis (EKV), epidermolytic hyperkeratosis (EHK), palmoplantar keratodermas, and a variety of ichthyoses. In addition, *the choice of retinoid drug may be influenced by the clinical scenario*. The aromatic retinoids have a relatively greater effect on volar skin leading to an advantage in the treatment of palmar plantar hyperkeratosis. For women of child bearing potential, exposure to retinoids are of concern because of their teratogenic effects. While isotretinoin is rapidly eliminated from the body after discontinuation of the drug, acitretin has a long half-life of elimination, and may persist in the body for months after the discontinuation of the drug. Isotretinoin, therefore, would pose a shorter duration of teratogenic risk and therefore may be preferred in female patients considering a future pregnancy.

Differences in toxicities between retinoids can sometimes be used as an advantage. While retinoid associated hair loss is generally considered completely reversible, it can be a troubling retinoid side effect for some patients. The frequency and extent of hair loss while on systemic retinoid treatment varies greatly

between patients. Compared to isotretinoin, acitretin associated hair loss is a more frequent side effect and tends to be more severe. Patients who do not tolerate the hair loss that occurs with acitretin may benefit by changing to isotretinoin.

Dose and Duration of Therapy

When starting retinoid for the first time, it may be advantageous to start at a low dose in order to allow the patient to gradually accommodate to side effects. Isotretinoin can be started at a dose of 0.5 mg/kg/day or lower then adjusted as needed; acitretin is usually started at 10–25 mg/day for adults.

Short-term treatment with systemic retinoids may be useful in the newborn period, and in patients with adult onset PRP, where the disease may undergo spontaneous remission. However, most patients with DOC's who do well on systemic retinoids will require long-term therapy.

Laboratory Monitoring

Prior to initiation of therapy, a baseline laboratory assessment, including complete blood count, liver function tests, fasting cholesterol and triglycerides, and pregnancy testing for women of childbearing potential is performed. Laboratory monitoring should be continued on a regular basis. Laboratory monitoring is discussed in Chapter 14.

Systemic Retinoids During Childhood

Etretinate, isotretinoin, and acitretin have been safely used for the DOC's during childhood. (11,14,22). The treatment of children is discussed in Chapter 9.

ICHTHYOSES

Autosomal Recessive Congenital Ichthyosis

Autosomal recessive congenital ichthyosis (ARCI) typically presents in the newborn period as the severe collodion presentation of ichthyosis. As the disease progresses it may have the form of LI with thick plate-like scale or CIE with generalize redness and scaling, or have features of both.

The collodion baby is born encased in a transparent, parchment-like membrane, which can interfere with respiration and sucking. The birth is often premature. Over the first two weeks of life, the membrane breaks up and desquamates. There may be impairment of the epidermal barrier function with susceptibility to infection, water loss and difficulties in thermal regulation. During this time, management should include careful monitoring of temperature, hydration, and electrolytes.

Therapy includes measures aimed at keeping the skin soft and pliable and encouraging desquamation, such as a humidified incubator and bland lubricants.

Advances in neonatal intensive care together with facilitating desquamation with judicious use of systemic retinoid therapy have led to improvements in survival.

Areas of membrane that are firm and dry can harden and constrict underlying structures leading to secondary problems, such as inhibition of respiration, and distal swelling of limbs. The most severe and often fatal form of congenital ichthyosis is the harlequin baby. This child, often born prematurely, has skin, which is characterized by thick, shiny plates of stratum corneum separated by deep red fissures. They may have poorly developed ears, and marked ectropion and eclabium. These infants are at risk of fluid and electrolyte imbalance because of abnormal water loss through the skin and poor temperature regulation. They are at increased risk of infection. The taut skin may restrict respiration. Infants with severe collodion or harlequin presentations who have experienced difficulty with membrane shedding have been treated with systemic retinoids to facilitate shedding of the membrane. Lawlor and Peiris reported treatment of a harlequin infant with etretinate and described good results (27–30). Subsequent reports with etretinate or acitretin confirmed their initial findings that systemic retinoid enhanced survival of these severely affected infants (31–39). While collodion babies are less severe than the extreme harlequin presentation, some collodion infants who are compromised by the membrane have been treated with systemic retinoid with good results.

In one report, two collodion babies, one with LI another with CIE were treated with acitretin (1 mg/kg/day) with clinical improvement and good tolerance to the drug (40).

The skin of babies with a collodion presentation can develop into a wide spectrum of ichthyoses from the mild self-healing phenotype to the more severe LI. Harlequin babies who survive usually have the appearance of a severe CIE.

LI is characterized by the presence of large, plate-like scales, which appear to be arranged in a mosaic pattern. The scales tend to be largest over the lower extremities. Involvement of the palms and soles is variable. Thick stratum corneum and tautness of the skin leads to pulling out of the eyelids (ectropion) and lips (eclabium). The thick stratum corneum can lead to compromise of the adnexal structures with scarring alopecia and compromise of sweat gland function resulting in hypohidrosis. Treatment with oral retinoids can improve and prevent some of these outcomes. There may be increased sweating with improved heat tolerance. LI is often caused by mutations in TGM1, the gene that encodes transglutaminase 1, an enzyme important for crossliking proteins to form the cornified cell envelope (41–43). During the formation of the stratum corneum, intracellular lipids are extruded to form a coat around the cell while intracellular proteins are crosslinked to form a brick-like mass.

In contrast to LI, patients with CIE typically have fine white scale and more prominent erythema. The degree of ectropion, eclabium, and alopecia are variable. As with LI, patients may have decreased sweating and some patients have severe heat intolerance. Mutations in the lipoxygenase genes ALOXE3 and

ALOX12B have been found in some patients with CIE (44). In other patients, mutations in the ABCA12 gene, which encodes an ATP-binding cassette (ABC) transporter protein have been found (45). This protein is part of a superfamily of membrane proteins, which bind ATP for the transport of molecules across cell membranes. In a small number of CIE families, mutations in TGM1 have been found (46,47).

LI and CIE respond to both the aromatic retinoids (etretinate, acitretin) and to isotretinoin; however, LI may respond more completely to the aromatic retinoids. Retinoids induce a decrease in skin thickness and scaling, which begins about one to two weeks after the initiation of therapy. Thickening recurs after the retinoid is discontinued. For some patients, the keratolytic and thinning effect of the systemic retinoid enhances the penetration and efficacy of topical keratolytics, such that even after the retinoid is discontinued, the topicals continue to maintain improvement. It may be that for some patients, the topicals are not very effective at inducing a large improvement, but may be better at maintaining improvement previously induced by retinoid.

There is a wide clinical spectrum of phenotypes between LI and CIE. LI patients with very thick hyperkeratosis and scale can improve substantially, but usually not completely, and are typically left with considerable residual involvement. Compared to LI, some patients with CIE may respond more completely and at lower doses (23). Since the systemic retinoid therapy is likely to be used long term, it is wise to keep the dose as low as practical. Patients need to continue aggressive use of other therapeutic measures to control scaling and hyperkeratosis including baths, moisturizers, and keratolytics.

Ophthalmologic Manifestations

Patients with LI/CIE who have ectropion often have blepharitis or conjunctivitis, and if eyelid closure is compromised, they are at risk for exposure keratitis. Local compromise of eyelid function, failure of the lids to close completely, and local factors from scaling around the eyes make these patients susceptible to bacterial colonization and infection. While blepharitis and conjunctivitis are well known retinoid side effects, these drugs are usually well tolerated by patients with ectropion. The ability of systemic retinoids to decrease the thickness of scale often leads to a decreased tendency for ectropion to progress. Patients with ectropion should have careful attention to eye care with liquid tears and eye lubricants, particularly a night, when failure of the lids to close fully during sleep can lead to exposure keratitis. Topical ophthalmologic antibiotics may be necessary for episodes of bacterial conjunctivitis.

EPIDERMOLYTIC HYPERKERATOSIS

Epidermolytic hyperkeratosis (EHK, bullous CIE) is an autosomal dominant disorder characterized by hyperkeratosis and often blistering. EHK has a wide

spectrum of clinical involvement and at least six clinical phenotypes have been described (48). EHK is caused by mutations in the genes encoding keratin 1 or keratin 10 (49). Involvement of palmar/plantar skin has been associated with mutations in keratin 1 (48).

The more severe forms of EHK are characterized by marked hyperkeratosis, which often is arrayed in cobble-stone like, or porcupine (hystrix) like spines. These are often hard, dark in color and thickest over joints. Because of the tendency towards blistering, recurrent infection is common. In patients with severe involvement, the thick hyperkeratotic spines can catch on clothing and external objects. This mechanical traction can lead to intraepidermal tearing and blistering. Systemic retinoid therapy leads to a reduction of these spines and other areas of hyperkeratosis and the result is often improvement in both the hyperkeratosis and the frequency of infection.

One side effect of systemic retinoid therapy is skin fragility. EHK skin is fragile and prone to blistering and this tendency can be enhanced by retinoids. It is therefore important to start EHK patients at low doses of retinoids to avoid exacerbating the blistering. For EHK patients with palm and sole involvement, it is important to recognize that the different retinoids may have different effects on volar skin. Compared to isotretinoin, the aromatic retinoids have a greater effect on volar skin. It has been suggested that retinoid therapy may be more effective in patients with mutations in keratin 10 compared to those with mutations in keratin 1 (50), i.e. patients with volar skin involvement (48).

Ichthyosis bullosa of Siemens (IBS) is an autosomal dominant disorder due to mutations in the gene encoding keratin 2e, which is expressed in the granular layer of the epidermis. The skin in IBS is hyperkeratoic and there may be areas of loss of the superficial epidermis, which have been called molting. IBS can respond well to low dose of systemic retinoids (51).

PITYRIASIS RUBRA PILARIS

Pityriasis rubra pilaris (PRP) is characterized by follicular hyperkeratotic papules, which coalesce into plaques. Patients may have typical areas of scaling with a salmon-red erythema and islands of sparing. Palms and soles may be involved with scaling and hyperkeratosis, which in some patients, can be severe. Five clinical types have been described (52). Systemic retinoid therapy can improve several aspects of PRP (13,53–57). In some PRP patients, palmar-plantar hyperkeratosis can be severe with decrease in finger sensation, limitation of finger motion, and painful fissuring. Systemic retinoids can decrease the hyperkeratosis, improve finger motion, and minimize the development of painful fissuring. The aromatic retinoids etretinate and acitretin tend to have more palm/sole effect than isotretinoin and may lead to a greater improvement than isotretinoin.

It may be useful to separate the response of PRP to systemic retinoids according to two different parameters: (*i*) improvement and (*ii*) remission.

Improvement

As PRP progresses, areas of follicular hyperkeratosis extend and coalesce into plaques. These may coexist with large areas of scaly salmon-red erythroderma. Involvement may be severe on the scalp with intractable itching. Palmar plantar keratoderma may be severe with progressive thickening, loss of finger motion, and the development of fissuring. Systemic retinoid can reduce the hyperkeratosis causing symptomatic improvement. In addition, existing areas of involvement may undergo clearing. Upon discontinuation of retinoid therapy, this may be followed by an increase in the area and severity of involvement.

Remission

Some patients with PRP undergo spontaneous remission of the disease. Several studies have reported disease remissions coincident with systemic retinoid therapy, with prolonged or permanent clearing after the discontinuation of therapy. Isotretinoin and the aromatic retinoids (etretinate and acitretin) have been associated with PRP remissions. Some reports have suggested that etretinate (which is no longer available) might be more likely to induce remissions of PRP (55).

A few reports have described efficacy of systemic retinoid with either narrow band UVB (58) or UVA 1 (59) for the treatment of PRP.

DARIER DISEASE

Darier disease (keratosis follicularis) is an autosomal dominant disorder characterized by hyperkeratotic follicular papules. The disease usually presents in late childhood to early preteen years, undergoes exacerbations and remission, and tends to be progressive over time. It is now known to be caused by mutations in ATP2A2 gene, which encodes a calcium pump (sacro/endoplasmic reticulum calcium ATPase pump-SERCA2) (60). Darier disease has been reported to respond to etretinate, acitretin, and isotretinoin (10,12,17,26,61–63). However, some patients with Darier disease who have responded well to treatment with etretinate have been reported to have less benefit from acitretin (64).

The skin in Darier disease may consist of dry, brown areas of hyperkeratosis, In contrast, some patients have areas of red skin, which is inflamed and infected, often with marked interetriginous involvement. The dry, hyperkeratotic type of Darier tends to be more responsive to retinoids compared to moist, infected Darier skin. When large areas are involved, Darier skin may be colonized by large numbers of organisms and prone to repeated infection. Over time, the skin may develop thickened, indurated areas, which tend to be frequently inflamed. Some patients have repeated episodes of infection with a variety of organisms and require topical and systemic antimicrobials. Systemic retinoid is effective in reducing the areas of hyperkeratosis. Topical and systemic antibacterials are helpful to manage acute and chronic areas of skin infections. In patients with localized areas that are resistant to treatment, local removal or destruction of the superficial skin can result in a scar that is resistant to recurrence of Darier in that area (65–67).

HAILEY-HAILEY DISEASE

Hailey-Hailey disease is an autosomal dominant disorder characterized by epidermal acantholysis. It is caused by mutations in the gene ATP2C1, which encodes a secretory pathway ATPase (SPCA1) that is found in the Golgi apparatus (68,69). Retinoids are not usually of benefit in Hailey-Hailey disease and may worsen the condition (19).

ERYTHROKERATODERMIA VARIABILIS

Erythrokeratodermia variabilis (EKV) is an autosomal dominant disorder characterized by hyperkeratosis and erythema. Patients can have generalized involvement with brown hyperkeratosis and migratory red patches. A localized type of EKS has fixed, sharply demarcated hyperkeratotic plaques. Both types have migratory red patches that can develop after trauma or change in temperature. Volar skin may be involved with hyperkeratosis. The disease is caused by mutations in GJB3 or GJB4, the genes that encode the gap junction proteins connexin 31 or 30.3, respectfully. Involvement of these connexins suggests that defective intercellular communication is the basic functional defect in EKV (70–72).

EKV responds very well to treatment with systemic retinoids and has been thought to be one of the most retinoid responsive genodermatoses (73–77). EKV often responds well to low doses of either acitretin or isotretinoin, however, the response to the aromatic retinoid acitretin may be more complete.

KERATITIS–ICHTHYOSIS–DEAFNESS SYNDROME

Keratitis–ichthyosis–deafness (KID) syndrome is characterized by keratitis, ichthyosis, and sensorineural deafness. Patients may have widespread or generalized scaling, but the more distinctive skin involvement is characterized by well-demarcated, red to orange, fixed plaques, which may be symmetrical, often involving the face.

There may be prominent follicular hyperkeratosis that leads to a scarring alopecia. In some patients, the plugged follicles predispose to infection with the development of cysts, abscesses, and sinus tracts. There may be an increased occurrence of bacterial, viral, and fungal infections.

KID syndrome is caused by muations in GJB2, the gene that encodes the gap junction protein connexin 26 (78). While the hyperkeratotic lesions may respond to retinoid (acitretin) (79,80); involvement of the cornea and hearing do not improve and in some patients corneal neovascularization may worsen with systemic retinoids (81–83).

PALMAR PLANTAR KERATODERMAS

The palmar plantar keratodermas (PPK) are a diverse group of DOC's that include epidermolytic and nonepidermolytic types. In some types, involvement is confined

to volar skin, while others also have involvement of the skin in other body locations. There is a wide clinical spectrum of severity. Volar involvement can be mild to severe, discrete or confluent, and thickly hyperkeratotic to blistering/peeling. Severe forms may include scaring, contractures and constriction of the digits, sometimes with loss of parts of the distal digits (84). For several of the inherited types of PPK, mutations in a variety of different genes have been found. There are also acquired types, such as the PPK that can occur in PRP. Systemic retinoids can reduce the hyperkeratosis in many forms of PPK, but the degree of clinical improvement is very variable. Some patients with thick volar hyperkeratosis develop fissures from their disease, which can be painful and limit mobility or use of hands. Resolution of deep fissuring is an objective sign of improvement. Other patients may have limited objective improvement.

In epidermolytic PPK, retinoids can enhance shedding and blistering and may be poorly tolerated (19,85,86). This is similar to the situation in patients with generalized EHK who have volar involvement. The effect on volar skin may be beneficial at low doses but enhance blistering and be intolerable at higher doses.

In addition, there is great variability in the degree to which the patients perceive benefit. There may be release of contractures, improvement in fine finger movement and tactile sense, which the patient appreciates even when the appearance of improvement is modest.

MISCELLANEOUS DISORDERS OF CORNIFICATION

Many uncommon DOC's marked by hyperkeratosis may also improve with retinoid therapy. A three-year-old male with ichthyosis follicularis, alopecia and photophobia (IFAP) syndrome treated with acitretin had improvement in cutaneous features and corneal erosions, but no change in alopecia or photophobia (87). A seven-year-old boy with Papillon–Lefèvre (due to a mutation in the cathepsin C gene) treated with acitretin, keratolytics and trimethoprim-sulfamethoxazole had marked improvement in palmoplantar keratoderma and periodontal disease (88).

TOXICITY

Patients with DOC's who respond well to systemic retinoid therapy are likely to require the retinoid for many years. In this patient population, long-term systemic retinoid side effects become a concern. Long-term retinoid toxicity affects mainly the skeletal system (89). The primary pathology is an enthesopathy, involving the tendons and ligaments around joints. This manifests clinically as stiffness, and radiologically as bony spurs (osteophytes) and tendon and ligament calcification around joints. The primary areas affected are the spine, heels, and hips, but other locations may also be involved. Early lesions of the spine are seen radiographically as bony spurs along the anterior spinal ligament, which when progressive can lead to bony bridging. These changes are common in the general population. One study found that 100% of 400 spine specimens from skeletons of individuals

older than 40 years of age showed some degree of anterior osteophyte formation, which increased in size with age (90). Another study found similar changes in up to 65% of individuals younger than 20 years of age (91). Because of the high frequency of these changes in the nonretinoid treated population, it is likely that most adult patients will have some degree of these changes at the onset of retinoid therapy. Therefore, for patients who are tolerating therapy well, a radiographic survey obtained soon after the start of treatment can be valuable to establish the extent of baseline involvement. A typical survey could include a lateral view of the cervical and thoracic spine, lateral view of the calcaneus (heel), and posterioanterior view of the pelvis. For patients on long-term treatment, a bone survey can be repeated periodically, and would include any symptomatic areas. Chronic toxicity can be minimized by keeping the retinoid dose as low as practical. During the treatment of children who are still growing, there is a risk for premature epiphyseal closure, therefore the retinoid dose should be as low as feasible and growth should be monitored with a standardized growth chart (14).

The systemic retinoids have been used since the late 1970s for DOC's and many patients have been maintained on therapy for decades. However, few systematic long-term evaluations of these patients have been reported. A recent retrospective review of 23 patients with a variety of DOC's treated with the aromatic retinoids etretinate or acitretin included four males treated for over two decades and one female treated for approximately one decade. While systematic radiographic surveys were not done, only one of these patients was reported to have symptoms and radiographic evidence of diffuse idiopathic skeletal hyperostosis (DISH). Due to degree of efficacy, this patient was reported to have continued acitretin treatment at least four years after the diagnosis of DISH with no deterioration on annual radiological assessment of the spine (20).

Hypervitaminosis A and some retinoid drugs have been associated with osteoporosis (92–95). While short-term retinoid exposure in active, healthy young adults may not increase the risk for osteoporosis (96) the risk during long-term treatment may be greater. For patients receiving long-term systemic retinoid therapy, particularly those who may be at risk to develop osteoporosis for reasons such as family history, prior or current predisposing medications, or physical inactivity, periodic bone densitometry (DEXA) evaluations may be useful.

SUMMARY

Systemic retinoids have been used in the long-term treatment of a variety of DOC's since the 1970s. These drugs have clearly improved the quality of life for patients who have a spectrum of difficult to treat disorders. Careful selection of patients and regular attention to monitoring for toxicity can help to insure a good clinical outcome. Some patients have been safely treated for over two decades with a manageable degree of toxicity. While challenges to drug development, most importantly teratogenicity, have slowed their continued development, there is a great need for new and even more effective retinoid drugs.

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9

Oral Retinoid Therapy in Children and Infants

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INTRODUCTION

After the advent of glucocorticosteroids, oral synthetic retinoids have probably been the most important breakthrough in dermatologic therapy in general and in keratinization disorders in particular. Although none of the available retinoids have been approved by the United States Food and Drug Administration for use in children, severe disorders of keratinization typically affect children, often from birth, and oral retinoids have been for many of them the best if not the only effective therapy available.

Precedence for the use of oral retinoids in children comes from a number of published clinical trials starting in 1977 by our group (1), and followed by others since 1983 (2). The treatment of alterations of keratinization or cornification in both, pediatric and adult patients can not be understood without the inclusion of oral retinoids. In adult patients, the therapeutic efficacy of oral retinoids for each condition and their side effects are relatively well known. In pediatric patients, due to the rarity of most diseases and to the fear of side effects, the therapeutic experience has been frequently limited to single case reports and follow-up of a few months.

The purpose of this publication is to report 29 years of experience of the Department of Dermatology of the National Institute of Pediatrics in Mexico City, with the use of oral retinoids in 53 pediatric patients, 34 treated with etretinate, 17 with acitretin and 2 more with isotretinoin. The diseases treated represent the

vast majority of keratinizing disorders seen in pediatric patients. Minor forms of ichthyosis, such as ichthyosis vulgaris and sex-linked recessive ichthyosis rarely need to be treated with oral retinoids and will not be discussed here. A few other patients with nonkeratinizing conditions are briefly discussed. Since pediatric patients, from newborns to adolescents are subject to an active process of growth and development, any therapeutic intervention should be considered in this context for its potential long-term impact.

Oral retinoid treatment of our patients has always been given after informed consent of parents or tutors. Potential side effects have been carefully monitored. Some of our patients today are offspring of our previous patients. In the past, the decision to treat or not to treat with oral etretinate was based on factors related to diseases causing severe physical, psychological, and social incapacity. At present, the decision to prescribe acitretin has been primarily based on "harm-benefit" considerations. We feel that the vast majority of patients treated with oral retinoids now enjoy a better quality of life.

In this chapter, we describe our experience with etretinate and acitretin in children, often from the first months of life into adulthood. The most worrisome side effect of oral retinoids: teratogenecity is, in prepubertal children, not relevant. Other potentially important side effects (musculoskeletal, neurological) were, in our patients, minor and without long-term consequences.

For How Long, and at What Dose?

At present, the choice of oral retinoids for use in pediatric patients is dependent on availability, therapeutic efficacy, and side effects.

In developing countries, the high price of oral retinoids was a frequent deterrent to their prescription, once the patents expired their use increased. This was especially true for isotretinoin which at present may be bought for a fraction of what it cost a few years ago. The most effective oral retinoid for keratinization disorders and for psoriasis is acitretin. However, due to problems of commercial availability and high cost isotretinoin is often prescribed instead.

How long treatment should be maintained will depend on the natural history of the disease and of patient's tolerance to its side effects. Once the disease has been under control with over 80% improvement, the general trend has been to prescribe lower and increasingly spaced doses allowing for oral retinoid "vacations" in which the patient may be retinoid free for one to three months. In case of minor relapses, observed in severe keratinization disorders, such as lamellar ichthyosis (LI) and epidermolytic hyperkeratosis (EH), topical emollients, urea, vitamin D- analogs and, if required, antibiotics may be used.

INDICATIONS FOR ORAL RETINOIDS IN INFANTS AND CHILDREN

The efficacy of systemic retinoid therapy in a number of dermatologic diseases is well established for disorders of keratinization, psoriasis, and acne. However, retinoids have also been used to treat and prevent cutaneous malignancies

complicating some genodermatoses, such as xeroderma pigmentosum (XP), nevoid basal cell carcinoma, and epidermodysplasia verruciformis.

Main Disorders of Keratinization

Lamellar Ichthyosis and Nonbullous Congenital Icthyosiform Erythroderma

Lamellar ichthyosis (LI) and nonbullous congenital icthyosiform erythroderma (NBIE) are inherited as autosomal recessive traits. They are caused by mutations in genes encoding transglutaminase-1, icthtyin, and several other proteins (see Chapter 8). In both instances, patients are born as "collodion babies." After a few weeks, the collodion membrane is shed. In LI large and dark plate-like scales with universal involvement are observed, while in NBIE the entire body is red covered by finer scales. Topical treatments with moisturizers, retinoid creams, or descalers are not enough for elimination of the scales.

Perhaps the most impressive improvement obtained with oral retinoids is in LI. We had the opportunity to treat a number of patients with etretinate, beginning in the 1980s, and witness the spectacular physical and mental improvement of patients, some followed for more than 20 years. After one or two months of treatment, the skin lost most of the scales (Fig. 1), and the ectropion and eclabium were considerably reduced. Lower doses are required for long-term therapy to maintain the skin with thinner scales. For detailed information about treatment see Chapter 9.

Lacour et al. (3) published the largest analysis of children on acitretin for the treatment of severe inherited disorders of keratinization. The mean optimal dosage for acitretin was 0.47 mg/kg/day. The overall improvement was

Figure 1 (*See color insert*) Lamellar ichthyosis before and after one month treatment with oral etretinate 1.5 mg/kg/day.

considerable, with only three patients responding poorly. Nine patients with LI had a mean follow-up of 13 years. Three children, with moderate involvement had an excellent response to a low dose of acitretin (<0.4 mg/kg/day). The other six children, who were more severely affected, had a moderate to marked improvement with doses of acitretin ranging from 0.45–1.0 mg/kg/day. In 22 patients, the clinical response, as well as side effects, in individual patients, did not qualitatively change between etretinate and acitretin.

Combination therapy with topical keratolytics and hydration of the stratum corneum is recommended. In otherwise healthy children, control liver function tests and serum lipids must be performed before treatment and monthly thereafter for three months. In most patients tests remain normal. When laboratory changes are present they are generally mild, transient, and nonsignificant.

Children with NBIE present finer and whiter scales than classic LI and a more prominent erythroderma; they respond poorly to acitretin probably because of increase of the erythematous component. The best effects are observed on the ectropion and the eclabium.

Collodion Baby

Collodion Baby (CB) does not represent a disease entity, but a phenotype commonly present at birth in several severe congenital disorders of keratinization and less frequently in other forms of ichthyosis. CB appears enveloped by a keratin membrane that resembles a dried film of collodion. Ectropion, eclabium, and hypoplastic ears are constant features. Oral acitretin treatment in CB has been reported and suggests that early management could prevent life-threatening events such as hyperthermia, disturbance in electrolyte and fluid balance, and infection (4). In contrast, Van Gysel et al. (5) reported 17 cases of collodion baby; infections were observed in 9, hypothermia in five and hypernatraemic dehydration in four cases. Skin infection was more frequently observed in CB treated with emollients, therefore they recommend treating CB in a humidified incubator, if necessary with intravenous rehydration, and not to use emollients. None of their CB received oral retinoids and the final outcome in the 17 patient was good, 14 developed some form of ichthyosis, and 4 showed normal skin.

In our opinion, newborns with CB phenotype do not necessarily need oral retinoid therapy, because the collodion membrane will spontaneously be shed. CB patients must be treated in an intensive care unit, with special attention to temperature, electrolyte balance, prevention of infections and percutaneous drug toxicity. All of our patients with CB phenotype survived without receiving oral retinoids.

Harlequin Ichthyosis

Harlequin Ichthyosis (HI) (Harlequin fetus) is an extremely rare condition caused by abnormal keratinization. The condition has been reported in dizygotic twins (6). Several patients with a history of one or more involved siblings (7,8) suggest that HI is determined by an autosomal recessive gene. However, it has been suggested that in families with recurrent cases, HI may result from a new dominant

mutation with parental mosaicism (9). Recent studies have revealed inactivating mutations in the ABCA12 gene as the most common cause of HI (10). At birth, HI newborns appear covered by an extremely thick and massive keratinous caparace with large fissures. Ectropion, eclabium, poorly developed ears, and contractures of the hands and feet give the newborns a grotesque appearance. HI formerly was universally fatal in the neonatal period. At present, due to a better perinatal care and possibly to oral retinoids, survival is the rule.

The apparent benefit of early retinoid therapy in HI is however difficult to assess, since not all survivors received retinoids and a controlled study is virtually impossible to conduct in this rare and life-threatening disease (11). Most surviving HI patients develop an erythrodermic ichthyosis resembling a severe (NBIE) (12), while in others there is diffuse, thick, silvery scaling, alopecia of the scalp, scarce eyelashes and eyebrows, and a marked ectropion and eclabium. Ears show a malformed pinna and fingers may present shortening and contractures. Fissuring of the thick skin is frequent (13). Differences in the clinical characteristics are not related to retinoid therapy, but may be linked to the genetic defect.

In our series, only one male Cuban patient presented with HI and survived without oral retinoids in childhood. When he was born, doctors and parents decided not to do anything else but normal feeding. Soon after, his appearance was quite the same as that of the first HI who survived with etretinate, treated by Lawlor and Peiris (14). The whole body in our patient was covered by fine and white scales over an erythrodermic skin. At 13 years of age he had almost total alopecia, scarce eyebrows and eyelashes, ectropion and deformed ears. Both hands presented retraction of fingers, without constriction; movements were difficult and scarce. He had never attended normal school. In an attempt to liberate the fingers, surgery was performed in one hand, however, three weeks after surgery, all fingers regressed to its former position. Treatment with acitretin was started at a dose of 0.5 mg/kg/day and was well tolerated. Improvement was most satisfactory for scaling and ectropion, but the erythrodermic component of the skin remained unchanged. After improvement, acitretin was reduced by half of the initial dose.

Epidermolytic Hyperkeratosis or Congenital Bullous Ichthyosiform Erythroderma

This autosomal dominant disorder is characterized by hyperkeratosis and blistering; the skin surface is not covered by scales, but by thick hyperkeratotic plaques (Fig. 2). Epidermolytic hyperkeratosis (EH)/congenital bullous ichthyosiform erythroderma (CBIE) is due to mutations in the genes encoding the intermediate filament proteins keratin 1 and 10. There are both localized and generalized forms; the former includes plaques, linear distribution and palmo-plantar hyperkeratosis. In newborns, generalized EH is characterized by erythroderma, numerous fragile bullae, and denuded skin, which in infancy is progressively replaced by thick and

Figure 2 (*See color insert*) Epidermolytic hyperkeratosis before and after two months of oral retinoid therapy 0.5 mg/kg/day.

spiny hyperkeratosis with a characteristic foul odor. Controlling the odor in EH is an ongoing issue; the use of antibacterial washes, absorbing powders, and masking fragrances (15) is of little help. A monthly intramuscular injection of benzathine penicillin is useful to prevent secondary streptococcal infection, which contributes to foul odor. Topical moisturizers and descalers are of little help in EH. Retinoid creams are not well tolerated and oral retinoids may control hyperkeratosis, but can cause increased blistering.

In our patients, treatment with 1 mg/kg/day of etretinate caused bullae formation, skin fragility and burning sensation. Recently, we treated five children with a daily dose of 0.5 ± 0.2 mg/kg/day of acitretin. In three patients, a 70% to 80% of improvement of the hyperkeratotic lesions was obtained after a few months. Two patients developed blisters and erosions and were lost for follow-up. Similar side effects have been reported in other series (3) with etretinate as well as with acitretin. Virtanen et al. (16) found that patients with EH due to KRT 1 mutation invariably had palmo-plantar keratoderma (PPK) nonresponding to acitretin, while patients with EH due to KRT 10 mutation did not have PPK, but responded

Figure 3 (*See color insert*) Palmo-plantar keratoderma before and after one month of oral retinoid therapy 1 mg/kg/day.

to retinoids, possible because they are less vulnerable to a down-regulation of K2e by retinoids.

Miscellaneous Disorders of Keratinization

Palmo-plantar Keratoderma

PPK is characterized by focal or diffuse thickening of the palms and soles. It may be an isolated defect or be part of a syndrome, such as keratitis, ichthyosis, and deafness (KID) syndrome. Hyperkeratotic lesions in PPK usually desquamate or detach in large sheets after two to three weeks of acitretin therapy, leaving behind a red, finely scaled, and tender surface (Fig. 3). Emollients and hydration are required. Maintenance therapy must be individually adjusted according to efficacy and occurrence of adverse reactions. In two of our cases treatment with low dosages of acitretin (0.2–0.5 mg/kg/day) given continuously or periodically were effective. Once the thick hyperkeratotic epidermis detaches, acitretin should be replaced by emollients and mild keratolytics.

Blanchet-Bardon et al. (17) treated four pediatric patients for four months with acitretin at 0.42–1.16 mg/kg/day. Three patients with Papillon-Lefevre PPK showed marked improvement while one patient with Thost-Unna responded poorly.

Pityriasis Rubra Pilaris

Pityriasis Rubra Pilaris (PRP) may be an acquired or a genetically determined disorder of keratinization. Clinically, PRP is characterized by follicular, hyperkeratotic lesions with a tendency to form plaques; palmo-plantar keratoderma is usually present. In children, self-limited cases are frequent, the natural evolution time from three months to seven year, may shorten with oral retinoids to two to three weeks with no recurrences. In chronic cases, a maintenance acitretin dosage (0.3–0.5 mg/kg/day) during four to six months is required. We treated five cases with severe nonhereditary PRP, three cases responded swiftly, but two required a weekly maintenance dose.

Darier's Disease

Darier's disease (DD) (diskeratosis follicularis) is a genetic disorder usually beginning at puberty (18). In our series, a young patient with DD received acitretin for more than 20 years with good response and tolerance. Routine liver function tests have been within normal limits. No abnormalities in vision or bone structures have been observed.

Papillon–Lefevre Syndrome

Papillon–Lefevre syndrome (PLS) is an inherited autosomal recessive disorder presenting with PPK and juvenile periodontitis followed by premature shedding of both deciduous and permanent teeth. Recently, a seven-year-old boy with PLS who suffered from PPK and chronic gingivitis since the age of three months was

reported (19). After one year of treatment with acitretin 10 mg daily and trimethoprim-sulfamethoxazole, the patient's skin was almost lesion-free, new teeth erupted, and periodontal disease disappeared. A maintenance dose of 0.5 mg/week is usually sufficient to keep the disease under control.

Erythrokeratoderma Progressiva Symmetrica and Erythrokeratoderma Variabilis

Erythrokeratoderma progressiva symmetrica (EPS) is a very rare condition of unknown etiology, its inheritance is probably autosomal dominant with variable penetrance (20), although sporadic cases and presumably new mutations have been reported. Macfarlane et al. (21) described two affected sisters: the youngest developed erythrokeratoderma variabilis (EKV) at the age of 17 months, whereas the oldest developed EPS at age six years. This report suggests that EKV and EPS may be different phenotypic expressions of a single genetic disorder. Connexin mutations have been found to be responsible for most cases of EKV, but some cases without demonstrated connexin mutations exist, suggesting genetic heterogeneity.

EPS usually appears in early childhood. It is clinically characterized by well-demarcated erythematosquamous plaques with an almost perfect symmetry and a reddish-orange or brownish color. Lesions commonly occur on the cheeks, limbs, buttocks, shoulders, and fingers. The chest and abdomen are not involved.

The characteristic skin lesions of EKV usually start during the first year of life, but may start later in childhood. They are characterized by irregularly shaped, erythematous plaques, distributed at any site and with a tendency to migrate slowly. Lesions can last from a few hours to few days leaving behind a fine scaling.

In our series, three patients with EPS were treated with an initial dose of 1.5 mg/kg/day of etretinate, after one month dosage was tapered to 0.5 mg/kg/day. In seven additional cases, acitretin was used at a dose of 0.5 mg/kg/day. All 10 patients had an excellent response (Fig. 4).

Psoriasis

Most cases of psoriasis in pediatric patients are of the guttate type and need not to be treated with oral retinoids. Acitretin has been considered among the treatments of choice for severe "en plaque," erythrodermic and pustular psoriasis in children (22). However, acitretin as a monotherapy for plaque psoriasis is less effective, and therefore preferably used in combination with other systemic psoriasis therapies, especially ultraviolet B or psoralen plus ultraviolet A phototherapy, to increase efficacy (23). Combination treatments may minimize toxicity by using lower doses of each medication.

In erythrodermic and pustular psoriasis, acitretin monotherapy is indicated also in children. Both forms of psoriasis respond in a few days to dosages in the range of 0.2–0.3 mg/kg/day. After a month, if required, the dose can be increased to 0.5 mg/kg/day.

Figure 4 (*See color insert*) Erythrokeratoderma progressiva symmetrica before and three weeks after oral retinoid therapy.

Other Uses

Malignant and premalignant conditions in children have also be treated with oral retinoids. The purpose is to decrease the level and rate of development of cutaneous malignancies.

Xeroderma Pigmentosum

XP is a rare autosomal recessive photosensitive disorder, which results in development of multiple basal cell carcinomas, squamous cell carcinomas and melanomas since an early age. Four of our cases with XP were treated with acitretin with fair results.

Better results have been reported with a combination of three days a week application of imiquimod 5% cream and oral acitretin for four to six weeks (23). Acitretin has also been considered effective as a maintenance therapy for psoriasis in patients developing squamous cell carcinomas as a result of psoralen ultraviolet A (PUVA) therapy (24).

Lymphomatoid Papulosis

Lymphomatoid papulosis (LP) is a chronic recurrent self-healing papulonodular skin eruption with histological features of a CD30+ cutaneous T-cell lymphoma. Lesions frequently become necrotic, ulcerated and leave scars. In our series, a 14 year-old female with LP (CD 30+) was treated with PUVA with good response. As a single maintenance treatment, a daily dose of isotretinoin (20 mg/day) was prescribed. In a nine months follow-up, minimal relapses of the skin lesions have been observed. This is probably the first pediatric patient so treated.

Stern and Lebwohl (25) reported a patient with mycosis fungoides refractory to PUVA that was successfully treated with PUVA plus the new oral retinoid bexarotene.

SIDE EFFECTS OF ORAL RETINOIDS IN PEDIATRIC PATIENTS

Treatment was well tolerated by most patients. Mucocutaneous symptoms disappeared after several months of continuous treatment. None of our patients had serious side effects, transient increase of liver enzymes was the main alteration observed, nonrequiring interruption of therapy. Irreversible side effects did not occur. (26).

The most common side effects associated with short-term treatment were cheilitis, xerosis, and dry nose, which are dose-dependent.

In long-term treatments with etretinate, it is generally accepted that retinoid-induced skeletal abnormalities predominantly affect the spinal column. Exclusively extra-spinal abnormalities have been reported occasionally (27). In our series of 39 patients treated with etretinate, two patients presented fractures as a result of minor trauma and six minor hyperostosis and osteoporosis. In other series of children continuously treated with lower doses of etretinate, no evidence of skeletal toxicity was found (28).

As a result of etretinate withdrawal from the market, all of our patients have been treated with acitretin with similar excellent results and good tolerance. In contrast with etretinate, no bone abnormalities have been observed with acitretin. Lacour et al. (3) reported 22 patients with various inherited disorders of keratinization who have had previous treatment with etretinate and switched to acitretin. Efficacy and tolerability with both retinoids were similar.

Thrombocytopenia may be associated with isotretinoin therapy. The exact mechanism is not clearly understood. Only one of our adolescent patients with acne developed severe thrombocytopenia ($18 \times 10^3/\mu L$ platelets) after one year of treatment with oral isotretinoin, with a total cumulative dose of 150 mg/kg. He had no bleeding or bruises and was otherwise asymptomatic. His blood cell count, transaminases and lipids were within normal limits. Only four previous cases of isotretinoin-associated thrombocytopenia have been reported. In one case platelet counts required approximately two months after discontinuation of isotretinoin to normalize (29). The long recovery process is possibly a direct result of the long elimination half-life of the active metabolites of isotretinoin.

TREATMENT MODALITIES

Continuous Therapy

Initially, all of our patients received uninterrupted oral retinoid therapy. In order to minimize the risk of chronic systemic toxicity, as soon as improvement was observed, usually after one or two months, the daily dose was reduced by half or given intermittently every other day or every two days.

Progressive Therapy

In those conditions with an erythrodermic skin, such as NBIE, EH, HI, PRP or pustular and erythrodermic types of psoriasis, systemic retinoids may exacerbate the inflammatory component. Retinoids are more effective and better tolerated when doses are progressively increased to reach the optimal effective dose that corresponds to the maximum tolerated dose. The dose is adjusted according to tolerance and not to standardize doses.

A new formulation, micronized isotretinoin has a better absorption, and tolerability, and milder side effects (30).

CONCLUSIONS

Severe cornification disorders are fortunately very rare and oral retinoid therapy seldom indicated in children; we have treated 53 patients in 29 years. In some conditions, our experience is limited to one or two patients. We are aware that our experience only represents "a point of view" that should be contrasted with that of other authors.

Why do we include etretinate, which has long ago been retired from the market in most countries? First, because etretinate shares with acitretin practically all its pharmacological characteristics, with the exception of the time it remains in the fat tissue which is much longer for etretinate. Second, because we consider it extremely interesting and useful to study the long-term side effects of etretinate in patients who were treated when they were babies and are now adults, some of them bringing their babies for consultation.

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Retinoids in Cutaneous T-Cell Lymphomas

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INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) are a heterogeneous group of T-cell lymphoproliferative disorders presenting as skin lesions. Mycosis fungoides (MF), characterized by a clonal and epidermotropic skin infiltrate of atypical CD4+CD45RO+ helper/memory T-cells, and its leukemic and erythrodermic variant, the Sézary syndrome (SS) are the most commonly encountered, lymphomatoid papulosis-CD30+ anaplastic T-cell lymphoma spectrum is second, and the least common are the NK-T cell lymphomas, the subcutaneous panniculitic T-cell lymphomas and the otherwise unclassified peripheral T-cell lymphomas (1). MF has an incidence rate of 0.45 per 100,000 person-years based on the most recent epidemiological data from 1992 (2). The clinical evolution of MF may be long and chronic in duration, especially when the involved area is <10% of the total cutaneous surface, and the rate of disease-related death is low overall, except in patients with tumors or lymph node or blood involvement (3). The poor prognosis in late-stage patients results from their immunocompromised status with death from opportunistic infection or sepsis (4,5).

The pathogenesis of MF and other CTCL is unknown, although defects in CD95/Fas Ligand (Fas/FasL) induced apoptosis and activation induced cell death, and inactivation of p16 have recently been proposed (6–8). Tan first proposed that MF may arise from chronic antigen stimulation (9), which could result in the accumulation of skin-homing CD4+ memory T-cells with dysregulated apoptosis in response

to chemokine receptors and growth factors (6,7,10). This is further supported by a histocompatibility locus antigen (HLA) association with DR5 and DQB*03 (11) and DR5 may show increased sensitivity to staphylococcal superantigen stimulation, which is a frequent colonizing organism in erythrodermic patients (12).

Treatment of MF/SS is based on the stage of the patient (13). Skin-directed therapies are used sequentially in stage I patients beginning with topical steroids or retinoids, phototherapy [ultraviolet B (UVB) or psoralen with ultraviolet A (UVA) irradiation, i.e., psoralen plus ultraviolet light A (PUVA)], topical chemotherapy with mechlorethamine (nitrogen mustard) or carmustine (BCNU) and finally total body electron beam radiation. For stage IB, patients who are refractory to topical agents and erythrodermic patients, biological response modifiers (retinoids and cytokines) are combined with skin directed therapy. For patients with nodal involvement, denileukin diftitox or chemotherapy with either single agent, such as gemcitabine, doxil, or methotrexate or combined regimens are often used. Patients with blood involvement are started on multimodality therapy with low doses of retinoids, interferons, and photopheresis. Advanced patients who respond should be considered for allogeneic bone marrow or stem cell transplantation (13).

In this chapter, the authors will focus on the clinical use and future perspectives of retinoids alone and in combinations for treatment of MF/SS.

CLINICAL USE OF RETINOIDS IN MYCOSIS FUNGOIDES/SÉZARY SYNDROME

Systemic Retinoic Acid Receptor Retinoid Therapy

Anecdotal reports of oral retinoids as single agents for treatment of MF appear in the literature as far back as 1983. There were clinical trials using retinoids as monotherapy or in combination that were reported from Europe in the late 1980s and in the United States in the 1990s, as previously reviewed by Zackheim (14).

Three traditional retinoids, isotretinoin, etretinate, and acitretin were the first retinoids tested for activity in CTCL. These retinoic acid receptor (RAR)-selective retinoids can induce IFN-γ production, partially via interleukin-12 (IL-12) induction in CTCL (15). Both IFN-γ and IL-12 are indicative of Th2 or cellular immunity, which is lost as MF advances as a result of Th2 cytokine secretion by MF cells (16,17) to Th1 cells. Retinoids also upregulate Langerhans cell antigen presentation and surface expression of HLA-DR and CD11 that are critically involved in T-cell activation (18). Combinations of IFN and retinoids have also been studied based on this model of the immunity of CTCL, and these are discussed subsequently.

Cumulative data from studies of isotretinoin and etretinate therapy estimate overall response rates (OR) from 43% to 100% with a median response duration ranging from 3 to 15 months (19–29) (Table 1). Etretinate appears to be equal in efficacy to isotretinoin in response and in toxicity. However, etretinate has been replaced by the retinoid, acitretin, with a shorter half-life, reducing prolonged risk

Table 1 Oral Systemic Retinoids in Cutaneous T-Cell Lymphomas

Type of retinoid	Patient no.	Clinical response (overall and complete)	Reference
RAR retinoids			
Isotretinoin	7	43% OR	Warrell et al., 1983 (19)
(13-*cis* RA)	4	100% OR	Kessler et al., 1983 (20)
	20	80% OR, 30% CR	Thomsen et al., 1984 (21)
	1	1 patient CR	Fitzpatrick and Mellette, 1986 (22)
	25	44% OR, 12% CR	Kessler et al., 1987 (23)
	39	59% OR, 21% CR	Molin et al., 1987 (24)
	6	100% OR	Neely et al., 1987 (25)
	1	1 patient CR	Leverkus et al., 2005 (26)
Etretinate	12	58% OR, 5% CR	Claudy et al., 1983 (27)
(Tigason)	1	1 patient CR	Wargon and Downie, 1984 (28)
	29	55% OR, 17% CR	Molin et al., 1987 (24)
Acitretin (Neotigason)	1	1 patient CR	Tousignant et al., 1987 (29)
RXR retinoids			
Bexarotene (Targretin)	Early I, IIA	300 mg/m^2 54% OR 650 mg/m^2 67%	Duvic et al., 2001 (35)
	Late ≥ IIB	300 mg/m^2 45% OR 650 mg/m^2 54%	Duvic et al., 2001 (36)

Abbreviations: CR, complete response; OR, overall response; RAR, retinoic acid receptor; RXR, retinoid X receptor.

of teratogenicity in women (24). Only one case report showed that acitretin is effective as monotherapy for one patient with MF (28). Recently, one case report showed that isotretinoin may be as an additional therapy for follicular MF, particularly for the treatment of residual cysts and comedones (26).

The side effects of traditional RAR retinoids are covered in chapter 14. However, their ability to induce dryness and irritation with topical application may exacerbate MF.

Systemic Retinoid X Receptor-Selective Retinoids, "Rexinoids"

Bexarotene is the first synthetic, highly selective RXR retinoid to be studied in humans (30). It was first approved in the United States in 1999 as an oral treatment for early stage CTCL patients who are not able to tolerate other therapies, or who have refractory or resistant disease, and for refractory, advanced-stage patients (31). In vitro studies from the present authors' laboratory have shown that bexarotene treatment, at clinically relevant concentrations, causes apoptosis of

CTCL cell lines by decreasing the protein levels of its cognate receptors [retinoid X receptor (RXR)-α and RAR-α] and antiapoptotic protein (survivin), activating caspase-3, and cleaving poly (ADP-Ribose) polymerase (PARP) (32). Rook has recently reported that bexarotene may influence adhesion molecules governing T-cell trafficking, such that treatment may shift T-cells from skin to periphery (33). Bexarotene also increases CD8 T-cell count in responders (34).

Two phase II-III, multicenter, open-label trials were conducted on 58 early stage MF (35) and 94 late-stage CTCL patients (36) who had failed or were refractory to other therapies (Table 1). The trial conducted in early stage patients (IA-IIA) evaluated three randomized dose levels (6.5, 300, and 650 mg/m^2/day), but the 6.5 mg arm was abandoned because of a statistically lower response rate (37) and the 650 mg/m^2/day starting dose was reduced to 300 mg/m^2. Similarly, advanced stage patients (IIB-IVB) were started at 650 mg/m^2, which was reduced to 300 mg/m^2. In both studies, the dose-limiting toxicity was severe hypertriglyceridemia leading to pancreatitis, in spite of use of gemfibrizol. In the early stage trial, patients on the 6.5, 300, and 650 mg/m^2/day doses achieved OR of 20%, 54%, and 67%, respectively. In heavily pretreated and advanced patients, doses of 300 or 650 mg/m^2/day produced ORs of 45%, and 55%, respectively. A dose of 300 mg/m^2/day was considered as providing a balance between response and safety issues, with an OR of 48% in the combined group of all patients. Although only 4% of patients had complete responses (CR), 23% had a 75% to 100% improvement in disease representing significant benefit. Furthermore, several patients achieving CRs had durability of up to five years and were able to taper their doses to 75 to 150 mg per day (37).

Oral bexarotene showed therapeutic efficacy even in the late stages of disease, and exhibited activity in tumor and lymph node reduction, although not in visceral disease in a small group of patients. The response rate was identical among patients who were retinoid naïve or who had received prior RAR therapy (36). Bexarotene was also effective in treating some patients with large-cell transformation in tumors, and in controlling, stabilizing or resolving the erythroderma of Sézary and erythrodermic MF patients, including some long-standing complete responders.

The side effects of bexarotene differ from RAR retinoids (Chapter 14). They are most commonly limited to laboratory abnormalities, are usually dose-dependent, and can be often managed without discontinuing the treatment. The most common significant side effects experienced were hypertriglyceridemia (82%), hypercholesterolemia (30%), central hypothyroidism (29%), and leukopenia (11%) (35,36). Hypertriglyceridemia can be managed successfully using fenofibrate alone or in combination with HMG-coA reductase inhibitors or "statins" (37). Gemfibrozil, while effective for isotretinoin-induced hypertriglyceridemia, was associated with higher bexarotene levels, hypertriglyceridemia, and increased risk for pancreatitis. In managing patients on bexarotene, the response rates are higher when the triglycerides are adequately managed (37). This may require combining of two lipid-lowering agents, such as Tricor® (Abbott Laboratories, North Chicago, Illinois) at 160 mg and Lipitor® (Warner-Lambert

Export Ltd., Dublin, Ireland) up to 80 mg daily as well as keeping the free thyroxine level in the normal range. With this combination, there is a remote possibility of rhabdomyalysis as a potential consequence, so monitoring symptoms and CPK levels is advised (37). We have therefore recommended starting bexarotene at a lower dose (75–150 mg) and checking weekly fasting triglyceride levels, as well as starting lipid-lowering agents one week prior to starting bexarotene. Generally, triglyceride levels up to 400 mg/dL are well tolerated, and pancreatitis does not develop until the levels are over 1000 mg/dL. Patients with pre-existing hyperlipidemia or diabetes appear to be most susceptible.

The true incidence of bexarotene-induced central hypothyroidism was underestimated in the clinical trials. It results from suppressed thyrotropin secretion, and is manifested by a very low thyroid stimulating hormone (TSH) value throughout therapy that subsequently reduces the free thyroxine levels (38). In our experience, all patients taking bexarotene will have decreased TSH levels, but whether they become hypothyroid with symptoms of fatigue depends on the dose and duration of bexarotene. We have found that fatigue and hypertriglyceridemia improve on thyroid replacement to give normal free T4 values (37). The hypothyroid condition is reversible upon discontinuation of therapy. Thyroid supplementation requires from 0.025 to 0.250 mg of synthroid, but results in more rapid lipid clearance and an easier time controlling the triglyceride elevations (37,38).

Recent data retrospectively comparing all-*trans*-retinoic acid (ATRA) to bexarotene suggests that ATRA may be as effective as other retinoids in MF, with a slightly different toxicity profile consisting on more mucocutaneous side effects and lower frequency of laboratory abnormalities (39).

Topical Retinoid Therapy

Topical bexarotene gel was evaluated in a phase I-II dose-ranging study of concentrations of 0.01% to 1% (40), and in a placebo-controlled, phase III study (41) (Table 2). The phase I-II trial involving 67 stage IA-IIA patients demonstrated CR in 21% of subjects and partial responses (PR) in 42% of patients for an OR of 63% (42). The median time to response was approximately 20 weeks, and a median duration of response was 99 weeks. Patients who had not previously been treated with any agent had a higher response rate of 76%. Topical bexarotene displayed a dose-response effect with greater efficacy at higher concentrations and frequencies of application. In the phase III trial of refractory stage IA, IB, and IIA patients, bexarotene gel 1% was applied topically every other day with increasing frequency up to four times a day as tolerated, and demonstrated a 44% OR with an 8% CR rate (41).

We have used bexarotene gel as adjuvant therapy for the treatment of refractory MF skin tumors (42). Others have found bexarotene gel is effective in combination with intralesional interferon (IFN) for MF tumors of the scalp (43). Bexarotene gel also reverses alopecia mucinosis (44). Bexarotene gel as well as oral capsules may shorten the duration of lymphomatoid papulosus (45).

Table 2 Topical Retinoids in Cutaneous T-Cell Lymphomas

Retinoid	Patient no.	Clinical response (overall and complete)	Reference
Bexarotene gel (1%)	50	44% OR, 8% CR	Heald et al., 2000 (41)
(Targretin®)	66	63% OR, 21% CR	Breneman et al., 2002 (40)
Tazarotene gel (0.1%) (Tazorac®)	20	58% OR, 35% CR	Apisarnthanarax et al., 2004 (49)

Abbreviations: CR, complete response; OR, overall response.

Similar to other topical retinoids, bexarotene induces local irritation in approximately 70% of treated patients; most cases are mild to moderate in severity (40,41). Since the redness from irritation induced by the retinoid may interfere with determination of the clinical response, it may be necessary to stop drug application to adequately assess the response. Irritation is easily managed by reducing the frequency of application, or by adding or alternating with a low- to mid-potency topical corticosteroid. Since topical corticosteroids are the most widely used treatment for early MF (46), the combination of topical retinoid/corticosteroid treatment should allow steroid sparing, decrease atrophy, as well as counteracting the irritancy of using topical retinoids.

The approval of the RAR-β/γ selective synthetic retinoid, tazarotene, as a gel and a cream for psoriasis (47) and acne (48) paved the way for testing topical retinoids for activity in MF lesions. We designed and conducted a pilot study of 20 stage IA and IB MF patients with refractory MF lesions who applied tazarotene gel 0.1% for six months (49). The response rate in these lesions was similar to bexarotene gel, achieving an OR of 58% with 35% CR as adjuvant therapy in patients with refractory disease (Table 2). Clinical response to tazarotene gel 0.1% is associated with a decrease of CD45RO+ memory T cells and an increase of CD8+ T cells in skin lesions (49). Tazarotene also caused mild to moderate local irritation in 84% of patients, which is slightly higher than that reported with topical bexarotene gel for MF lesions. Although tazarotene may be an alternative to topical bexarotene gel, a side-to-side comparison study would be helpful to evaluate durability of response in lesions and to compare irritation profiles in MF patients. Other topical retinoids may be useful in MF lesions, but have not been systematically tested.

Retinoid Combination Therapies

To improve overall response, length of remission, and side effects of retinoids in CTCL, they are often used in combination with other active therapies including PUVA, extracorporeal photopheresis (ECP), and other biological response modifiers such as IFN-α with higher response rate from 58% to 100% (50–62)

Table 3 Retinoid Combination Therapies in Cutaneous T-Cell Lymphomas

Regimen	Patient no.	Clinical response (overall and complete)	Reference
Isotretinoin			
+ PUVA	69	73% OR	Thomsen et al., 1989 (50)
+ IFN-α	7	57% OR, 29% CR	Knobler et al., 1991 (51)
+ IFN-α + TSEB/CT	28	82% OR	Duvic et al., 1996 (52)
Etretinate			
+ PUVA	16	92% OR	Mahrle and Thiele, 1987 (53)
	56	68% OR	Serri et al., 1990 (54)
+ IFN-α	7	86% OR, 14% CR	Thestrup-Pedersen, 1988 (55)
	13	77% OR, 54% CR	Altomare et al., 1993 (56)
	1	1 patient PR	Torii et al., 1994 (57)
+ TSEB	23	100% OR, 70% CR	Jones et al., 1992 (58)
+ CT	12	58% OR, 25 % CR	Molin et al., 1987 (59)
+ CT + NM	10	100% OR, 80% CR	Zachariae et al., 1982 (60)
+ ECP	5	100% OR	Lim et al., 1995 (61)
Acitretin			
+ PUVA	98	38% CR	Stadler et al., 1998 (62)
Bexarotene			
+ PUVA	8	100% OR, 63% CR	Fiza Singh et al., 2004 (63)
	1	1 patient CR	Huber et al., 2004 (64)
+ IFN-α	3	3 patients CR	McGinnis et al., 2004 (65)
+ PUVA/IFN-α	2	1 patient PR	Talpur, et al., 2002 (37)
+ ECP	8	75% OR, 13 % CR	Talpur, et al., 2002 (37)
+ ECP/IFN-α	4	2 patients PR	Talpur, et al., 2002 (37)
+ ECP/IFN-α/PUVA	1	1 patient PR	Talpur, et al., 2002 (37)
+ IFN-α/PUVA/NM	1	1 patient PR	Talpur, et al., 2002 (37)
+ LLA	10	70% OR	Talpur, et al., 2002 (37)
+ Ontak	9	75% OR	Divenuti and Foss, 2001 (67)

Abbreviations: CR, complete response; CT, chemotherapy; ECP, extracorporeal photopheresis; LLA, lipid lowering agent; NM, nitrogen mustard; OR, overall response rate; PR, partial response; TSEB, total body skin electron beam.

(Table 3). The benefit of combining IFN-α with retinoids while attractive, may lead to leukopenia or an increased risk of chemical hepatitis. Accutane at 1 mg/kg and 3 million units of IFN-α were compatible as an induction therapy for a combined modality therapy regimen that also included total-body electron beam radiation and, in advanced patients, six courses of combined chemotherapy (52).

Although the response rates of PUVA plus retinoids do not differ from PUVA alone (CR = 73%), RAR selective retinoids have the advantage of allowing lowered PUVA dosage requirements and producing longer remissions with

retinoid maintenance therapy (50,53,54,62). Systemic chemotherapy plus retinoids has also been evaluated, although no significant differences in response rate could be determined when compared to systemic chemotherapy alone (62).

Bexarotene combined with other active CTCL therapies including IFN-α, PUVA, and ECP also achieved higher clinical response rate from 50% to 100% in patients with advanced CTCL, without unacceptable side effects (37,63–65) (Table 3). The combination therapy of bexarotene reduced the number of abnormal CD4+ cells in the blood and down-regulated surrogate markers of disease such as soluble IL-2 receptors and lactate dehydrogenase (LDH) (37). However, our phase II trial of bexarotene-combined interferon showed that the major response rate for their combination is similar to that for bexarotene alone and they may not be synergistic (66).

An interesting combination of bexarotene with denileukin diftitox (Ontak®, Ligand Pharmaceuticals, San Diego, California) has reported to give a 75% OR in nine patients with CTCL in a pilot phase I trial (67). In vitro, bexarotene induces expression of the high-affinity IL-2 receptor on T-cells, suggesting a rationale for giving bexarotene prior to denileukin diftitox (Ontak) administration to increase target cells (68).

Future evaluations of bexarotene should focus on determining the optimal efficacy of the drug in combination with existing therapeutic modalities as maintenance therapy following irradiation or chemotherapy, and in combination with other newly developed classes of therapy currently under investigation.

STRATEGIES TO IMPROVE RETINOID EFFICACY

Although clinical studies have demonstrated the efficacy of retinoids for MF/SS, disease relapses are common, and side-effects have limited their long-term use. Understanding that retinoids work through specific nuclear receptors to modulate gene expression should lead to the identification or synthesis of novel, receptor-selective agonists that are more effective in specific diseases with less toxicity in the future (69). For example bexarotene, although an RXR-selective retinoid, has RAR activity at higher concentrations, but nonetheless, appears to exhibit a better therapeutic index than ATRA (70).

Secondly, a combination of various receptor-selective retinoids may be better to induce apoptosis and differentiation, and inhibit proliferation of tumor cells. For example, treatment of HL-60 cells with a combination of 4-(E-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-nephthalenyl)-1-(propenyl) benzoic acid (TTNPB), a RAR-selective retinoid, with LG100268, a RXR-selective retinoid, is synergistic in inducing cellular differentiation followed by apoptosis, which is not seen with either alone (71). Our in vitro studies have similarly shown that bexarotene, only at higher concentrations (>1 μM), induces apoptosis of CTCL cell lines. Thus, RAR activity seen at higher bexarotene concentrations may be required for apoptosis in CTCL cells (32). Retinoids antagonists have been reported that have the ability to inhibit unwanted side effects (72).

Retinoids may be combined with the ligands of other nuclear receptors. The action of bexarotene and a PPAR-γ ligand (CDDO) was superior to that of either agent alone in inducing apoptosis of CTCL cells (73). Retinoids plus vitamin D may also be effective in combination.

Drug metabolism inhibitors such as liarozole, fluconazole, and ketoconazole may be used to inhibit the rapid metabolism of retinoids that leads to insufficient levels in target tissues. An in vitro study showed that liarozole greatly potentiated the ability of low concentrations of RA to inhibit carcinogen-induced neoplastic transformation (74). This is a complex issue since a phase I clinical trial to evaluate the combination of ATRA and ketoconazole in adults with solid tumors did not show enhanced plasma ATRA levels or objective tumor responses (75). Encapsulating retinoids in liposomes can be used to slow down their metabolism, and to enhance their stability and efficacy with lower toxicity (76). Ganglioside D2 (GD2)-targeted immunoliposome with trapped *N*-(4-hydroxyphenyl) retinamide (4-HPR) was shown to enhance target selectivity for neuro-ectodermal tumors and provide another strategy for site-selective delivery of retinoids (77).

SUMMARY

Although retinoids have long been used alone or in combination with other agents for the treatment of CTCL, the first rexinoid for CTCL was only recently approved. Use of retinoids in future long-term clinical trials and their eventual application in CTCL regiments will require strategies to decrease the side-effects of existing retinoids, or the identification of more effective retinoids with few or no side-effects. The structure-activity relationship studies of RA and its first-generation analogs have resulted in the recent discovery of receptor-subtype-selective retinoids, such as bexarotene, with a better therapeutic index. Combination with other agents may also enhance the clinical efficacy of retinoids. More mechanistic studies are needed to identify new target genes and clarify the molecular details of retinoid action that could be used as surrogate end-points to develop novel function-selective retinoids.

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11

Retinoid Therapy and Autoimmune Skin Disease

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INTRODUCTION

Autoimmunity, defined as immunity against self, is a factor in the etiopathogenesis of a wide variety of skin disorders. While many diseases that have an autoimmune component, such as psoriasis, are reviewed in other chapters of this book, the following chapter will focus on lichen planus, lichen sclerosus et atrophicus (LSA) as well as lupus erythematosus. Retinoids have shown some beneficial effects in these conditions and may be a reasonable therapeutic option in the right circumstance. Other autoimmune disorders, such as autoimmune bullous diseases, have not been reported to respond to retinoids in any large, controlled trials.

LICHEN PLANUS

Lichen planus is a cutaneous disorder characterized by multiple polygonal violaceus papules on the extremities, trunk and mucous membranes. Although the exact etiopathogenesis is unknown, the lichenoid reaction pattern seen in skin biopsies of lichen planus, with its intense, band-like infiltrate of lymphocytes and deposition of immunoglobulin in the papillary dermis, suggests a cutaneous hypersensitivity reaction in which autoimmunity may be responsible for continued disease (1). While corticosteroids and phototherapy are often used to treat lichen

planus, many patients are resistant to these treatments and need alternative therapies, such as retinoids.

A double-blind, placebo-controlled study in 65 patients with lichen planus was performed with oral acitretin (2). The patients were treated with acitretin 30 mg per day or placebo for eight weeks. After the eight week double-blind treatment period, all patients were treated during an eight week open treatment phase. Improvement was determined by assessing the extent of disease and the intensity of pruritus, papules, and erythema. Sixty four percent of the acitretin group had remission or marked improvement at the end of the eight week treatment phase versus 13% of the placebo group. A further improvement of the acitretin group was obtained during the eight week open phase of the trial giving a total of 83% of patients with remission or marked improvement. Those treated with placebo during the double-blind phase showed a good response to acitretin during the open label phase, with 74% showing remission or marked improvement. Even mucous membrane lesions showed marked improvement in 74% of patients receiving acitretin. Adverse effects, including dry lips, mouth, nose, eyes, and skin were common, occurring in 88% of acitretin-treated patients versus 52% of patients on placebo. Scaling of the palms and soles occurred in 44% and hair loss in 16% of these patients, but none withdrew due to side effects.

Other retinoids have been used for lichen planus, but there are no controlled studies available. A report of oral tretinoin, 10 to 30 mg/day in 18 patients treated up to 19 months resulted in complete remission in 72% and marked improvement in 22% (3). Cheilitis was seen in four patients, otherwise side effects were rare.

Oral lichen planus is one of the most common diseases of the mucous membrane seen in dermatology and oral medicine clinics. A systematic review of 11 randomized controlled trials in oral lichen planus showed that there is weak evidence for the effectiveness of any modality due to the lack of well-controlled studies (4). A report of six patients with oral erosive lichen planus treated for eight weeks with isotretinoin up to 1 mg/kg/day showed that five of six patients had slight subjective and objective improvement. Because of the minimal improvement and adverse side effects, no patient desired retreatment with isotretinoin (5). A more recent study using topical tazarotene gel 0.1% twice daily for oral lichen planus showed a significant reduction in active lesions compared to controls (6). Transient burning and taste abnormalities were the only side effects.

Overall, there is good evidence for the use of acitretin in the treatment of lichen planus, while such evidence for other retinoids is much weaker. Side effects from retinoid treatment must be balanced with any potential benefit.

LICHEN SCLEROSUS ET ATROPHICUS

LSA is characterized by multiple ivory-white macules and plaques, which are atrophic in appearance. This condition often affects the vulva in women and causes multiple symptoms, including burning, pruritus, and dyspareunia. Similar to lichen planus, the etiology is unknown. Potent topical steroids and testosterone creams have been used effectively for LSA, and are the mainstay of treatment;

however, some patients require more aggressive therapy due to resistant disease. A double-blind, placebo-controlled study was performed in 78 patients with LSA of the vulva, in which patients received acitretin 30 mg/day or placebo for 16 weeks (7). Fourteen of the 22 patients in the acitretin group analyzed for efficacy responded versus 6 of 24 in the placebo group. Most patients were partially or completely satisfied with the treatment. Thus, acitretin may be a reasonable option in a patient with vulvar LSA.

LUPUS ERYTHEMATOSUS

Cutaneous lesions in patients with lupus may be generally divided into two subsets: lupus specific skin disease and lupus nonspecific skin disease. Nonspecific skin findings in lupus may be seen in association with other disease processes and include such entities as vasculitis and oral ulceration. In contrast, the lupus-specific skin diseases, including acute cutaneous lupus erythematosus (ACLE), subacute cutaneous lupus erythematosus (SCLE), and discoid lupus erythematosus (DLE) are seen exclusively in patients with a lupus diathesis, and share specific histopathologic features (8). It is this subset of patients who may respond to either oral or topical treatment with retinoids.

Newton, Jorizzo et al. were among the first to suggest a role for oral retinoids in the treatment of cutaneous lupus. In an open label study, they reported that eight patients with discoid or subacute cutaneous lupus experienced an excellent clinical response without significant side effects on 80 mg/day of oral isotretinoin for 16 weeks (9). A similar study documented successful treatment in six patients with recalcitrant cutaneous lupus. The authors observed rapid clearing of skin lesions on 1 mg/kg/day of isotretinoin. They were also struck by rapid recurrence of the lesions upon discontinuation of the medication (10).

Ruzicka et al. corroborated this finding, describing successful treatment of cutaneous lupus with acitretin. They noted that 15 of 20 patients with discoid lupus or subacute cutaneous lupus had either total clearing or marked reduction of all lesions (11). The same group subsequently performed a randomized double-blind study comparing the efficacy of acitretin (50 mg daily) with hydroxychloroquine (400 mg daily), evaluating improvement of erythema, infiltration, and scaling of lesions. Overall improvement with acitretin was roughly equivalent in terms of efficacy when compared with hydroxychloroquine, but was more toxic (12). No placebo arm was used in this study, however.

Hypertrophic discoid lupus, a subtype of discoid lupus that occurs on the sun-exposed extremities and has a verrucous, hyperplastic surface, has been reported in particular to respond to oral retinoids. Three separate case reports document clearing of recalcitrant lesions in a relatively short period of time (13–15). Callen, in a recent review of treatment for cutaneous lupus, notes that isotretinoin and acitretin "are particularly helpful in patients with hypertrophic skin lesions" (16). The effectiveness of oral retinoids in hypertrophic DLE lesions may in part be related to a combined antiproliferative and anti-inflammatory mechanisms of action. Another case report documents clearing of recalcitrant

localized discoid lupus with topical tazarotene (17). The authors note that they elected to treat their patient with this treatment because hypertrophic DLE, similar to psoriasis, is a disease that involves inflammation as well as abnormal proliferation of keratinocytes.

POTENTIAL MECHANISMS OF ACTION OF RETINOIDS IN AUTOIMMUNE SKIN DISEASE

Although the precise mechanism of action in the treatment of autoimmune inflammatory skin diseases is unclear, it is likely that retinoids exert their effect in lichen planus and lupus skin disease via several different pathways. Retinoid receptors' involvement in the control of inflammation is largely mediated through their ability to down-regulate (transrepress) expression of proinflammatory transcription factors, especially, activator protein-1 (Ap-1), a protein heterodimer comprising c-jun and c-fos. Examples of genes regulated by AP-1 are matrix metalloproteinases and collagenases, which have been implicated in a variety of different rheumatic diseases. This is similar to glucocorticoids, which we now know have broad-reaching anti-inflammatory effects mainly because of their ability to down-regulate inflammatory gene expression of the transcription factors nuclear factor-κB (NF-κB), signal transducers and activators of transcription (STATs), and nuclear factor of activated T cells (NFAT). Other AP-1 dependent genes include transglutaminase I, involucrin, and keratin 6, which are involved in hyperproliferative conditions including psoriasis. The observed efficacy of retinoids for the hypertrophic forms of lichen planus and lupus may be due to such an effect (18).

Apoptosis of keratinocytes, lymphocytes, and fibroblasts, and/or ineffective removal of apoptotic debris is likely a key inciting event in the development of autoreactivity in autoimmune processes, such as lupus and lichen planus. Retinoids are known to modulate immune responses via inhibition of apoptosis via up regulation of Fas ligand in skin fibroblasts. In one study, etretinate was given to MRL/lpr mice, which are prone to develop lupus-like skin lesions and are characterized by aberrant apoptosis due to defects in Fas antigens. The treated mice had significantly decreased apoptotic cells in the dermis compared to controls. The authors concluded that etretinate suppressed the appearance of skin lesion by inducting apoptosis and perhaps regulation of cytokine expression (19).

CONCLUSIONS

Oral retinoids may be effective second or third line treatment in recalcitrant autoimmune/inflammatory conditions, such as lichen planus, lichen sclerosis, and lupus-specific skin disease. There are potential mechanistic explanations for their observed efficacy. Avoidance of toxicities, especially those related to teratogenicity must be taken when treating these diseases, which are common in women of child-bearing potential.

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Retinoids in the Prevention and Treatment of Skin Cancer

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INTRODUCTION

Skin cancer is the most common malignancy in the United States, with an estimated one million new cases diagnosed annually. It is estimated that one in six Americans will develop a skin cancer at some time in their lives (1). Among skin cancers, basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) are by far the most frequent types, and the incidence of these cancers continues to rise (2). Traditional treatments of surgery, cryotherapy and topical medical therapies with flurouracil and imiquimod are used to manage patients with few lesions. However, genetic conditions predisposing patients to high risk for multiple and aggressive nonmelanoma skin cancer (NMSC) are well known, and, with increasing patients immune-suppressed in the course of organ transplant treatment, alternative approaches to treating these cancers are being pursued. Although retinoids are well known to dermatologists for their significant efficacy in the treatment of disorders of keratinization, one of the most exciting effects of retinoids is in skin and mucosal cancer prevention and treatment.

Peck (3) who first used retinoids for acne first suggested that the preventive and therapeutic effects of retinoids for actinic keratoses and skin cancer may prove to be the most significant application of these drugs. In the first human study, published in the *New England Journal of Medicine* in 1986, Hong et al. (4) in a

randomized controlled study of 44 patients, concluded that 13-*cis*-retinoic acid, even in short-term use, appeared to be an effective treatment of oral leukoplakia with an acceptable level of toxicity. Predating this human clinical trial, multiple articles of clinical observations, murine studies and theoretical effects of retinoids in cancer appeared. Incongruous results characterized the early studies. Prutkin (5) was the first to report on the role of tretinoin in cutaneous tumors noting that topical tretinoin enhanced development of keratoacanthomas induced by dimethylbenzanthracene in rabbit ears, but also induced regression of these same tumors when 5-fluorouracil was combined with tretinoin. Other studies showed retinoids enhanced as well as inhibited chemically induced experimental cancers (6,7). Many of the studies that showed enhancement of tumors by retinoids involved very high doses (8).

Most cancers occurring in human skin are induced by UVB radiation. Epstein (9) showed acceleration of cutaneous carcinogenesis in hairless mice treated with 0.3% tretinoin three times weekly after UVB radiation over 10 months. Forbes et al. (10) accelerated development of cutaneous tumors induced by UVB in hairless mice with applications of 0.01% tretinoin. Despite these and other similar findings, other studies showing the opposite effect started to be reported in the early 1980s. Epstein and Grekin, in 1981 (11), published the first study showing inhibition of ultraviolet skin carcinogenesis by topical all-*trans*-retinoic acid. Hairless mice were divided into four groups. Each received standardized UVB three times a week. After each exposure, group I was treated with 0.05% tretinoin, group II with 0.025%, group III with 0.005%, and control group IV the vehicle alone. No significant difference in tumor incidence or growth occurred in groups II or III, but tumors developed significantly more slowly and tumor incidence was significantly less in group I than in controls. Kligman and Kligman (12) subsequently compared the effects of applying 0.001% tretinoin concomitantly with UVB radiation and both 0.01% and 0.001% tretinoin started after 30 weeks of UVB to hairless mice. Tretinoin appeared to stimulate the regression of UVB-induced tumors when applied after discontinuing the radiation. In January 1983, Conner et al. (13) published a murine study showing that UVB followed by one application of tretinoin did not significantly decrease tumor incidence but did reduce tumor growth in their mouse system. In contrast, five applications of tretinoin over a four hour period after UVB radiation did inhibit tumor formation resulting in fewer mice with skin tumors, fewer tumors per mouse, smaller tumor diameters and slower growing tumors than control groups. They hypothesized inhibition of ornithine decarboxylase induction was significant to the mechanism of action. In September 1983, Kingston et al. (14) published an early clinical observation that the oral retinoid RO13-6298 caused a statistically significant reduction in the mean number and area of actinic keratoses and/or SCCs in 16 patients.

In a supplement to the *Journal of the American Academy of Dermatology* devoted to retinoids and cancer treatment published in 1986, Epstein (15) noted retinoids were of interest for prevention and treatment of skin and other cancers.

Meyskens et al. in 1986 (16) published a pilot trial of topical tretinoin in dysplastic nevus syndrome, which resulted in regression of some treated lesions to benign nevi showing minimal or no dysplasia. Three patients with at least three biopsy proven dysplastic nevi each, treated the individual residual lesions daily for 10 to 12 weeks with 0.05% tretinoin cream occluded with Blenderm tape. Post-treatment biopsies in two of the patients showed benign compound nevi without dysplasia, and the third patient's were all read as benign compound nevi with minimal dysplastic change. Four months later, repeat biopsies of the first two patients remained unchanged; follow up biopsies on the third patient were not obtained.

Widespread interest and attention to retinoids has continued throughout the 1990s and in the 21st century. Spectacular responses to retinoids in acute promyelocytic leukemia (17), though thought to probably not be replicated as dramatically in other cancers, stimulated research and study of retinoids in other cancers. Meyskens in 1982 (18), reported on investigations into topical retinoid use in secondary prevention of cervical dysplasia; adjuvant treatment of resected high-risk stage I and II malignant melanoma with bacilli Calmette Guerin plus or minus oral vitamin A; topical vitamin A acid therapy for cutaneous metastatic melanoma; and oral isotretinoin as an anticancer agent. Studies of retinoids used in the prevention and treatment of other cancers have followed. Retinoids have been effective in superficial bladder cancer, second primary tumors associated with head and neck and lung cancers, and had a possible protective effect against ovarian cancer (19). Others hypothesized retinoids would be effective for neuroblastoma and breast cancer. The development of receptor-selective retinoids and selective inhibitors of retinoid metabolism were hypothesized to lead to their use in both the chemoprevention and treatment of cancers (20). The chemoprevention of cancer with retinoids has been the subject of a large number of translational research studies.

RETINOIDS IN PATIENTS AT HIGH RISK FOR SKIN AND MUCOSAL CANCERS

Research in the field is exploding with studies on the mechanisms and effect of retinoids on cancer with many, albeit far from congruent, results. A number of predisposing conditions are known to increase the incidence of NMSC. In these patients, clinical management requires aggressive therapy resulting in reports of the use of retinoid therapy. Reviewing these high-risk situations and studies provides an excellent introduction to the effect of retinoids on skin cancers.

Increased sun exposure, sunburns, and fair complexion are all undisputed contributors to NMSC. In addition, other conditions give patients a predisposition to increased NMSC. Patients with recessive dystrophic epidermolysis bullosa are at high risk of developing SCC on or after mid adolescence, and most patients die of metastatic SCC within five years of diagnosis of their first SCC. Several retinoid studies, not reviewed here, have shown safety and some efficacy in this setting (21).

Patients with psoriasis treated with oral psoralen and UVA have an increased risk of SCC development. In one nested cohort study oral retinoid use was associated with a 30% reduction in SCC incidence in these patients (22).

High skin cancer risk occurs in the autosomal recessive inherited disorder, xeroderma pigmentosum, caused by mutations in DNA repair enzymes leading to abnormal repair of UV-induced DNA damage, resulting in early onset of actinic keratoses and all major types of skin cancers (23) and mutations in the sonic hedgehog pathway crucial in the development of BCCs (24). Kraemer et al. in the *New England Journal of Medicine* in 1988 (25) published a three year controlled prospective study of oral isotretinoin in five patients with xeroderma pigmentosum. Patients received 2 mg/kg/day for two years and were followed after treatment cessation for another year. In the two years before treatment, patients had a total of 121 tumors (mean 24). During treatment there were 25 tumors (mean 5). After treatment, tumor frequency increased a mean of 8.5 fold over the frequency during treatment. ($P = 0.007$), showing high-dose oral isotretinoin was effective in chemoprophylaxis of skin cancers in patients with xeroderma pigmentosum. Basal cell nevus syndrome, caused by mutations in the sonic hedge hog pathway affecting the tumor suppressor gene PTCH (26), located on chromosome 9q22.3-q31, has an autosomal dominant inheritance with complete penetrance but variable expressivity and is characterized by numerous nevoid BCCs of the skin appearing at a young age. Case reports by Peck in 1987 (27), Hodak et al. in 1987 (28), and Goldberg et al. in 1989 (29), all showed effectiveness of isotretinoin or etretinate in basal cell nevus syndrome on existing BCCs and a prophylactic effect in inhibiting new tumor formation during treatment.

Retinoids in Skin Cancer Prevention in Organ Transplant Recipients

Clinical reports, studies and reviews of the use of retinoids in treatment and prevention of NMSC now span three decades and are mainly limited to studies of organ transplant recipients (OTRs). These patients are at a significantly increased risk of developing skin cancer compared to immunocompetent individuals (30,31) and have approximately two times more SCC's than BCC's compared to nonimmunocompromised patients in whom BCC is four times more common than SCC (32,33). Increased incidence has been noted in liver transplant recipients (34).

Ong et al. (35) reported cardiac transplant patients had an incidence of skin cancer of 31% at 5 years and 43% at 10 years with an SCC:BCC ratio of 3:1. They also showed Caucasian origin, increasing age at transplantation, and duration of follow-up was significantly associated with skin cancer. Long-term immunosuppression medication is thought to play a role in skin cancer development, either by decreased immune surveillance and reduced eradication of precancers or via direct carcinogenesis (36–38). The duration and intensity of immunosuppression appear to increase the risk of developing skin cancer in these patients (39), with increasing levels and duration of immunosuppression correlating with a higher frequency of skin cancers, with the prevalence of NMSC reaching 7% after 1 year,

45% after 11 years, and up to 75% after 20 years (31,40,41). Cessation of immunosuppression results in a reduction in tumor incidence (42). Skin cancers can also appear at a younger age often appearing three to five years after transplantation (43,44).

Skin cancers in OTRs tend to be more numerous and more aggressive than those in the general population, with a higher likelihood of metastasis and mortality in association with cutaneous neoplasms (35,45,46). The rate of metastasis of SCCs in OTRs ranges from 6% to 9%, resulting in a disease specific survival of approximately 50% in those patients with metastasis (47–49). Recurrence rate is also high, occurring in 14% of renal transplant patients following initial therapy.

Mortality of SCC can be significant in transplant patients, with 5.2% of OTRs in one series dying from skin cancer, 63% of which were SCC (50). In a group of Australian heart transplant recipients 27% of deaths occurring four or more years after transplantation were due to skin cancer (35).

Human papilloma virus (HPV) may also play a role in the development of SCC, especially OTRs in whom there is an increased prevalence of HPVs in their SCCs (51). Patients with epidermodysplasia verruciformis (EV) also have an abnormal genetically determined susceptibility to widespread and persistent HPV skin infection and malignant changes accentuated on sun-exposed areas. Retinoids have been reported to be effective in the treatment of EV related SCC and Bowen's disease. Gubinelli et al. (52) reported a 43-year-old female with EV who developed multiple SCC in the oral and genital mucosae in the four years prior to their treatment. Her wart and cancer lesions harbored HPV24 and a novel HPV sequence. Acitretin (0.2 mg/kg/day) and peginterferon alfa-2b subcutaneously (1 mg/kg/wk) given for one year markedly decreased her verrucous lesions within three months, and no mucosal cancer occurred. After one year, interferon was discontinued, but a Bowen's disease lesion developed in the perianal region two months later. Acitretin was increased with no clinical signs of cutaneous or mucosal carcinoma during the six-month follow-up.

At least 12 studies of the effects of systemic retinoids on NMSC in 186 OTRs have been published. Only three of these have been randomized, controlled trials; the majority are case series and case reports.

In 1995, Bavinck et al. (53) reported a randomized, double-blind, placebo-controlled trial to test the effect of acitretin 30 mg/day on skin cancer prevention in renal transplant patients. Forty-four renal transplant patients with at least 10 hyperkeratotic lesions, consisting of warts or solar keratoses, of the forearms and hands were enrolled and were randomized, 21 to acitretin and 23 to placebo. Thirty-one patients finished the six-month treatment period. There were no significant differences between the two groups in history of skin cancers, sex or age of the patients, time after transplantation, number of keratotic lesions, or immunosuppressive therapy. During the six month treatment period, 9 of 19 patients in the placebo group developed a total of 18 new skin cancers (47%): 15 SCC, one Bowen's disease, and two BCC. In the acitretin group, 2 of 19 patients developed two new SCC (11%). The most profound effect was in the group of patients with

a history of skin cancer. Of 10 patients with a history of skin cancer who were treated with placebo, seven developed new skin cancers, compared with one of nine in the acitretin group (P = 0.009). After the six-month double-blind treatment period, patients were monitored for an additional six months. Patients tended to relapse when treatment was discontinued. No significant side effects on the grafted kidney occurred at this dosage. Mild side effects of retinoid treatment occurred including mucocutaneous dryness and hair loss. Three had increased serum cholesterol or triglycerides above pretreatment levels, and none had a change in liver function. The authors concluded acitretin 30 mg/kg/day over six months had significant effect in prevention of SCC and keratotic lesion development in renal transplant recipients. This effect was most pronounced in those who had a history of previous skin cancers.

In 2002, George et al. (54) reported a prospective, two-year open randomized crossover trial evaluating the efficacy of acitretin for chemoprevention of SCC and BCC in renal allograft recipients. Twenty-three patients with a history of previous NMSC were enrolled and randomly allocated to two groups and crossed over at the end of one year. Patients allocated to the active treatment group received acitretin, 25 mg orally, once daily with food. The dose was decreased to 25 mg on alternate days if they developed adverse side effects, but was increased to 50 mg daily at the end of three months if there were no side effects. Demographics in the two groups were comparable. A total of 109 NMSC were diagnosed in these patients after transplantation prior to trial entry. Thirteen patients (56.6%) had more than three NMSC and 10 (43.5%) had one to three NMSC in the previous five years. Eighty-eight were SCC and 21 were BCC, ratio 4:1. Nineteen patients (82.6%) had more than 10 actinic keratoses at the time of entry into the study. The incidence of SCC was significantly lower in patients receiving acitretin. Patients who had fewer than 10 actinic keratoses at the start of treatment had complete regression of lesions. Side effects were a major limiting factor in the study, including rash, elevated serum cholesterol, nausea, headache and epistaxis. Mucocutaneous side effects were well tolerated but musculoskeletal symptoms and headaches were not and accounted for early withdrawals. There were no adverse effects on renal function. One patient developed an excessive number of SCC after acitretin was ceased; eight had no tumors in control or follow-up period, suggesting rebound increase in skin cancers is a concern but is variable. The authors concluded acitretin is effective in chemoprevention in renal transplant patients, and thought long-term treatment with the drug will help in reducing the morbidity associated with NMSC, particularly SCC, in these patients. However, they found side effects are a major limiting factor.

De Sevaux et al. in 2003 (55), reported the effects of a randomized trial comparing two doses of acitretin on premalignant and malignant skin lesion prevention in renal transplant recipients. Twenty-six long-term renal transplant recipients were randomized to one-year acitretin 0.4 mg/kg/day throughout the whole year or 0.4 mg/kg/day during the first three months followed by 0.2 mg/kg/day for the remaining nine months. Due to mucocutaneous side effects, they had

to lower the intended dose in most patients. Nevertheless, doses remained significantly different between the groups at all time points. A rapid decrease in the number of keratoses occurred, significant beyond two months in both groups ($P < 0.0001$). Thickness of keratotic lesions decreased significantly in both groups ($P < 0.01\%$ for comparison with baseline at all time points beyond one month). Skin cancers developing during treatment compared to the year before treatment were nonsignificant: SCC 30 versus 28; BCC one versus three; Bowen's disease nine versus 20; and keratoacanthomas 7 versus 16. There were no significant differences in the incidence of SCCs, BCCs, Bowen's disease or keratoanthomas between the two dosage groups during the study period. The authors concluded that acitretin reduces the number of actinic keratoses in renal transplant patients at 0.2 mg/kg/day, and patients' contentment with their skin increased significantly. In contrast to earlier studies, no reduction in the number of new skin malignancies occurred. The authors call for additional studies to clarify whether retinoids have merely a cosmetic effect or really change the process of malignant degeneration of actinic keratoses in renal transplant patients.

The remaining nonrandomized, noncontrolled studies and case reports are summarized in Table 1. In 11 of the 12 studies, treatment with systemic retinoids resulted in a decreased incidence of NMSC, and all studies noted decreased keratotic lesions. Although the studies vary in type of patient and dose of retinoid, the risk of developing premalignant and malignant cutaneous neoplasms in patients with solid organ transplants who are on immunosuppression is decreased by use of systemic retinoids. Of the retinoids, acitretin and etretinate were used most frequently. Beneficial prophylactic effects seem to be quickly lost on discontinuation of retinoid therapy, requiring long-term use. Optimal dosing and indications for initiation of retinoids have not been delineated.

MECHANISM OF ACTION

Although retinoids exert chemopreventive and chemotherapeutic effects, mechanisms of action for chemoprevention and cancer treatment are not yet well understood, as they can affect a variety of cellular and biological processes controlling growth, differentiation and development.

Retinoid molecules mediate their effects through two families of receptors: retinoic acid receptors (RAR) alpha, beta, and gamma, and retinoid X receptors (RXR) alpha, beta, and gamma. Through binding to these retinoid receptors, retinoids mediate their effects on cellular differentiation (56) and on programmed cell death (57) through the regulation of gene transcription and interaction with transcription factors. Reduction in cutaneous levels of RAR alpha and RXR alpha have been associated with cutaneous tumors (58,59). Interactions with retinoid receptors can result in a wide range of biological activities (60–62), shown in Table 2.

The ability to regulate the expression of proapoptotic genes and to sensitize keratinocytes to apoptosis may play a role in retinoids' prevention of NMSC in

(*Text continues on page 216.*)

Table 1 Oral Retinoid Use in Organ Transplant Patients

Year	Authors	Study design	Transplanted organ	Number of patients	Drug, dose, mean, duration	Response	Post treatment	Side effects	Authors' conclusions
1988	Shuttleworth D, et al. (80)	Case series	Renal	6	Etretinate, 1mg/kg/d 6 mos	4 patients had almost complete resolution of lesions. 1 had partial response. 1 developed new lesions	6 mos after treatment, 1 pt new BCC; 13 new SCC & 1 BCC; 4 no new lesions		Anti-tumor effect of etretinate not due to cytostatic action and does not require intact immune system
1991	Kelly JW, et al. (37)	Case series	Renal	4	Etretinate 50mg/d/1yr	Comparing year prior to treatment with treatment year, SCC decreased from mean of 5.75 to 1.5 per patient; BCC from mean of 1.5 to 0.75 per patient. AK improved in all patients within three mos of treatment	Within 1 yr after treatment 34 SCC, 14 BCC and recurrence of AKS	Mild mucocutaneous side effects; thrombocytopenia in 1 patient	Etretinate effective in decreasing SCC, BCC and AKS during treatment, but significant rebound effect
1992	Vandeqhinste N, et al. (81)	Case report	Renal	1	Acitretin 0.5mg/kg/d/15mos	6 yrs before treatment, 1SCC, 3 Bowen's, 3 BCC, and 1 KA; during treatment no SCC, Bowens, BCC, KA or KA	Within 4 mos after treatment recurrence of Bowen's and many verrucous lesions	Mucocutaneous side effects	Acitretin very effective during treatment

1995	Bavinck JN, et al. (53)	Randomized double-blind, placebo controlled trial	Renal	44	Acitretin 30mg/kg/d 6 mos	Prior to study patients had at least 10 keratotic lesions on hands and forearms. During treatment, new SCC developed in 4790 placebo (mean 0.95) vs. 1190 treated (mean 0.11)	Within 6 mos after treatment, NMSC and keratotic lesions greater than pre-treatment levels	Mucocutaneous, hair loss, 3 had increased serum cholesterol or triglycerides above pretreatment levels. No change in liver function	Acitretin 30mg/d over 6 mos had significant effect in prevention of SCC and keratotic lesion development Effect was most significant in patients with a history of skin cancer
1995	Yuan ZF, et al. (82)	Case reports	Renal	15	Acitretin 10–50mg/d variable time periods	All 15 Patients reported skin become softer and smoother. AKs and warts improved or disappeared in 4 of 6 treated more than 12 mos, the number of malignancies decreased		Cutaneous side effects, requiring reduced dosage in 5 and discontinuation in 4. No disturbance of liver or renal function or lipid profile	Most patients treated with acitretin experienced subjective improvement and AKs and warts improved or disappeared Effect on skin malignancy variable

(*Continued*)

Table 1 Oral Retinoid Use in Organ Transplant Patients (*Continued*)

Year	Authors	Study design	Transplanted organ	Number of patients	Drug, dose, mean, duration	Response	Post treatment	Side effects	Authors' conclusions
1998	Gibson GE, et al. (83)	Case series	Renal	11	Etretinate 0.3mg/kg/d up to 18 mos	Statistically significant reduction in NMSC during treatment compared with pretreatment at 3 and 6 mos, with trend towards fever skin cancers at 12 and 18 mos periods. One patient was non-evaluable due to exponential tumor development, despite etretinate, necessitating marked decrease of his immunosuppressive medications	Within 1 yr after treatment 34 SCC, 14 BCC and recurrence of AKS	Mucocutaneous, skin fragility, hyperhidrosis well tolerated. No lab abnormalities	Low dose etretinate is safe, well-tolerated and partially effective in chemophrophylaxis of skin cancer in renal transplant patients
1999	McKenna DB, Murphy GM (84)	Case series	Renal	16	Acitretin 0.3mg/kg/d 5 yrs	Significant reduction in total number of tumors excised during treatment		Mucocutaneous, alopecia, headache, epistaxis,	A significant chemophrophylactic effect was

						compared with immediate pre-treatment interval: 21:77(p=0.007) 50% remained tumor free when on drug; 12 of 16 had fewer tumors excised compared to same pre-treatment interval. Significant reduction in number of new nonmelanoma skin cancer at 1 yr, 2 yrs, 3 years, and 4 years compared to pretreatment. Few also at 5 yrs but not statistically significant.	hyperhidrosis; hematologic & liver function tests unchanged, one hand triglyceride and cholesterol increase	shown for up to 4 yrs of treatment Patients with 5 or more tumors prior to acitretin benefited the most. The introduction of low dose acitretin proved to be a useful strategy in the long term reduction of skin cancer in RTR
2002	McNamara IR, et al. (85)	Case series	Heart	5	Acitretin 10 or 25 mg/d (mean 15 mos)	All experienced subjective improvement in skin (softer, smoother, more supple, less aged)	Hepatotoxicity mild to moderate and generally resolved regardless of continuation or dose adjustment.	10 and 25 mg/d doses acitretin significantly reduces development of new NMSC

(*Continued*)

Table 1 Oral Retinoid Use in Organ Transplant Patients (*Continued*)

Year	Authors	Study design	Transplanted organ	Number of patients	Drug, dose, mean, duration	Response	Post treatment	Side effects	Authors' conclusions
2002	McNamara IR, et al. (85) (*cont.*)					3 experienced marked decrease in NMSC per year form >30 to <10. 2 of 5 experienced a moderate decrease		Mucocutaneous, alopecia in 1 patient.	in heart transplant recipients. Those with prior skin cancer history benefit more. Tolerated well with at least partial efficacy decreasing morbidity of NMSC
2002	George R, et al. (54)	Randomized, controlled 2 period crossover after 1 yr	Renal	23	Acitretin 25mg/d to QOD 1 yr	Incidence SCC significantly lower in patients receiving acitretin	One patient developed an excessive number of SCC after acitretin was ceased; 8 had no tumors in control or follow up period. Suggest rebound variable	Mucocutaneous, headache, elevated cholesterol, musculoskeletal symptoms. No adverse effect on renal function	Acitretin is effective in chemopreven-tion. Long term with the drug will help in reducing the morbidity associated with NMSC SE are a limiting factor

2003	De Sevaux RGL, et al. (55)	Randomized controlled trial 2 doses	Renal	26	Acitretin 0.20–0.4 mg/kg/d 1 yr	Actinic keratoses by almost 50%, but the number of NMSC was similar to the number in the year before the study. Thickness of keratoses decreased significantly in both groups. Patients contentment with skin appearance improved	Dose had to be reduced due to mucocutaneous side effects	Acitretin reducers the number of AKs in RTRs at 0.2mg/kg/d Call for additional studies to clarify whether retinoids have merely a cosmetic effect or really change the process of malignant degeneration of AKs in RTRs
2005	Harwood CA, et al. (86)	Retrospective review	Various	32	Systemic retinoids at 0.2 to 0.4 mglkg/d for 1 to 16 yrs			Low dose systemic retinoids significantly reduced SCC development in OTRs for the first 3 yrs; the effect may be sustained for 8 yrs with a generally well tolerated side effect profile

Abbreviations: AKS, actinic keratoses; BCC, basal cell carcinoma; KA, keratoacanthoma; NMSC, non melanoma skin cancer; QOD, every other day; SCC, squamous cell carcinoma; SE, side effect.

Table 2 Retinoid Effects Relevant to Chemoprevention and Cancer

Apoptosis of tumor cells or induction of apoptosis
Inhibition of tumor differentiation and proliferation
Induction of normal cellular differentiation (87,88)
Effects on keratinocyte differentiation and patterns of keratin expression (59,89–98)
Effects on cell cycle control
Influences on multiple transcription factors
Protein phosporylation and protein modification
Inhibition of ornithine decarboxylase
Alteration of gap junctional intercellular communication
Immunomodulation including increased density of Langerhans cells (87–99,100)
Preferential retinoid induced growth inhibition of HPV 16 immortalized keratinocytes compared with noninfected cells (50)
Suppression of telomerase activity, which is crucial for the continued growth and progression of cancer cells (101)

Abbreviation: HPV, human papilloma virus.

transplant patients and patients with DNA-repair deficiencies. All-trans-retinoic acid treatment strongly increases mRNA and protein expression of p53 and caspase-3,-6,-7,-9 which are key regulators of apoptosis (63). It could therefore interfere with the tumor promotion phase by inducing apoptosis, impeding proliferation and stimulating differentiation (64).

Tazarotene, a synthetic RAR beta and gamma retinoid, has been shown to be effective in treatment of hyperproliferative disorders, acne, psoriasis, icthyosis, and BCCs. It activates RAR beta and gamma and induces a number of downstream antiproliferative genes. It modulates expression of p73 gene in immortalized keratinocyte cell lines by inducing proapoptotic and antiproliferative TAp73 isoforms and by repressing antiapoptotic and proproliferative delta Np73 isoforms (65). CD437 produces slight apoptosis of normal human keratinoctes but pronounced apoptosis of their malignant counterparts. This induction of apoptosis in proliferating but not post mitotic cells is mediated via AP-1 activation with c-jun overexpression and is p53, a tumor suppressor gene, independent (66). Marcheti et al. (67) attribute CD437 apoptotic activity to mitochondrial dysfunction via the induction of mitochondrial permeability transition. These authors showed acute cytotoxic effects of CD437 are contingent on mitochondrial respiration. They speculate that CD437 promotes these effects by inhibiting some segment of the electron transport chain. This inhibition results in rapid decrease in respiration and apoptotic cell death.

Retinoids also modulate the expression of a variety of cytokines, including down regulation of IL-2 and IFN (68,69). Cystolic retinoid binding protein II (CRABPII) has been shown to be increased in keratoacanthoma and SCC cells but normal in seborrheic keratoses and BCCs. All types of tumors have shown moderately increased levels of cellular retinoid binding protein (70). Different effects

on different lines and cancer induced by different mechanisms or stimuli indicates the protective effect of retinoic acid on skin carcinogenesis is not universal.

Gap Junction Intercellular Communication

The effect of retinoids on gap junction intercellular communication (GJIC) and the expression of connexins, the protein subunits of gap junctions, have also been studied (71). Gap junctions are transmembrane channels formed by hexameric connexin proteins that facilitate passage of small molecules between the shared cytoplasm of neighboring cells. Retinoic acids inhibit both cell growth and GJIC in at least one line of SCC cells. Restoration of GJIC in tumor cell lines by transfection with connexin genes retards tumor cell growth following transplantation into nude mice (72,73). GAP junctions may affect growth controlling or stimulating signals (74), but are decreased in cancer and many tumor promoters inhibit GJIC (75).

Tazarotene Inducible Gene-3

In 2000, Duvic et al. (76) demonstrated that the expression of a retinoid-inducible tumor suppressor, tazarotene inducible gene-3 (TIG-3), is significantly decreased in psoriasis and in both basal and squamous cell skin carcinoma compared with adjacent and overlying normal skin. Furthermore TIG-3 expression is significantly less in aggressive SCC tumors compared with nonaggressive SCC tumors. The assumption that TIG-3 acts as a tumor suppressor and controls normal epithelioid differentiation is supported by the finding TIG-3 is expressed in differentiated epidermis, hair follicles and sebaceous glands of normal control skin, and is most pronounced in the suprabasal layers. It stains equally in all layers of thinner normal skin. By contrast, TIG-3 mRNA expression is significantly reduced in psoriatic epidermis compared with paired normal, uninvolved skin and is likewise significantly decreased in both basal and SCCs compared with adjacent and overlying normal skin. Topical tazarotene upregulated TIG-3 expression in psoriasis lesions and in BCCs in parallel to clinical response (77,78). DiSepio et al. (79) showed TIG-3 has significant homology to the class II tumor suppressor gene, H-rev 107. Tazarotene increases TIG-3 expression in human keratinocytes, correlating with decreased proliferation. These studies suggest that TIG-3 is a retinoid—inducible antiproliferative/class II tumor suppressor gene. Whereas loss of TIG-3 may be an important event leading to skin cancer, expression of TIG-3 in suprabasalar epidermis induced by retinoids may help to regulate normal terminal differentiation and may be a very significant mechanism in NMSC chemoprevention and treatment.

CONCLUSION

When NMSC becomes a cause of morbidity and mortality in high-risk patients, treatment and prevention are urgent. With numbers of high-risk patients increasing,

largely due to iatrogenic immunosupression, retinoid use for chemoprevention and chemotherapy of NMSC is increasing. Although complicated and variable medical status of patients at high risk for NMSC will require individualized retinoid treatment regimens, consensus currently is to start with a lower dose and increase as possible, thus increasing patient acceptance by decreasing side effects. To date, no data support any particular interval after high-risk status is identified to start retinoids. A number of reports do suggest patients with the highest propensity to skin cancer, or the higher the incidence of skin cancers before treatment, the more significant the response. When used for skin cancer chemoprevention, retinoids generally follow the acknowledged side effect profile of this class of drugs, discussed in detail in Chapter 13.

One of the current concerns is the rapid increase in new NMSC seen in some patients when retinoids are discontinued. Some propose that stopping retinoids does not increase the incidence of NMSC but allows the lesions suppressed while on retinoids to emerge. They suggest during treatment possible precancerous hyperkeratotic lesions are being made less keratotic and thick without being cured; when retinoids are discontinued, rebound includes back accumulation of these lesions now transforming to NMSC. Thus the question arises of retinoid treatment being a cosmetic effect or actually a change in process of malignant degeneration of AKs. Clinically, whichever the case, for the duration of treatment, the patients are happier with their skin appearance and clinicians can follow for NMSC more easily against a smoother background.

Adjuvant treatment with retinoids is one of great potential, including use with topical agents used for skin cancer prophylaxis and treatment and various oral adjuvant therapies. No doubt numerous studies will look at a number of agents to enhance the effect and decrease the dosage of retinoids needed in these patients.

As speculated by many involved in retinoid research in skin cancer chemoprevention and treatment, this may be retinoids' most valuable contribution to medicine.

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13

Side Effects and Pitfalls in Retinoid Therapy

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INTRODUCTION

Vitamin A is involved in the biosynthesis of the visual pigment rhodopsin (1), in embryogenesis (2), as well as in cell growth and differentiation (3). It exists in interconvertible forms as retinol, its esters (the main storage form in the body) and retinaldehyde, whereas the latter is irreversibly converted to retinoic acid, the biologically active form of vitamin A that binds to nuclear receptors and modulate gene expression (Fig. 1) (4,5). Retinoic acid exists as three *cis*/*trans* isomers: all-*trans*-, 9-*cis*- and 13-*cis*-retinoic acid; all-*trans*-retinoic acid (retinoic acid, tretinoin) binds to the nuclear receptors such as retinoic acid receptor (RAR)-α, β, and γ with a high affinity, 9-*cis*-retinoic acid (alitretinoin) binds to RAR-α, β, γ and retinoid X receptor (RXR)-α, β, γ with a high affinity, whereas 13-*cis*-retinoic (isotretinoin) does not bind to nuclear retinoid receptors with significant affinity (6,7).

Lack of vitamin A causes hyperkeratosis of the skin and squamous metaplasia of mucous membranes, immunodeficiency, xerophthalmia and blindness, anemia and increased sensitivity to infections (8). On the other hand, excess of vitamin A is associated with alopecia, amenorrhea, anorexia, birth defect, bone pain, cheilosis, fatigue, gastrointestinal disturbances, headache, hepatomegaly,

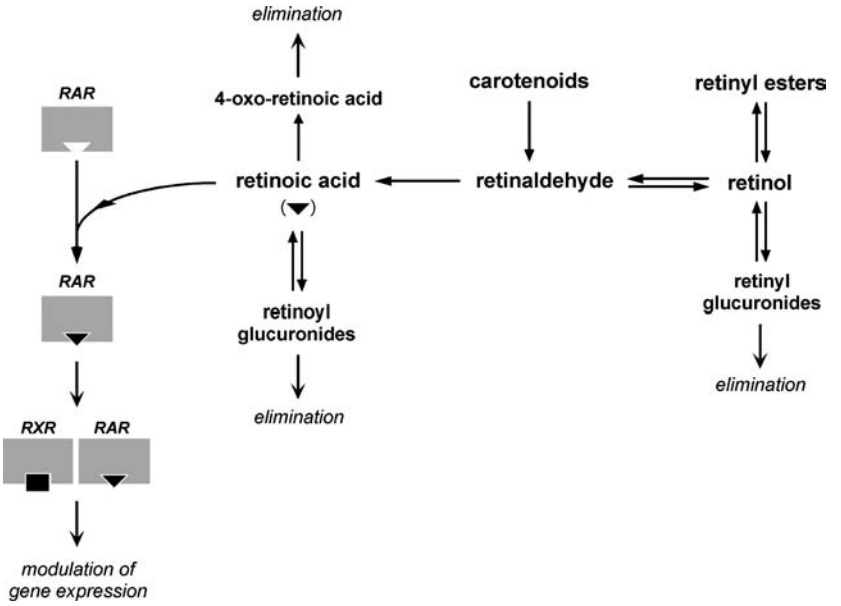

Figure 1 Metabolism of endogenous retinoids. Retinol and retinaldehyde are interconvertible, retinaldehyde is oxidized to retinoic acid, and the latter binds to nuclear receptors (RAR). The retinoic acid-RAR complex forms a heterodimer with a ligand-(RXR) complex and modulates gene expression. Retinol and retinoic acid are eliminated by conjugation to glucuronate. *Abbreviations*: RAR, retinoic acid receptor; RXR, retinoid X receptor.

hydrocephalus, itchy dry skin, nausea and vomiting, splenomegaly and general weakness (9,10).

Early evidence that retinoids play a role in epithelial differentiation came from observations of vitamin-A deficiency in human beings and animals (10,11,12). This property of vitamin A led pioneers von Stuettgen and Bollag to administer topical and systemic retinoids to treat disorders of keratinization. Because of a narrow therapeutic window, a large program was launched to engineer synthetic retinoids that would have the highest therapeutic activity with the lowest level of toxicity. Three generations of retinoids have been developed. The first generation consists of nonaromatic natural retinoids, such as tretinoin and isotretinoin, as well as their *cis/trans* isomers. The second generation is represented by acitretin and etretinate: these monoaromatic retinoids are formed by replacing the cyclic end group of retinol with different substituted and unsubstituted ring systems. The third-generation includes polyaromatic retinoids, also called arotinoids, which are produced by cyclization of the polyene side chain; these include adapalene, bexarotene, and tazarotene (Fig. 2) (13).

As any efficient therapeutic agent, oral and topical retinoids have undesirable effects; a thorough knowledge of their biological actions and their therapeutic

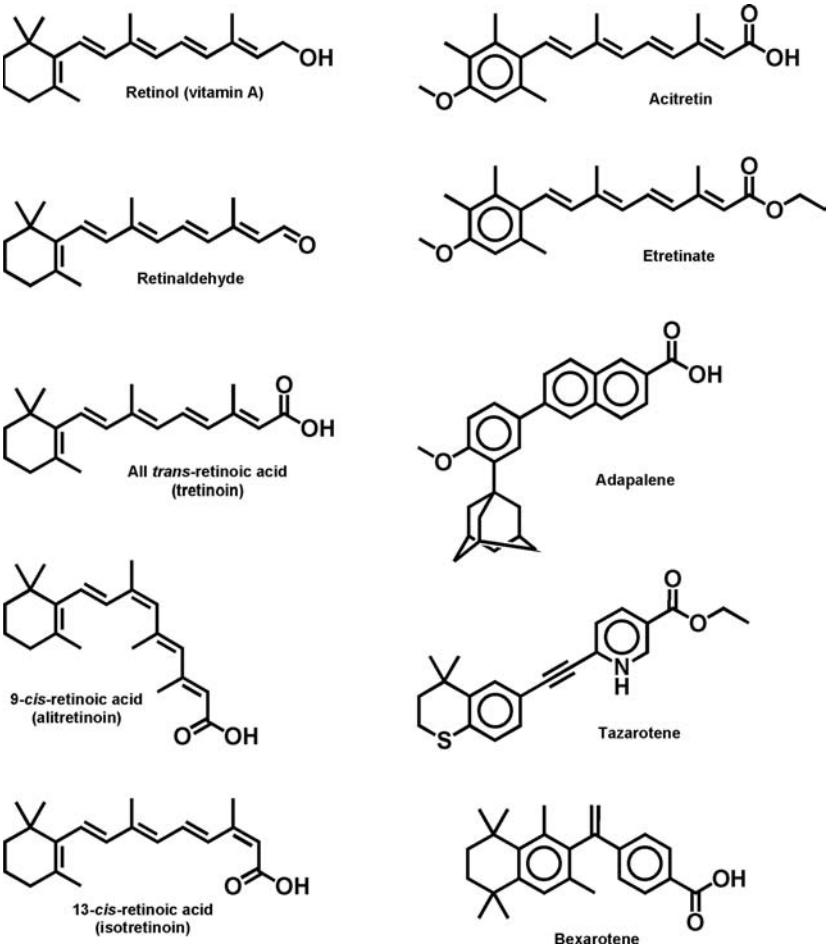

Figure 2 Structure of natural and synthetic retinoids.

window is thus required to use them adequately. In this chapter, we review the side effects of retinoid therapy in general and focus on the use of retinoids in dermatology.

SYSTEMIC AND CUTANEOUS RETINOIDS

Tissue Distribution and Metabolism of Retinoids

Endogenous Retinoids

Bioavailability, plasma transport and tissue distribution of oral retinoids depend on their physicochemical properties. Retinoids bind strongly to plasma proteins: free retinol binds to a specific serum retinol-binding protein (RBP), the free acids

tretinoin and its *cis-trans* isomers isotretinoin and alitretinoin, as well as acitretin, bind to albumin, and retinoid esters (retinyl esters, etretinate) to lipoproteins (14,15). The free acids are metabolized through phase I and phase II enzymes, which convert the lipophilic parent drugs to more hydrosoluble metabolites (hydroxyl and oxo derivatives) or glucuronide conjugates. Retinoid esters are first metabolized to retinol or the active retinoid acid before being further metabolized as described previously.

In the general population, the mean plasma value for retinyl esters is 150 to 300 nM, that of retinol, 2 to 2.5 μM, whereas plasma concentrations for retinaldehyde and retinoic acids are in the range of 1 to 10 nM (16–19). Vitamin A is stored in the body as retinyl esters and retinol, which account for more than 99% of total endogenous retinoids. The liver contains approximately 90% of total body vitamin A, with a concentration of approximately 1 μmol/g, whereas other tissues, such as adipose tissue, lung, kidney or testis contain approximately 20 nmol/g, and the skin approximately 1 nmol/g (20–23).

Oral Retinoids

Oral tretinoin is mainly used to treat acute promyelocytic leukemia, in which a chromosomal translocation produces a chimerical protein between RAR-α and a protein called promyelocytic leukemia protein (PML). PML-RAR-α works as a dominant negative receptor in the leukemia cells, interfering with the normal function of RAR-α and/or PML, which in turn results in the arrest of cell maturation at the stage of promyelocytes (6,24). The half-life of oral tretinoin following initial dosing is in the range of 0.5–2 hours. Since oxidation to 4-oxotretinoin by phase I enzymes (cytochromes P450 such as CYP2C8 and CYP26) is critical in the catabolism of this drug (3,25–27), long-term oral treatment is associated with a progressive decrease in plasma drug concentrations leading to resistance in patients with acute promyelocytic leukemia. Retinoic acid metabolism blocking agents (RAMBAs), such as liarozole, rambazole or R115866, as well as liposome-encapsulated tretinoin and combined therapy, seem promising to counteract this phenomenon (28–30).

Oral isotretinoin is the most effective anti-acne compound. As a *cis*/*trans* isomer of tretinoin, it has a different geometry, which explains its higher half-life of approximately 20 hours. It can be converted to tretinoin, and oxidized to 4-oxo-isotretinoin: thus tretinoin, isotretinoin and their respective 4-oxo metabolites are found in plasma following oral isotretinoin treatment (14,17).

Etretinate, the ethyl ester of acitretin, is converted to its active metabolite acitretin. It is much more lipophilic than acitretin, thus it accumulates in adipose tissue during long-term oral treatment, and has a long terminal elimination half-life of approximately 120 days, as compared to 50 hours for acitretin in continuous therapy (14,31). However, acitretin is also partly converted to etretinate in the presence of ethanol, especially during continuous therapy, thus increasing the elimination half-life of acitretin (32,33). Both acitretin and etretinate are reversibly

interconvertible to 13-*cis*-acitretin and 13-*cis*-etretinate, which account for less than 10% of the parent compounds.

Tazarotene is an acetylenic arotinoid, which is metabolized to tazarotenic acid by ethyl ester hydrolysis and which binds selectively to the nuclear receptors RAR-β and RAR-γ (34). Oral tazarotene is in clinical development for the treatment of psoriasis and advanced cancer (35,36). Following oral administration, tazarotene was rapidly absorbed and underwent extensive hydrolysis to tazarotenic acid, the major circulating species in the blood that was then excreted unchanged in feces. A smaller fraction of tazarotenic acid was further metabolized to an inactive sulfoxide that was excreted in the urine (35).

Bexarotene, an aromatic retinoid (arotinoid), is a selective agonist for retinoid X receptors (RXRs) (rexinoid). After oral administration, bexarotene is absorbed with a T_{max} of two hours and has a terminal half-life of seven hours. Four metabolites have been identified in plasma: 6- and 7-hydroxybexarotene, and 6- and 7-oxobexarotene. In vitro studies suggest that CYP3A4 is the major enzyme responsible for its oxidative metabolism, and that oxidized metabolites may be conjugated to glucuronate. Bexarotene is thought to be eliminated through the hepatobiliary system (37).

Topical Retinoids

Application of topical natural retinoids has been shown to induce a manifold increase of the basal epidermal concentration (22,38–45), although no significant increase of plasma retinoids was observed (46–49); this indicates that topical retinoids are potential selective therapeutical agents for the skin devoid of teratogenic potential. However, although all biochemical and clinical studies demonstrate that the daily variations of plasma retinoids are larger than those occurring under topical retinoid treatment, the scientific and ethical discussions regarding teratogenicity of topical retinoids are still open, and any topical retinoid treatment during pregnancy should be carried out under medical control (50).

Topical tretinoin has been widely used for decades to treat acne and photoaging. The globally attainable concentrations of tretinoin in the different layers from therapeutically efficient formulations two hours following application (from 1 nmol/g in the dermis to 3 μmol/g in the epidermis) account for a biological action mediated by its binding to nuclear receptors (22,38).

Topical retinaldehyde appears as a good precursor for both storage (retinol, retinyl esters) and functional (retinoic acids) forms of epidermal retinoids, in particular when combined to glycolic acid (Antille et al., unpublished data), thus confirming previous studies in mice (51–53).

Adapalene, a stable arotinoid that binds to the nuclear receptors RAR-β and RAR-γ, is used in topical formulations to treat acne vulgaris (54). Pharmacokinetic studies demonstrated that significant amounts of adapalene were present in the epidermis and the dermis following application of 0.1% gel, although only 10 ppm of the applied dose penetrated through the skin (55). These properties render adapalene suitable for topical retinoid therapy.

Topical tazarotene is prescribed for plaque psoriasis and facial acne. The pharmacokinetic profile of tazarotene ensures that systemic exposure to the drug and its metabolites is minimal. First, percutaneous penetration is limited, with less than 6% of the applied drug being absorbed into the bloodstream. Second, tazarotene is rapidly metabolized into tazarotenic acid and other polar metabolites. Third, tazarotene and its metabolites are rapidly eliminated from the blood in the urine and feces. These pharmacokinetic properties indicate that systemic effects of topical tazarotene are comparable to those of endogenous retinoids, which suggests that the risk of teratogenic effects is minimal. Moreover, topical tazarotene is not associated with contact sensitization, phototoxicity, photoallergic reactions, mutagenicity, or carcinogenicity (56).

SIDE EFFECTS IN RETINOID THERAPY

Systemic Retinoids

The major side effect of retinoids is their teratogenicity, which precludes the use of any therapeutically active systemic retinoid to pregnant or breast-feeding women, or to women contemplating becoming pregnant. Relative contraindications of systemic retinoids are leucopenia, moderate-to-severe hyperlipidemia, significant hepatic (especially bexarotene) or renal dysfunction, hypothyroidism (especially bexarotene), young children, depressive symptoms or suicidal ideation, as well as pseudotumor cerebri (13,57,58). Furthermore, patients should be advised to avoid excessive vitamin A supplementation, since this can induce a dysregulation of the tight control of the production of biologically active retinoids from vitamin A (59–61).

Teratology

Retinoids have been demonstrated to be teratogenic in a number of experimental mammals. The evidence that humans are susceptible to retinoid teratogenesis is of concern in dermatology because retinoids belong to the drugs prescribed for the treatment of skin diseases and various cancers (62). As for any teratogenic agent, the teratogenicity of retinoids depends on the developmental stage of the embryo or fetus at the time of exposure. The embryo is highly sensitive to teratogenesis during organogenesis (from week 3 to week 8 of human gestation), and there is a gradual decrease in teratogenic sensitivity from the end of week 8 until parturition (62). Basically, the human malformations caused by retinoids appear to be induced by perturbations of the neural crest cells and the central nervous system. Other cells are probably sensitive at higher retinoid doses. The cranial neural crest is primarily responsible for defects of the craniofacial, thymic, and cardiovascular systems, although different populations of cranial crest cells are differentially sensitive to retinoids (63). This explains that women who received oral isotretinoin during pregnancy gave birth to children with growth and mental retardation, hydrocephaly, heart defects, or ear and eye abnormalities (64–66).

Unlike thalidomide, which mostly induce characteristic limb reduction defects, these malformations do not seem to be induced by retinoids, although one case of limb reduction was reported for a child whose mother has taken isotretinoin during the first trimester of pregnancy (67). Although the mechanism of action of the retinoid-induced teratogenesis is not well understood, compelling evidence indicates a receptor-mediated mechanism, indicating that the teratogenic potential of nonligand precursors, such as retinol, retinyl esters, retinaldehyde and retinoyl β-glucuronide depends on their conversion to RAR- and/or RXR-active ligands (48,62,63,68,69).

Skin and Mucous Membrane Adverse Effect

Dose-dependent mucocutaneous toxicity is frequently observed during oral retinoid therapy, and it mainly reflects a decreased production of sebum, reduced stratum corneum thickness, and altered skin barrier function. Dry lips or cheilitis is the earliest and the most frequent sign that appears after starting therapy. Dryness of the mouth accompanied by thirst, and dryness and fragility of the nasal mucosa leading to epistaxis are also frequently observed (13,70,71).

Xerosis of the skin, associated with pruritus and peeling, especially of the palms and soles, is a frequent side effect. Skin fragility and fissuring of the fingertips may create a specific problem for those who must perform manual labor. Photosensitivity may be observed, in particular with isotretinoin, and probably reflects a reduction in the thickness of the stratum corneum. Xerophthalmia due to decreased meibomian gland secretion may prohibit the use of contact lenses and can lead to blepharoconjunctivitis, with varying degrees of severity. Corneal ulceration or opacity can occur as a complication in some patients (13,72).

The mucocutaneous side effect profiles of systemic retinoids depend on the nature of retinoids. For instance, isotretinoin causes more mucosal dryness, and acitretin has been associated with higher incidences of alopecia and palmoplantar peeling, whereas bexarotene induces milder mucocutaneous and ocular side effects than other classes of retinoids (13).

Bone Toxicity

Bone pain is frequently observed with oral retinoids, although only few transient objective evidences of bone abnormalities have been reported, especially with synthetic retinoids, such as isotretinoin and etretinate. Specific findings include anterior spinal ligament calcification, osteophyte formation, extraspinal calcifications, and bony bridges, but without a narrowing of the disk space (13,73,74). These changes resemble those observed in the disorder known as diffuse idiopathic skeletal hyperostosis (DISH) or Forestier's disease, a variant of osteoarthritis without the degenerative intervertebral discs and joints observed in classic osteoarthritis (75–77). The likelihood to develop DISH-like hyperostosis increases with age. Besides retinoids, DISH has been associated with hypertension, obesity, dyslipidemia and coronary artery disease (75). Osteoporosis has been observed with hypervitaminosis A and after long-term retinoid therapy, in particular with

etretinate (78). Subsequent prospective studies have shown that the effect of retinoids on bone, if present at all, is likely to involve worsening of pre-existing skeletal overgrowth rather than induction of de novo changes (79).

According to a recent study, a 16- to 20-week course of isotretinoin treatment at the recommended dose for severe acne had no clinically significant effect on lumbar spine and total hip bone mineral density in the adolescent population, that is, in growing patients who are most susceptible to suffer from severe acne (80).

Effects on Muscles

Muscular pain and cramps can be observed in patients taking etretinate or acitretin; however, these symptoms are associated primarily with isotretinoin, especially in individuals involved in vigorous physical activity. Sometimes elevated creatine kinase levels may be observed (81). Increased muscle tone as well as axial muscle rigidity and myopathy were reported to be related to etretinate and acitretin therapy, respectively (82,83). Acitretin therapy has been associated to a dysfunction of predominantly sensory nerve fibers in some individuals. Although in the investigated patients this dysfunction remained subclinical, it seems reasonable to suggest that neurological and neurophysiological evaluation of peripheral nerves should be added to the list of investigations that are routinely performed in patients receiving oral acitretin (84).

Effects on Central Nervous System

Central nervous system side effects are rare. Although individual signs of increased intracranial pressure such as headache, nausea, and vomiting are occasionally observed, the complete syndrome with papilloedema (pseudotumor cerebri) and blurred vision, also observed in certain cases of hypervitaminosis A (85), is very rare (86). Concomitant use of other drugs associated with intracranial hypertension (e.g., tetracycline, doxycycline, or minocycline) is a major risk factor for developing pseudotumor cerebri and should therefore be avoided (87). Examination for papilloedema should be performed immediately when a patient receiving retinoid therapy complains of persistent headache, especially if it is accompanied by visual changes, nausea, or vomiting, or when pseudotumor cerebri is otherwise suspected (13).

There have been anecdotal reports suggesting a causal association between isotretinoin therapy in acne patients and severe depression, psychosis, and suicide attempts (88). New studies have now addressed this question: whereas one study reports a (slight) positive relationship between headache and depression for several kinds of drugs, including isotretinoin (89), another one concludes that the use of isotretinoin in the treatment of moderate to severe acne in adolescents did not increase symptoms of depression (90). Although the first study by Wysowski and Swartz was limited by the inability to determine the precise chronology of headache, drug treatment and depression (89), these data should prompt physicians to monitor patients with depressive symptoms or suicidal ideation carefully.

A possible explanation for the retinoid effects on the central nervous system could involve a developmental dysfunction of the expression of brain RAR receptors. The expression of RAR-α is increased in the dentate gyrus from schizoprenic patients, as compared to nonpsychiatric controls (91), whereas RAR-β has been shown to be related to locomotion, dopamine signaling and cortical synchrony in mouse (92–94).

Ophthalmologic Side Effects

As for the skin and mucous membranes, the most common ocular retinoid effects are dryness and irritation (see also section "Skin and Mucous Membrane Adverse Effect"). Alterations in visual function, mainly nyctalopia, excessive glare sensitivity, and changes in color perception have also been reported, especially with isotretinoin and fenretinide (95). This apparent paradox, since hypovitaminosis A predisposes to nyctalopia and xerophthalmia (96), could be explained by a competitive inhibition of retinal (ocular) retinol dehydrogenase by exogenous retinoids, resulting in decreased rhodopsin formation (13,97).

Hypothyroidism

Clinical and biochemical central hypothyroidism occurred in 40% of patients in the cutaneous T-cell lymphoma (CTCL) trials with the RXR-ligand bexarotene, and it was rapidly and completely reversible with cessation of therapy without any clinical sequel (98,99). This effect is probably mediated through suppression of thyrotropin-β subunit (TSH-β) secretion by the thyrotrope cells of the anterior pituitary, which express RXR-γ, and increase of thyroid hormone clearance (99,100).

Gastrointestinal Side Effects

Uncommon nonspecific gastrointestinal complaints (e.g., abdominal pain, nausea, diarrhea) have been reported in association with retinoid therapy or carotenoid consumption (101). Synthetic retinoids have been temporarily linked with other toxicities, such as inflammatory bowel disease, on rare occasions; however, no cause-and-effect relationship has been established (102).

Renal Effects

Renal toxicity is not characteristic of retinoid therapy. Isotretinoin has been safely administered to patients with end-stage kidney disease who were undergoing hemodialysis. Moreover, retinoids were shown to reduce renal damage in animal models (103,104). However, case reports of reversible renal dysfunction (with elevated creatinine levels) during etretinate therapy have been described. Therefore, renal function should be monitored during retinoid therapy in patients with a history of renal disorders (105).

In patients suffering from chronic renal failure, etretinate has been shown to require a longer time for complete elimination (106); this should be kept in mind when giving retinoids to these patients.

Patients receiving immunosuppressant drugs following organ transplant showed a higher incidence of precancerous or cancerous cutaneous lesions. Retrospective studies assessed the efficacy of a preventive retinoid treatment (in particular acitretin) on the risk of cutaneous carcinoma. Acitretin (0.4 mg/kg) seems to decrease the incidence of actinic keratoses, but this dose is too high to be used for long-term therapy. Lower doses are better tolerated but less efficient too (107,108).

Dyslipidemia

The most frequently observed systemic side effect of oral retinoids is a dyslipidemia characterized by an increase of triglyceride and total cholesterol levels and a decrease of high density lipoprotein (HDL) plasma concentrations (13,109). This subject is covered in Chapter 15 of this book.

Liver Toxicity

Transitory abnormal elevations in serum transaminases have been reported in approximately 20% of patients treated with etretinate or acitretin, occurring much less frequently during isotretinoin or bexarotene therapy. Circulating levels of alkaline phosphatase, lactate dehydrogenase, and bilirubin may also become elevated during retinoid therapy. Liver function abnormalities, mostly mild, usually occur between two and eight weeks of starting therapy, and they return to normal within another two to four weeks, despite continued therapy (13). Acitretin therapy elicited no biopsy-proven hepatotoxicity in a two-year prospective study, thus suggesting that periodic liver biopsy should not be necessary (110). One study reported clinical or histological hepatitis possibly or probably related to etretinate therapy in about 10 (1.5%) of 652 American patients treated for psoriasis (111). No specific studies have evaluated the use of retinoids in patients with hepatic insufficiency. However, since retinoids are metabolized by the hepatic mono-oxygenases CYP26 and the CYP3A group, and undergo partial biliary elimination, significant hepatic insufficiency may be expected to interfere with drug elimination (26,112). Transaminase increases of more than three-fold the upper normal limit should lead to discontinuation of retinoid therapy. With two- to three-fold transaminase elevations, therapy should be withdrawn until normalization of tests of liver function (111).

Hematological Toxicities

A high incidence (28%) of dose-related leucopenia was reported in the studies of bexarotene in CTCL, occurring as early as two to four weeks, with a decrease in neutrophils rather than lymphocytes (113). Hematological abnormalities are much less common with other retinoids, but careful hematological monitoring in HIV-infected patients is required (13).

Interactions with Other Drugs

Most of the biological actions of retinoids are mediated by their binding to nuclear receptors RAR and/or RXR (6,7). The latter make functional heterodimers with

other nuclear receptors, such as vitamin D receptor (VDR) (114), thyroid hormone receptors (THR) (115), peroxisome proliferator-activated receptors (PPAR) (115,116), pregnane X receptors (117), or liver X receptors (oxysterol receptors, LXR) (118). Thus any drug acting as a ligand for one of these nuclear receptors is potentially able to interact with retinoid action, as it was demonstrated for the RXR agonist alitretinoin (9-*cis*-retinoic acid) and calcitriol (1,25-dihydroxyvitamin D3) (119).

In a general manner, concurrent use of retinoids with alcohol or other medications having similar side effects may increase their incidence. The following associations should be avoided: (*i*) minocyclines and tetracyclines (risk of intracranial pressure increase); (*ii*) alcohol (increased conversion of acitretin to etretinate and hepatotoxicity); (*iii*) methotrexate (synergy of hepatotoxicity with retinoids); (*iv*) vitamin A or provitamin A (retinyl esters, certain carotenoids) supplements (risk of hypervitaminosis A with retinoids) (13,32,33,58,120).

The usefulness of retinoids, in particular retinoic acid and its *cis*/*trans* isomers, is often limited by the emergence of acquired resistance involving, at least in part, excessive retinoid catabolism by cytochrome P450 enzymes such as CYP26. To counteract this phenomenon, the concept of RAMBAs has been developed. Ketoconazole, a general cytochrome P450 inhibitor, and liarozole, a more specific CYP26 inhibitor, are promising agents in the prevention of retinoic acid resistance (121,122).

Topical Retinoids

Teratology

As mentioned earlier, oral retinoids are potent teratogens. A critical point for the teratogenic potential of retinoids is the maximal plasma concentrations reached by biologically active retinoids following their application. Regarding topical retinoids, many animal and human studies addressed the question of the pharmacokinetics of plasma retinoids following topical application. Although topical retinoids penetrate easily through the epidermis, all these studies reported no significant increases of natural retinoids in plasma following topical application (46–49). In the case of the synthetic topical retinoids adapalene and tazarotene, the plasma concentrations following topical application at 0.1% concentrations were always below 3 nM, that is, similar to or lower than endogenous tretinoin, which suggests that the teratogenic potential should be negligible (56,123,124). For these reasons, topical retinoids are considered no teratogenic.

Irritation

The most common side effect of topical retinoids is skin irritation characterized by erythema, scaling, pruritus, burning, stinging, and dryness. This "retinoid dermatitis" occurs within the first month of treatment and tends to recede thereafter. It responds to a temporary reduction in the frequency or amount of retinoid

application and to application of moisturizers (13). Tretinoin-induced irritation, for example, is generally dose- and vehicle-dependent, and the level of irritation ranges from high to low with the solution, gel, and cream formulations, respectively, that is, from a polar and little penetrating to a lipophilic well penetrating formulation (125–127). Since efficacy of topical retinoids is dose-related as well, pharmaceutical research programs have focused on methods to minimize the irritation caused by topical retinoids, while preserving or improving their therapeutic benefits. Improved tretinoin delivery systems are now available, and include complexes of tretinoin with polymer molecules and incorporation of tretinoin in microsponge particles (127–130).

Interactions with UV Radiations

Although no photoallergic or phototoxic reactions have been proven in human studies for topical retinoids (131), many patients note a decreased tolerance to UV radiation shortly after sun exposure. This reaction is often accentuated by a sensation of heat, raising the question of involvement of infrared irradiation (13). A retrospective study including 61 patients who applied retinaldehyde 0.05% during 6 to 142 months evaluated the safety of long-term application of retinal in terms of photocarcinogenesis. It clearly appeared in this study, that long-term use of topical retinaldehyde was not associated with a higher risk of actinic keratoses or nonmelanoma skin cancers (132). Although topical retinoids display pro-oxidative or antioxidative properties, anticarcinogenic or procarcinogenic activities in different in vivo and in vitro models, it is interesting to underline that human studies showed prevention or no effect of topical retinoids on nonmelanoma skin cancers (133–136).

INTERACTIONS BETWEEN UV RADIATIONS AND RETINOIDS

Endogenous and synthetic retinoids have a molecular structure with a conjugated double bond system, which enable them to absorb strongly UV radiations in the UVA range (320–400 nm) with extinction coefficients of approximately 50,000 $(mol/L)^{-1} \cdot cm^{-1}$ (Fig. 3A). On the other hand, UVA penetrate the epidermis and a part of the dermis (Fig. 3B). As a consequence, cutaneous retinoids, especially in the epidermis, are photosensitive, a result clearly demonstrated in animal and human studies (52,53,137–142). Considering the good percutaneous absorption of topical retinoids, this implies the appearance in the skin (mostly the epidermis) of significant amounts of retinoid photoproducts when exposing the skin to the sun following an application of one of the numerous creams containing retinoids. The nature of these photoproducts appearing in the skin after UV irradiation is still unknown; according to studies aiming at analyzing retinol and retinyl palmitate photoproducts in solution or in liposomes, natural retinoids could form cationic, hydroxyl, epoxide, peroxide, and radical photoproducts (143–146). Although retinyl palmitate photoproducts obtained in solution were shown to be

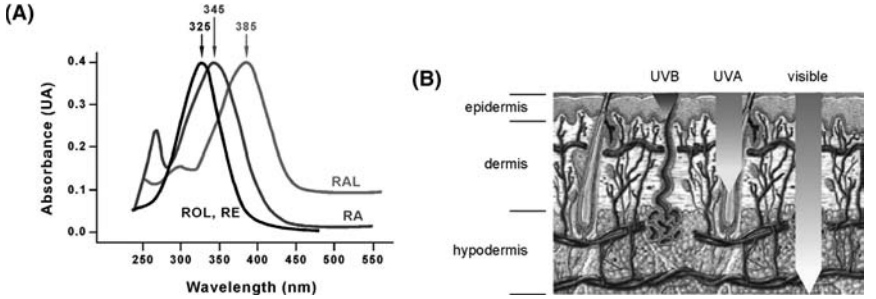

Figure 3 UV absorption by retinoids and the skin. (**A**) UV absorption spectra of retinol (ROL), retinyl esters (RE), retinaldehyde (RAL), and retinoic acid (RA). Their molar extinction coefficients are approximately 50,000 $(mol/l)^{-1} \bullet cm^{-1}$, that is, they strongly absorb UV radiations. (**B**) Penetration of visible light, UVA and UVB through the skin.

nonmutagenic in bacterial assays (144), in vivo studies are required to analyze the photoproducts formed in sun-exposed skin after topical retinoids, as well as their cutaneous and systemic toxicity.

In particular, the involvement of these potential photoproducts in the animal studies reporting an enhancement of photodamage by topical retinoids was never documented. Both human and animal studies report preventive or no effects of topical retinoids before exposing the skin to UV radiations (132–136,147,148), an effect probably mediated by the activation of the pro-apoptotic p53 gene and the repression of the transcription factor AP-1 (149–152). Conversely, only animal and in vitro studies report a potentiation by retinoids of UV-induced cellular damage (153–156).

On the other hand, the absorption of UV radiations by cutaneous retinoids has the advantage of decreasing the energy received by biomolecules that play a crucial role in the skin: cutaneous retinoids thus act as UV filters and could prevent UV-induced DNA damage and oxidative stress. This concept has been illustrated in an animal study reporting a similar filter effect of the various topical natural retinoids at a concentration of 0.05% (retinyl palmitate, retinol, retinaldehyde, and retinoic acid) on UV-induced apoptosis and DNA photodamage in mice (157). In a human study, topical retinyl palmitate 2% afforded a similar photoprotection as a sunscreen with a sun protection factor of 20, as demonstrated by the prevention of UVB-induced erythema and DNA photodamage (142).

RETINOIDS AND OXIDATIVE STRESS

Oxidative stress is defined as an imbalance between the overproduction of reactive oxygen species and/or free radicals and the ability of a biological system to neutralize them (158–162). Due to their cyclohexane moiety and their conjugated double bonds, retinoids can stabilize free radicals and scavenge singlet oxygen—a

highly reactive oxygen species—and thus act as antioxidants. However, an overproduction of radical retinoid species can lead to an oxidative stress in the absence of other antioxidants able to recycle retinoids to their nonradical state. Thus, as for UV radiations, the effects of retinoids on oxidative stress depend on the context (model, microenvironment, markers, etc.).

Almost all published studies aimed at analyzing the effects of retinoids on oxidative stress were performed in vitro. If most of them report a prevention by retinaldehyde, all *trans*- or 13-*cis*-retinoic acid (53,163–167), other studies show an increase of oxidative state by retinol (153,154), which emphasizes the dual properties of retinoids as both oxidants and antioxidants. However, it's important to bear in mind that an induction of oxidant species is not necessarily deleterious, since some physiological processes require the generation of oxidant intermediates (161,168–171).

The few studies performed in animals or humans reported a beneficial effect of retinoids on oxidative stress. For instance, a pretreatment of mice with topical isotretinoin prevented benzoyl peroxide-induced oxidative stress (163); vitamin A supplementation decreased the lipid peroxidation in blood of patients with pregnancy-induced hypertension (172); in a clinical trial enrolling former smokers, oral alitretinoin (9-*cis*-retinoic acid) induced an increase of serum vitamin E (α-tocopherol), the main endogenous antioxidant (173).

Two large clinical trials enrolling participants at high risk to develop lung cancer were conducted in order to assess the benefit provided by a supplementation with α-tocopherol and β-carotene (ATBC study) or β-carotene and retinol (CARET study) for the prevention of lung cancers. Both studies reported either no benefits or potential harmful effects of the preventive treatments (174,175). Since cigarette smoke is known to contain free radicals (176), the increase of lung cancer incidence by antioxidants in the ATBC study suggests that the supplemented antioxidants were not sufficient to stop the free radical chain reaction, leading in these conditions to an adverse effect of inadequate retinoid treatment.

CONCLUSION

Since their introduction in the late 1960s and late 1970s, respectively, topical and oral retinoids have greatly improved the conditions of many patients suffering from various dermatological diseases. The main successes of retinoids are certainly the treatments of severe acne, psoriasis, and photoaging. Although these diseases are not life threatening on an organic, objective point of view, these patients suffer from a dramatic decrease of their quality of life, and the availability of efficient medications thus constitutes an unquestionable progress. Future generations of retinoids are now promising for the treatment of various forms of cancers. In this context, bexarotene is already used in the treatment of CTCL.

However, as it is the case for any efficient medication, the use of both topical and oral retinoids is limited by their undesirable side effects. The knowledge of these adverse events and the factors that predispose to them is a requisite

condition to make the best utilization of these drugs by assessing the benefit/risk ratio for each patient. The main concern for oral retinoids is their potent teratogenicity. Thus, the absolute contraindication is pregnancy or women contemplating becoming pregnant before the plasma concentrations of retinoids return to a safe value. Regarding the teratogenic potential of topical retinoids, all studies performed in humans or animals demonstrated an absence of biologically significant retinoid concentrations in plasma, indicating that topical retinoids should not be teratogenic. However, in general practice, topical retinoid treatments to pregnant women are delayed when possible, and imperative topical treatments are associated with a strict monitoring of plasma retinoid concentrations.

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Retinoid-Induced Hyperlipidemia and the Risk of Atherosclerosis

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INTRODUCTION

Already during the early clinical trials with oral synthetic retinoids in the late 1970s, it was noted that a significant proportion of the patients developed hypertriglyceridemia and hypercholesterolemia. Subsequent studies of the plasma lipoproteins showed that there was a shift of cholesterol from the "good" high-density lipoproteins (HDL) to the "bad" low-density lipoproteins (LDL). This was a major concern because such lipid changes are epidemiologically strongly associated with an increased risk of ischemic heart disease (IHD) and stroke, i.e., diseases, which are secondary to atherosclerosis. However, hyperlipidemia is not the only culprit in the pathogenesis of atherosclerosis. Elevated blood pressure, increased blood clotting, smoking, and chronic or acute inflammation causing smooth muscle cell (SMC) proliferation in the blood vessels, have also been incriminated in this process. Recent findings indicate that some of the contributory factors, such as blood clotting and increased SMC proliferation, may in fact be favorably influenced by retinoids. Thus, the question as to whether or not atherosclerosis is promoted during retinoid therapy cannot be answered solely by looking at its effect on blood lipids. The purpose of this chapter is to review the pharmacodynamics of retinoids in hyperlipidemia and atherogenesis, beginning with a short recapitulation of the normal lipid transport in blood.

This chapter represents an update of a previous review in Retinoids 2004; 29(1):26–30.

LIPID TRANSPORTATION FROM THE GUT TO THE TISSUES

Although dietary fat is important for the intestinal uptake of vitamin A and synthetic retinoids, presumably acting via stimulation of bile secretion, there is no evidence for the reverse phenomenon, i.e., increased fat absorption during retinoid therapy. It is common knowledge that alimentary fat is transported from the gut in voluminous chylomicrons present in the draining lymphatics. The subsequent transport and turnover of plasma lipids is summarized in Figure 1. Initially, fatty acids are split off from the chylomicrons by means of lipoprotein lipases present in muscle and adipose tissues, leaving chylomicron remnants behind that are taken up by the liver (1). The very low-density lipoprotein (VLDL)-particles, which contain most of the serum triglycerides (TG) and some cholesterol esters, are solubilized by apo-B-lipoprotein produced by the liver. Other examples of lipoproteins synthesized by the liver are apo-CIII and apo-E, which will be addressed later in this chapter. Apo-B directs the VLDLs to the target tissues where lipoprotein lipases degrade the TG-core of the particle, resulting in the formation of cholesterol-rich, LDL. In the pool of circulating LDL particles, the smallest ones, i.e., those, which contain a low proportion of cholesterol relative to apo-B-protein, penetrate the blood vessel wall most efficiently and are therefore more atherogenic than larger particles. The essential reverse transport of superfluous cholesterol from the tissues is accomplished by HDL, which contain apo-A1 addressing cholesterol to the liver. Thus a high apo-A1 level, together with a low apo-B/apo-A1 ratio in plasma (normal value 0.7), shows that the net flow of cholesterol is toward the liver and not

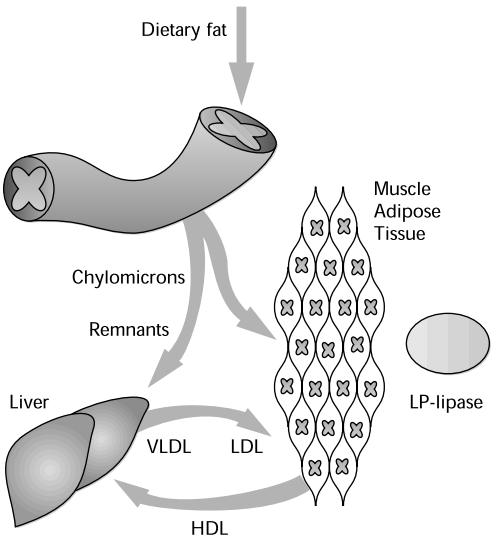

Figure 1 Schematic representation of the lipoprotein metabolism. *Source*: Retinoids & Lipids, 2004.

the peripheral tissues where it may be deleterious (1). Eventually, superfluous cholesterol is metabolized in the liver and excreted via the bile.

RETINOID-INDUCED HYPERLIPIDEMIA

Characteristic Features

In a typical responder, retinoid therapy increases the TG and VLDL blood levels within four to six weeks and concurrently accomplishes a redistribution of cholesterol from the HDL fraction to the LDL fraction. Retinoic acid receptor (RAR)-selective retinoids are particularly prone to cause this effect in a dose-dependent way, but the frequency of responders and the magnitude of changes vary for different drugs and between individuals. For example, in a cross-over comparison of isotretinoin and etretinate, the former drug was clearly a more potent inducer of lipid abnormalities (2). Thus, the average increase of VLDL-TG in psoriasis patients treated for eight weeks with comparable doses of either isotretinoin or etretinate was 37% and 10%, respectively, and the corresponding increments in the LDL/HDL-cholesterol ratio were 33% and 14%. In this study (2), all observed plasma lipid changes reverted to pretreatment values during a drug-free holiday of eight weeks between the two randomized retinoid treatments.

In clinical praxis, only TG and cholesterol levels are usually monitored and blood samples are not routinely drawn from fasting patients. This, together with a variable nutrition and different cut-off values used to define hyperlipidemia, limit the conclusions that can be drawn from population-based studies of the incidence of retinoid-induced dyslipidemia. For example, a recent meta-analysis of serum lipid levels during isotretinoin therapy for acne vulgaris in Northern California ($n = 13{,}772$) showed a cumulative incidence of new abnormalities as high as 44% for TG and 31% for cholesterol (3). The combined United States and European experience, looking at many years of treatment with acitretin and isotretinoin, is that hypertriglyceridemia (i.e., >2 mmol/L or >350 mg/dL) occurs at some point during therapy in about 30% of patients and that TG levels more than twice the baseline are rarely seen (4). Mild TG elevations are always asymptomatic, but could possibly increase the risk of future atherosclerosis if maintained over very long periods of time. More importantly, however, in a small group of patients that cannot readily be predicted (see subsequently), especially isotretinoin may cause acute hypertriglyceridemia (>10 mmol/L) (3,4), which in rare instances may precipitate eruptive xanthomas and pancreatitis (5).

Slightly elevated total serum cholesterol (6–7 mmol/L or 250–300 mg/dL) is commonly observed during retinoid therapy, but overt hypercholesterolemia (>7.5 mmol/L) is only rarely induced (<5% of patients) (2–4). However the LDL/HDL ratio is frequently raised during therapy, which was a major concern when discovered in long-term treated patients, because in large population studies a high LDL/HDL (and apo-B/apo-A1) ratio is strongly correlated to an augmented risk of IHD (1,6). Since especially etretinate, acitretin and bexarotene are used to

treat chronic skin disorders in elderly patients with pre-existing risk factors for IHD, the occurrence of retinoid-induced hyperlipidemia in such patients should always alert the physician. On the other hand, isotretinoin used for short periods to treat acne patients, who are otherwise healthy will probably add very little to the future risk of atherosclerosis even when hyperlipidemia is induced.

Mechanism of Action in Humans

The exact cause of the retinoid-induced lipid changes is not fully understood, but it probably reflects a series of disturbances in the complex lipoprotein metabolism. There is plenty of evidence suggesting that retinoids both increase the synthesis of TG-rich VLDL-particles and at the same time retard their elimination from the circulation (7–8). Although the molecular and cellular aspects of these effects have been mainly studied in animal experiments (see below), available human data suggest that retinoids primarily retard the elimination of serum lipids (9). Thus, when the fat elimination capacity was studied by an intravenous fat tolerance test, the clearance rate decreased especially in isotretinoin-treated patients, and this was associated with a decreased lipoprotein lipase activity in skeletal muscle (9). Interestingly, retinoids also increase the expression of apo-C-III, an antagonist of plasma TG catabolism in man; in one study, isotretinoin treatment (80 mg/day for five days) resulted in clearly elevated plasma apo-CIII (but not apo-E) concentrations (10). Likewise, human hepatoma HepG2 cells showed increased apo-CIII mRNA and protein production when stimulated with a retinoid X receptor (RXR) agonist (10). Several lines of evidence thus incriminate apo-CIII as contributory factor in retinoid-induced hypertriglyceridemia.

A matter of great concern is whether or not the hyperlipidemic response is genetically predicted and thus potentially avoidable. A clue in this direction comes from a recent study by Rodondi et al. (11). The authors hypothesize that familial combined hyperlipidemia, isolated or as part of the metabolic syndrome, might be the underlying genetic defect of retinoid-induced hyperlipidemia and that isotretinoin treatment is able to unmask this familial predisposition. From medical records of 613 persons with acne who had been treated with isotretionin for at least four weeks, Rondom et al. identified 601 individuals for whom fasting plasma lipid levels had been measured once before starting the therapy and monitored on a monthly basis. Of 583 persons without pretreatment hyperlipidemia, 117 were hyper-responders and 145 were nonresponders with respect to retinoid-induced hyperlipidemia. All participants were re-evaluated on average four years after completion of therapy (median age 26 years), showing that an elevation of $\geq$ 1.0 mmol/L of plasma triglyceride levels during isotretinoin therapy was associated with an increased risk for future hypertriglyceridemia, hypercholesterolemia, truncal obesity, and hyperinsulinemia. Both fathers and mothers of the hyper-responders had higher plasma triglyceride levels and a higher ratio of total cholesterol to HDL cholesterol than fathers and mothers of nonresponders. This suggests that the hyperlipidemic response is indeed genetically determined and

that individuals with a family history of hyperlipidemia should only be treated with isotretinoin—and probably other retinoids as well—on strong indication and under strict surveillance with regard to serum lipid elevations. It should be stressed, however, that pretherapy hyperlipidemia is not always associated with a genetically determined hyper-response to retinoids, but might reflect occult diseases, such as diabetes, renal disease and hypothyroidism. The latter example is intriguing because treatment with a RXR ligand, bexarotene, is known to induce central hypothyroidism, reduced TSH levels, and hyperlipidemia (see subsequently).

Animal Models

Research has benefited from the fact that retinoid-induced hypertriglyceridemia is qualitatively similar in rats and humans, viz. it primarily involves the VLDL fraction, it is readily reversible on withdrawal of treatment, and impaired clearance of TG seems to be a major cause (8,9). Hence rats are used to predict the hyperlipidemia-inducing potency of new retinoid derivatives.

In a recent comparative experiment (12), oral gavage of rats with all-*trans*-RA and 13-*cis*-RA (principal RAR agonists which can be converted to RXR agonists), and 9-*cis*-retinoic acid (a RAR/RXR pan-agonist), caused dose-dependent increases in serum TG at doses that did not cause weight loss or mucocutaneous toxicity. Ro 13-6298 and AGN 190121, two RAR-specific agonists, caused dose-dependent increases in serum TG, although Ro 13-6298 only induced hypertriglyceridemia at weight-suppressive doses. Hypertriglyceridemia induced by AGN 190121 was significantly inhibited by cotreatment with an RAR-selective antagonist, AGN 193109. Two RXR-selective agonists, LG100268 and AGN 191701, failed to induce hypertriglyceridemia up to the highest doses tested, and a structural isomer of AGN 190121 that does not activate RARs or RXRs, AGN 190727, did not induce hypertriglyceridemia.

Taken together, these data suggest that retinoid-induced hypertriglyceridemia is predominantly mediated via RARs, with RXR-specific agonists (rexinoids) being less active in this respect. However, this may not be the whole story; RXR obviously mediates the effects on apo-C-III (see previously) and at least one animal study incriminates RXR in the hyperlipidemic response. Davies et al. (13) have found that administration of a rexinoid, such as LG100268 to normal or diabetic rats results in a rapid increase in serum TG levels. LG100268 has no effect on hepatic TG production but suppresses post-heparin plasma lipoprotein lipase activity suggesting that the hypertriglyceridemia results from diminished peripheral processing of plasma VLDL particles. This effect is independent of changes in lipase mRNA. Somewhat surprisingly and contrary to this observation, another rexinoid (LGD1069 or bexarotene, Targetrin®) was recently shown to decrease triglyceridemia and to increase HDL levels in hypertriglyceridemic rats (14). Yet, one of its most common side effects in man is hypertriglyceridemia (82%) and hypercholesterolemia (30%) (14,15), which is probably secondary to central hypothyroidism (15,16).

Clearly there are discrepancies between animals and humans with respect to retinoid-induced hyperlipidemia and more research is needed before any firm

conclusions can be made about the possible advantage of using RXR- instead of RAR-selective retinoids

Clinical Management of Hyperlipidemia

In most patients with retinoid hyperlipidemia simple measures, such as weight reduction, appropriate dietary modifications (restrictions in fat and alcohol intake), more physical activity, and dose reduction are sufficient to reverse the triglyceride levels despite continued therapy. An adjuvant approach is to supplement the patient with ω-3 fatty acids (from whole fish oil) known to depress the production rate of VLDL (17).

In some patients, more vigorous action needs to be considered before a decision is made about stopping retinoid therapy due to a marked hyperlipidemia. One way of reducing the TG levels especially is to prescribe lipid-lowering fibrates, such as gemfibrozil, which (*i*) reduces hepatic TG production by inhibiting peripheral lipolysis and decreasing hepatic extraction of free fatty acids, and (*ii*) decreases VLDL production by inhibiting the synthesis and increasing the clearance of apolipoprotein B. Thus, in a double-blind placebo controlled study in 14 acitretin-treated psoriasis patients with hypertriglyceridemia, gemfibrozil (300 mg b.i.d. for eight weeks) was found to reduce the TG concentration from a mean value of 3.7 mmol/L to 1.9 mmol/L ($p < 0.01$) without any concomitant change in the LDL/HDL-cholesterol ratio (18). Reassuringly enough it was noted that neither gemfibrozil nor fish oil appears to interfere with the clinical effectiveness of retinoids. However, other lipid-lowering drugs, such as statins (e.g., simvastatin) and resins (e.g., cholestyramine) are probably more effective than gemfibrozil against retinoid-induced hypercholesterolemia, but have not been formally tested for this indication. Theoretically, there may be a risk for drug interactions, since both simvastatin and retinoids can cause myalgia and cholestyramine may interfere with the intestinal absorption of retinoids.

Bexarotene-induced hypertriglyceridemia can be managed successfully using fenofibrate alone or in combination with HMG-coA reductase inhibitors or "statins" (19). Gemfibrozil, on the other hand, was associated with higher bexarotene levels, hypertriglyceridemia, and increased risk for pancreatitis, which is puzzling in view of its good effect against acitretin-induced hyperlipidemia. In managing lymphoma patients treated with bexarotene, the response rates are higher when the triglycerides are adequately controlled, which may require combining of two lipid-lowering agents, such as Tricor® at 160 mg and Lipitor® up to 80 mg daily (19). With this combination, there is a remote possibility of rhabdomyolysis as a potential consequence, so monitoring symptoms and creatine kinase levels is advisable (19). Duvic et al. recommend starting bexarotene in lymphoma patients at a lower dose (75–150 mg) in addition to thyroid replacement and checking weekly fasting TG levels, as well as starting lipid-lowering agents one week prior to starting bexarotene (see Chapter 10).

The author's own advice on how to manage retinoid-induced hyperlipidemia are summarized in Table 1.

Table 1 Recommended Actions Against Retinoid Induced Hyperlipidemia[a]

Ask the patient for any family history of hyperlipidemia, metabolic syndrome or premature death in cardiovascular diseases.
Check pretherapy levels of triglycerine and cholesterol (fasting)—if cholesterol is increased do additional HDL analysis.
If any abnormal lipid values are encountered, check for other risk factors, such as diabetes, hypothyreosis and renal disease.
Repeat fasting blood lipid levels with 3–6 weeks intervals during therapy.
If no hyperlipidemia occurs after 2–3 months of adequate retinoid dosage, repeated check-up of the lipid status is only required with yearly intervals if therapy is continued.
If hyperlipidemia (elevated triglycerides or cholesterol or decreased HDL) is observed, during therapy the actions discussed in the text should be considered.
Check that blood lipid levels return to pretreatment levels within 1–2 months posttherapy.

[a]These recommendations represent the author's personal opinion.
Abbreviation: HDL, high-density lipoprotein.

DO RETINOIDS ALSO IMPEDE ATHEROSCLEROSIS?

Accumulating evidence collected over the last decade suggests that retinoids may exert antiatherosclerosis effects that could in theory mitigate the adverse effects of hyperlipidemia. Specifically, this research is focused on the effect of retinoids on arterial (SMC) proliferation and on the endogenous fibrinolysis system.

Effects on Arterial Smooth Muscle Cells

The growth and differentiation of SMC in the arterial wall are interrelated processes, which are highly relevant in the pathogenesis of vascular occlusive diseases including IHD. For example, stimulated growth of SMC due to inflammation or trauma of arteries may narrow the lumen of the vessels, especially when combined with other risk factors for atherosclerosis. Interestingly enough there is ample in vitro data demonstrating that retinoids antagonize growth factor-stimulated SMC hyperplasia and in some cases promote a more differentiated SMC phenotype (20), effects, which are probably mediated by retinoid receptor-mediated changes in the SMC transcriptome. Indeed, improved vessel wall geometry after vascular injury has been documented in retinoid-treated animals, and this effect includes attenuation in the neointimal mass, an outward remodeling of the vessel wall and accelerated re-endothelialization (20). This might prove to be one mechanism by which retinoids actually mitigate the pro-atherogenic effects of hyperlipidemia. However, more in vivo studies are needed to support this hypothesis.

Effects on Endogenous Fibrinolysis and Hemostasis

The fibrinolytic system is an important protective mechanism working against intravascular coagulation and thrombosis. Drugs that enhance the endogenous fibrinolytic activity might therefore be of value in preventing thrombosis-associated

diseases, such as atherosclerosis and IHD. Retinoids seem to have this propensity. The most dramatic example of their interference with the hemostatic systems comes from treatment of acute promyelocytic leukemia (APL) with all-*trans*-retinoic acid (ATRA). ATRA induces a complete remission in >90% of patients with APL and rapidly resolves the life-threatening intravascular clotting typical of this disease. As shown by several groups, ATRA interferes with various hemostatic mechanisms of both malignant and normal (endothelial and monocytic) cells (21) and counteracts the procoagulant action of cytokines on the endothelium as well as increases the expression of fibrinolysis proteins and the anticoagulant thrombomodulin by endothelial cells. Although, the effects appear to be most dramatic in APL, a preliminary study shows that ATRA also mitigates the hypercoagulable state in breast cancer patients treated with tamoxifen (22). But is there any evidence that retinoids also affect fibrinolysis in dermatological patients who are usually not in the hypercoagulable state? Yes, but this requires a short explanation about the fibrinolytic system.

Under normal conditions, the availability and activity of plasminogen activators (PAs) are key factors in determining the fibrinolytic capacity of plasma. Two distinct PAs are known, urokinase-type PA (u-PA) and tissue-type PA (t-PA). The local concentration of active (free) PA is determined, in part, by the local concentration of PA inhibitor type-1 (PAI-1). Stimulation of endogenous fibrinolysis could therefore be directed at pharmacological upregulation of t-PA or u-PA synthesis or down-regulation of PAI-1. Recently it was shown that several retinoids enhance fibrinolytic activity in cultured medium of human endothelial cells by increasing t-PA synthesis without affecting PAI-1 synthesis (23). The mechanism of this enhanced t-PA synthesis occurs most likely via nuclear receptor proteins (RARs).

In dermatologic patients treated with either isotretinoin or etretinate/acitretin, enhanced baseline t-PA plasma concentrations have been noted whereas the PAI-1 and von Willebrand factors were unchanged (24). This fits with the clinical observation that retinoid-treated patients often suffer from nose bleedings that are only partially explainable by mucous membrane toxicity. Thus, enhanced fibrinolysis might be another mechanism by which retinoids actually reduce the risk of thromboembolic complications associated with hyperlipidemia-induced atherosclerosis.

CONCLUSIONS

Retinoid-induced hyperlipidemia that is mainly due to a combination of decreased tissue lipase activity and increased hepatic production of triglycerides, is especially prevalent during treatment with RAR-selective retinoids. But drugs differ in their potency to induce such effects and the variation in the individual response is remarkable and possibly genetically determined. Specifically, hypertriglyceridemia is prevalent in tretinoin- and isotretinoin-treated patients, but also in bexarotene-treated patients who frequently develop a central hypothyroidism.

Admittedly, hypertriglyceridemia is less overtly coupled to IHD than is hypercholesterolemia, but an accumulation of triglycerides to more than five times the upper normal limit may elicit acute problems, such as pancreatitis and eruptive xanthomas.

Retinoid-induced hypercholesterolemia, although usually less dramatic than hypertriglyceridemia, is often associated with an increase in the LDL/HDL (apoB/apoA-1) ratio, which is epidemiologically strongly associated with an augmented lifetime risk of cardiovascular disease. The concern about retinoid-induced hyperlipidemia is therefore valid and should be taken seriously especially when a patient has other risk factors for atherosclerosis, such as pre-exisiting familiar hyperlipidemia, diabetes, hypertension, heavy smoking, obesity and chronic renal failure.

Reassuringly, retinoids also exert positive effects on some aspects of atherosclerosis. For example, they retard smooth muscle proliferation in the vessel wall and increase fibrinolysis via stimulation of the plasminogen activity. Consequently, retinoid therapy seems to elicit a combination of both a negative effect on blood lipids and a positive impact on other aspects of atherosclerosis. Presumably, the relative weights of these opposing effects will individually determine whether or not retinoid therapy will augment the risk of atherosclerosis in the long run (Fig. 2). Clearly, long-term studies comparing the risk of cardiovascular diseases in large cohorts of retinoid-treated patients and age- and sex-matched controls are needed to prove this theory.

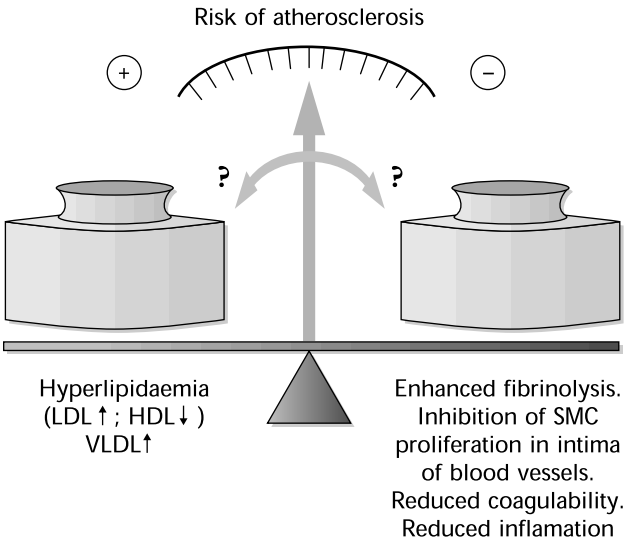

Figure 2 Summary of putative negative and positive effects of retinoid therapy that might influence the patient's future risk for ischemic heart disease and stroke. The weight of each factor varies from one person to another and the net-balance will probably determine whether the risk of atherosclerosis is increased or not. *Source*: Retinoids & Lipids, 2004.

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15

Carotenoids and the Skin: An Overview

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INRODUCTION

Carotenoids are a class of compounds which has never been identified as being essential for humans; there are no studies indicating that a completely carotenoid-free diet causes deficiency symptoms as long as preformed vitamin A is available in the food. Yet overwhelming evidence indicates that carotenoids play an important role in protecting the organism from many harmful reactions and noxious agents (for review see Chapters 16, 18 and 19). This applies also to the skin where carotenoids function both as potential provitamins A (e.g., beta-carotene and lutein) and as antioxidants or cellular signaling agents of particular importance in relation to UV irradiation, skin aging and carcinogenesis (see Chapters 17 and 18).

The visible proof that dietary carotenoids eventually accumulate in the skin is the yellow-orange discoloration (carotenoderma), which regularly accompanies hypercarotenemia due to excessive intake of carotenoids. Indeed, the color spectrum of carotenoderma is a reflection of the carotenoid composition in the diet. For example, massive ingestion of beta-carotene results in a carrot-like discoloration of the skin, whereas ingestion of a brownish carotenoid, such as canthaxanthin, results in a more agreeable hue that resembles suntan. Regrettably, oral canthaxantin has been shown to crystallize in the retina and impair night vision, which prohibits its use in humans. Apart from this exceptional toxicity, carotenoids are generally well tolerated even when given in high doses, thus contrasting with the more toxic retinoids.

The purpose of this chapter is to review current methods for analyzing carotenoids in the integument and to give a general background about the deposition and function of natural carotenoids in normal and diseased human skin.

ANALYSIS OF CAROTENOIDS

There are numerous reasons for analyzing carotenoids in the skin: (*i*) skin is the most accessible tissue in humans and its carotenoid content often parallels that of other internal organs, which can be useful when studying the epidemiologic relationship between nutrition and, for example, the risk of cancer, (*ii*) analysis of skin carotenoids following experimental exposure to various stress factors (e.g., topical oxidants and sunburn) may shed further light on their role in skin homeostasis, (*iii*) findings of abnormal carotenoid levels in association with skin disease might give valuable hints about the pathogenesis of the disease and suggestions for new therapies, and (*iv*) monitoring of carotenoid levels during oral or topical carotene therapy may establish the dose-effect relationships and improve our understanding of how carotenoids work in human skin.

The first identification of carotenoids in human skin was made in the 1970s using spectrometric analysis on saponified extracts of whole skin samples (1). A typical absorption peak with a broad maximum around 450 nm was observed also in subsequent studies in the 1980s (2,3), but no detailed information about the carotenoid composition was obtained by this crude approach. Nevertheless a normal "carotene" level of about 1 nmol/g wet tissue or 10 μg/g protein was soon established (1–3), but the individual values ranged from almost nil to more than 10 nmol/g (corresponding to 0.5–30 μg/g protein) (2).

The development of more sophisticated techniques, such as high-performance liquid chromatography (HPLC), made it possible to analyze carotenoids with high precision in nonsaponified skin extracts (4–7). When combined with a careful dissection of the sample into well-defined tissue compartments (subcutis, dermis and epidermis), HPLC is now the golden standard for quantifying individual carotenoids in the skin. However it is a laborious method which requires invasive sampling.

For ethical and practical reasons, noninvasive methods for measuring carotenoids in the skin are clearly preferable, especially when studying large cohorts of subjects. The earliest such methods were based on photoacustic depth profile analysis (8) and remittance measurement (= reflection spectrometry) of skin color (9,10). Only the latter method has been used lately and it demonstrates a good correlation between plasma and skin carotenoid levels (10). Alas, the information obtained about the carotenoid composition is meager and mainly reflects the superficial epidermis where absorption by melanin represents a confounding factor.

Raman spectroscopy, which is a sensitive and highly specific form of vibrational spectroscopy, represents the latest development in this field (11–12). Carotenoid molecules are especially suitable for Raman measurements because they can be excited with light overlapping their visible absorption band. This

makes detection of the characteristic vibrational energies possible also in complex biologic systems by using laser beams.

Also under field-like conditions, Raman spectroscopy can be used to detect carotenoids simply by applying a laser-equipped instrument to the skin surface and reading the result on a display. Advocates of the method (12) claim that individual carotenoids can be adequately quantified down to a depth of 250 μm, roughly corresponding to the thickness of epidermis in nonpalmoplantar skin. Opponents (13) say that: (*i*) the linearity of the method is not proven, (*ii*) the Raman signal will be proportionally greater for carotenoids located closer to the skin surface, and (*iii*) a depth of 250 μm is not constantly attained in various types of skin and will also represent widely different tissues when comparing, for example, plantar skin (with a several mm thick horny layer) to atrophic, nonglabrous skin (with an epidermal thickness of sometimes less than 100 μm). More research comparing invasive HPLC analysis of carotenoids and Raman spectrometry data obtained in the same skin samples is clearly needed to resolve this ambiguity.

DISTRIBUTION OF CAROTENOIDS IN THE SKIN

There are several independent studies showing that the most prevalent carotenoids in normal human skin are lycopene, beta-carotene and various types of xanthophylls (oxidized carotenoids), such as lutein and zeaxanthin (7,11,12). The structural formula of these compounds (see Chapter 16) disclose a series of conjugated double bonds and in some cases a terminal ring structure together explaining their variable antioxidant and provitamin A properties. The principal food source of lycopene is tomatoes, whereas beta-carotene is more abundant in other vegetables and fruits, e.g., carrots, spinach, and apricots.

By HPLC analysis, the concentrations of lycopene and carotene in human skin (epidermis plus dermis) are usually 0.2–0.3 nmol/g ww, whereas the xanthophylls occur at much lower levels (7).

The exact location of the carotenoid pigments within human skin is still a matter of debate. Old textbooks in dermatology usually say that carotenoids are secreted via sweat and hence "contaminate" the skin surface. However spectometric analysis of lipids collected from the skin surface show only minor amounts of carotenoids (2), clearly insufficient to account for a reverse transport of carotenoids from the skin surface as a major source of epidermal carotenoids. Instead, carotenoids should probably be regarded as natural ingredients of the skin delivered by low-density lipoprotein (LDL), which is the principal carrier of carotene in human blood (14).

A comparative analysis of carotene and vitamin A levels in various parts of human skin shows that both compounds accumulate in subcutis and epidermis, with substantially lower levels observed in dermis and blood (Fig. 1). Compared to vitamin A in subcutis, the concentration of carotenoids is relatively low, but clearly sufficient to explain the normal yellow color of human adipose tissue. This contrasts the white subcutis in rats and other rodents who do not absorb

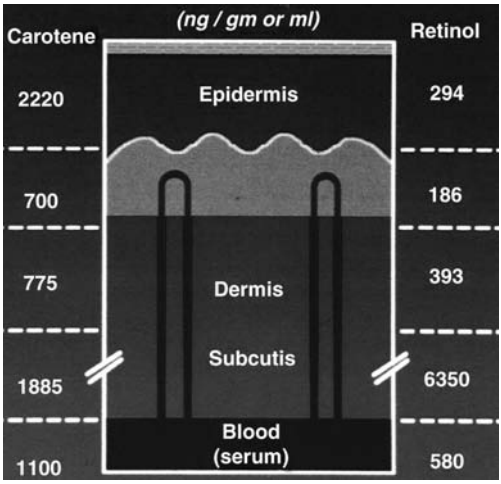

Figure 1 The distribution of carotenoids (in comparison to vitamin A) in different layers of human cadaver skin and blood. *Source*: From Ref. 2.

carotenoids from the gut. It is presently unknown whether human adipocytes actually require carotenoids or simply store them for other purposes.

Compared to the storage in subcutis, epidermal accumulation of carotenoids appears to be more specific and is probably of greater biological significance. Notably keratinocytes must take up carotenoids against a concentration gradient, because the resulting levels are two to four times higher in epidermis than in dermis (1,2). The latter tissue is mainly composed of extracellular matrix, and although dermal fibroblasts have been shown to accumulate carotenoids in vitro (15), they are few and scattered in vivo making them unlikely as storage sites for carotenoids.

When studying the role of carotenoids in the skin, the intraepidermal distribution profiles are of special interest, but regrettably little is known about this matter in humans. The fact that carotenoderma is most conspicuous in areas with a thick horny layer (e.g., palms and knees) (16), points to a skewed distribution of carotenoids with the highest values in upper epidermis. One plausible explanation is that the carotenoid uptake starts in basal cells and continues until cornification without any significant consumption in viable keratinocytes. This theory is supported by studies using the exceptionally thick cow snout epidermis as model and analyzing the tangentially cut slices by HPLC (17). Figure 2 shows that, in contrast to vitamin A, which is concentrated in the basal layer, the highest beta-carotene levels are observed in mid-epidermis where keratinocytes undergo terminal differentiation. The low concentrations of both vitamin A and beta-carotene in the keratotic (dry) layers of epidermis may be somewhat spurious because values are expressed per μg of tissue protein (instead of wet weight). Needless to say, human horny layer and cow snout stratum corneum also function

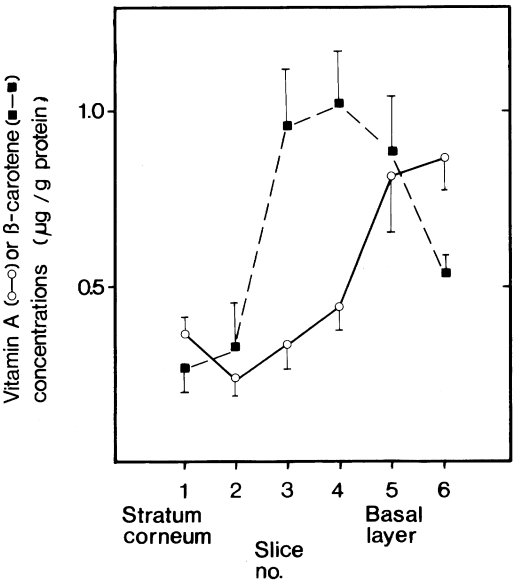

Figure 2 The concentrations of beta-carotene and vitamin A (retinol) in consecutive layers of horizontally cut pigmented cow snouts analyzed by high-performance liquid chromatography of skin extracts. *Source*: From Ref. 17.

quite differently and any extrapolation from the bovine to the human situation must be made with care.

While keratinocytes are undoubtedly the key players in epidermis, accounting for >95% of the total cell mass, pigment cells distributed along the basal membrane may also contribute to the uptake and storage of carotenoids. In fact melanocytes, and probably also melanin pigment released to adjacent keratinocytes, appear to have a special affinity for beta-carotene. Thus, its concentration is higher in pigmented versus unpigmented snout epidermis and in normal versus vitiligous human skin lacking melanocytes (A. Vahlquist, unpublished observation). Because melanin is a known scavenger for various xenobiotics, it may be speculated that beta-carotene is needed in a close proximity to quench noxious photoreactions.

In cell culture experiments with radio-labeled beta-carotene added to the medium, uptake of the tracer has been demonstrated in human melanocytes and keratinocytes (18). Interestingly, both cell types are also able to convert beta-carotene to retinol (18), a metabolic reaction catalyzed by 15,15-beta-dioxygenase. This pathway probably provides an alternative to retinol-binding protein (RBP)-mediated delivery of retinol to epidermis (19). In analogy, it may be speculated that lutein in human epidermis is converted to 3,4-didehydroretinol (vitamin A2) by the same mechanism, thus representing an alternative to its biosynthesis from

retinol (20). Additional evidence for a functional 15,15-beta-dioxygenase activity in human epidermis comes from the demonstration of increased retinyl ester concentrations after topical application of beta-carotene (21). However, under physiologic conditions, the enzymatic activity is probably under homeostatic control in order to prevent excessive formation of retinol in the skin. Thus, when oral beta-carotene was given to psoriasis patients (400 mg per day for six weeks) the epidermal carotene level increased on average 170% without any concomitant change in the vitamin A level (22).

CAROTENODERMA

Slightly yellow skin pigmentation (xanthoderma) is commonly observed in healthy Caucasians and usually reflects a high intake of food pigments, such as saffron, picric acid, mepacrine and mixtures of carotenoids (16). More seldom, xanthoderma signals a pathologic condition, such as hepatic disease with jaundice and icteric sclerae due to increased bilirubine levels in blood. Carotenoderma, on the other hand, has a different shade and there is no discoloration of sclerae. This condition should be suspected if a person is a vegetarian or takes tablets containing carotene (16). Overt cases of carotenoderma should however always be examined further, because a high intake of certain vegetables may be associated with leukopenia and hormonal disturbances due to food ingredients other than carotenoids (23). Thankfully, natural carotenoids per se have a low toxicity; even a massive intake of beta-carotene carries no risk of vitamin A toxicity because 15,15-beta-dioxygenase in the gut is rate limiting for the production of retinol. However, it should be recalled that this enzymatic process always involves retinaldehyde as intermediate, which can be further oxidized to retinoic acid and hence appear in the blood.

In the absence of an obvious dietary explanation for carotenemia and carotenederma, the following causes should be considered: (*i*) hyperlipidemia leading to an accumulation of LDL-bound carotenoids in the blood, (*ii*) hypothyreosis reducing the 15,15-beta-dioxygenase activity in the gut and allowing more beta-carotene to be absorbed unchanged in the blood, and (*iii*) a genetic deficiency of 15,15-beta-dioxygenase, which may even result in vitamin A deficiency unless preformed retinol is available in the food (16,24). The genetic case is a rare condition often presenting in early childhood as "yellow baby syndrome." It can easily be diagnosed by giving the patient a single dose of oral beta-carotene in the morning and monitoring the blood levels for up to eight hours (25). Compared to healthy controls, the patient's blood will show elevated beta-carotene values within a couple of hours. The accumulation of carotenoids in the skin is a slower process; however, it usually takes three to five weeks of high carotenoid intake before carotenoderma develops (9,10,26). The elimination of carotenoids from the skin is a slow process as well; after stopping an excessive intake, it usually takes many weeks before carotenoderma vanishes (9,26). The elimination of carotenoids occurs via oxidation to more water-soluble metabolites, which are then excreted in urine and bile.

CAROTENOID LEVELS IN PATHOLOGICAL SKIN

There are only few reports on carotenoid levels in association with skin disease. Most of them were performed in the 1980s and are based on spectophotometric analysis of skin extracts (3,22,27–28). The overall results show epidermal carotenoids levels to be either in the normal range (acne, eczema, ichthyosis, lichen planus, Darier's disease) or somewhat lower than normal (psoriasis, uremic pruritus). Furthermore, no significant differences were found between the patients' lesional and nonlesional skin, e.g., in psoriasis (22).

Recently, Bruch-Gerharz et al. (29) using HPLC demonstrated is an accumulation of lutein in skin amyloid deposits of systemic amyloidosis. Clearly, additional HPLC studies are needed to clarify a possible relationship between skin carotenoid levels and the pathogenesis of skin diseases.

Incidentally, the almost normal skin carotenoid values found in chronic renal failure patients (3) do not support the frequently expressed opinion that accumulation of "carotene-like pigments" explains the yellow hue in many hemodialysis patients. It is interesting to note in this context that UV-therapy for uremic pruritus lowers the epidermal levels of carotenoids by about 20% to 30 % (30). However, the UV-induced reduction of epidermal vitamin A is much more pronounced and probably of greater importance in both hemodialysis patients and healthy control skin (30,31).

CONCLUSIONS

Except for carotenoderma, which is usually a harmless symptom, there is no known skin condition or disease that is specifically associated with abnormal carotenoid nutrition. Yet carotenoids play an important role in the skin both as a potential precursor of vitamin A and as antioxidants of special importance in epidermis that is heavily exposed to UV radiation and oxidative stress.

Analysis of skin carotenoids can be helpful when monitoring a subject's nutritional status or when trying to decipher the function of skin carotenoids under various pathophysiologic conditions and during pharmacologic intervention with carotenoids. HPLC is currently the best method for analyzing carotenoids in biopsy samples, but noninvasive techniques, such as Raman spectrometry, are on the march and if proven reliable they will probably replace the more tedious HPLC analyses especially in studies of large cohorts of subjects. Meanwhile the increased interest in carotenoids as therapy or prophylaxis for skin diseases will hopefully result in new indications and more knowledge about their general role in skin biology.

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16

Antioxidative Effects of Carotenoids

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INTRODUCTION

There have been numerous reviews that have appeared in the last few years detailing various aspects of the antioxidant action of carotenoids (1–3), although the existence of an antioxidative effect has been questioned by some (4).

This chapter will focus on recent studies on the antioxidative actions of carotenoids by discussing the carotenoid–radical interaction, and interactions of carotenoids with other antioxidants. Currently available assays to determine carotenoid status and various biomarkers to determine antioxidative actions of carotenoids will be provided. In addition, evidence of auto-oxidation and co-oxidation of carotenoids will be discussed. Finally, clinical applications of carotenoids in skin are discussed in this review.

DETERMINATION OF CAROTENOID STATUS

High-Performance Liquid Chromatography

The carotenoid concentrations in biological samples can be determined by an high-performance liquid chromatography (HPLC) analysis (5). Advances in

technology allow analyzing various major carotenoid isomers present in both circulation and in skin adequately as shown in Figure 1. The process requires an extraction of the lipid components of the biological sample using organic solvents such as tetrahydrofuran and hexane prior to HPLC analysis.

It is well known that the levels of carotenoids in the circulation can be readily altered by either increasing or decreasing the number of servings of fruits and vegetables in the diet (6,7) or by carotenoid supplements (8–10). The specific biological roles of the oxygenated carotenoids, lutein and zeaxanthin, against eye diseases such as cataract (11) and age-related macular degeneration (12) have been intensively studied by identifying these carotenoids in the both macula (13) and lens (14,15) of the eye in humans.

Spectroscopy

There are several spectroscopic techniques that can be used to identify and quantify carotenoids in plasma/serum or tissues. These techniques include UV/VIS absorption spectroscopy, mass spectrometry, nuclear magnetic resonance spectroscopy, infrared analysis and circular dichroism. For the purposes of this chapter, the most important is UV/VIS absorption spectroscopy, from which one can derive the number of conjugated double bonds, the absence or presence of *cis* bonds found in *cis*-isomers, and the presence of conjugated carbonyl groups. Figure 2 presents the structures of some of the major carotenoids found in human tissues and plasma, as seen in the HPLC pattern in Figure 1. These compounds are 40-carbon hydrocarbons or alcohols, and the chromophore consists of the

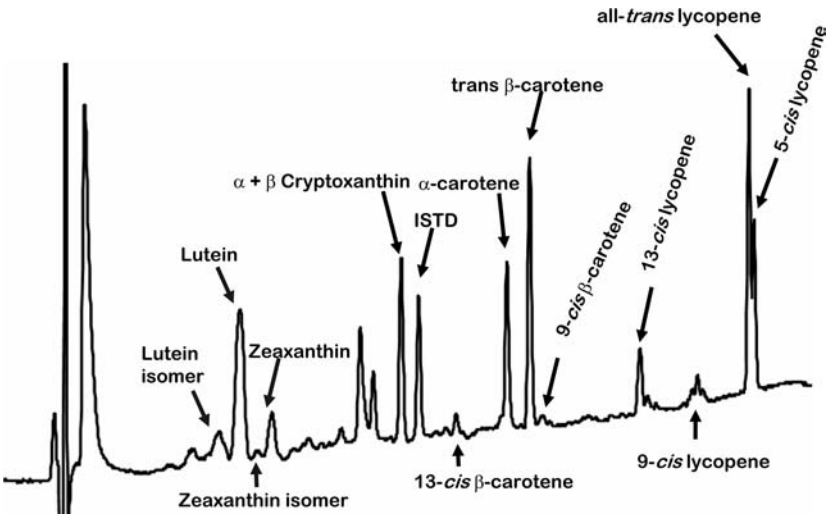

Figure 1 High-performance liquid chromatography profile of carotenoids in human serum.

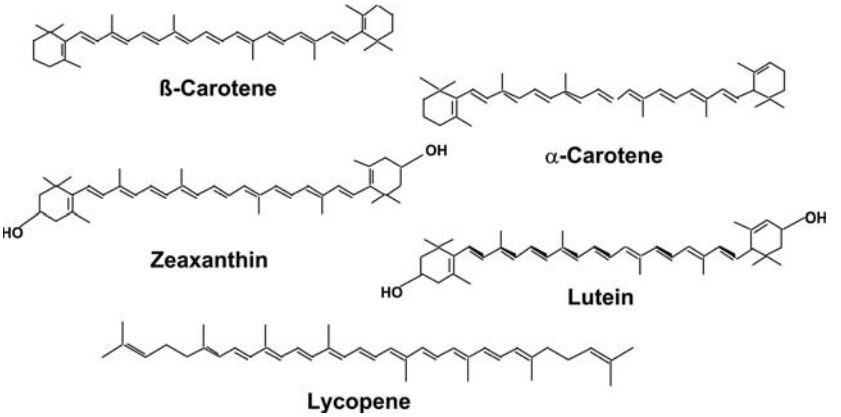

Figure 2 Carotenoid structures.

conjugated double bond system. Although many of the structures look very similar, it is possible to detect the difference between β-carotene or zeaxanthin, which each have a chromophore of 11 conjugated double bonds, and α-carotene or lutein, which have a chromophore of 10 conjugated double bonds, simply from the visible spectrum.

The spectrum of an 11 conjugated double bond carotenoid, zeaxanthin, is compared with the spectrum of a 10 conjugated double bond carotenoid, lutein, in Figure 3. Thus, it is possible to identify carotenoids based on their spectra, as well as on other important chemical properties.

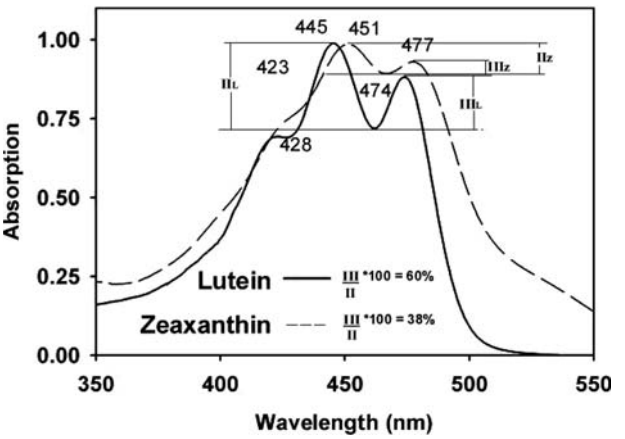

Figure 3 Absorption spectra of lutein and zeaxanthin. *Source*: From Ref. 112.

DETERMINATION OF ANTIOXIDANT CAPACITY/OXIDATIVE STRESS

An antioxidant is any compound that inhibits oxidations induced either spontaneously or by means of external oxidants. This is a relatively simple definition, but at times, it becomes very difficult to evaluate whether a compound actually has an antioxidant action, particularly in vivo. There have been many reports concerning the relative antioxidant efficacy of carotenoids, with varying results. Part of the problem with attempting to evaluate efficacy is that very different systems have been used to dissolve the carotenoids, initiate oxidative stress, and then evaluate efficacy. Therefore, there is probably no one system that would accurately determine the antioxidant/pro-oxidant activities of carotenoids.

Total Antioxidant Capacity

An assay to measure total antioxidant capacity in biological sample should consider the heterogeneity of plasma consisting of both hydrophilic and lipophilic compartments and containing water-soluble and fat-soluble antioxidants, respectively. In addition, the cooperative/synergistic interactions among antioxidants in biological samples cannot be overlooked.

Investigators have continuously failed to show a direct correlation between the physiologic consumption of dietary carotenoids and subsequent changes in antioxidant capacity in humans (16). It has even been suggested that carotenoids may not act as antioxidants in vivo (4). However, these suggestions derive from the lack of a proper analytical method for measuring antioxidant capacity. In as much as conventional methods, total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC), and so on, use primarily hydrophilic radical generators and measure primarily antioxidant capacity in the aqueous compartment of plasma, these methods are unable to determine the antioxidant capacity of the lipid compartment (17,18). This can be explained by considering that plasma carotenoids, being deeply embedded in the core of lipoproteins, are not available for reaction with aqueous radical species or ferric complexes used in these assays.

When the hydrophilic approaches were applied to determine antioxidant capacity in plasma, the majority of antioxidant capacity of plasma was accounted for by protein (10–28%), uric acid (7–58%), and ascorbic acid (3–27%), while the effect of vitamin E (<10%) was minimal from estimates reported earlier (19–21). That is, these assays measure the antioxidant capacity of the aqueous compartment only, since the radical inducers and probes are all hydrophilic. α-Tocopherol, which has its chroman head group oriented toward the boundary between the lipoprotein and the aqueous mileau, may participate somewhat in the antioxidant action through interaction with water-soluble antioxidants such as ascorbic acid. The lack of contribution of fat-soluble antioxidants can also be ascribed to the relatively lower amount of fat-soluble antioxidants in plasma as compared to that of water-soluble antioxidants, although it should be recognized that the antioxidant activity of fat-soluble antioxidants could be greatly enhanced by synergistic

interactions with water-soluble and fat-soluble antioxidants. In addition, it is noteworthy that there is considerable and consistent in vitro evidence for antioxidant function of carotenoids (3,22) and their geometrical isomers (23) when tested in solvent systems.

Lipid Peroxidation

For many years, determination of thiobarbituric acid-reactive substances (TBARS) such as malondialdehyde (MDA) was assumed to be a valid measure of lipid peroxidation, but this is a somewhat nonspecific biomarker. Nevertheless, changes in MDA levels have been used to evaluate the effects of added or deleted dietary or supplementary nutrients such as carotenoids where an oxidative stress might arise (24). Kikugawa et al. have demonstrated (25) that the oxidation of β-carotene by either nitrogen dioxide or oxygen itself results in measurable TBARS activity. Dixon and her associates (24) put women on carotenoid-deficient diets, and observed an increase in plasma MDA levels. This effect could be reversed when the diets were supplemented with a mixture of carotenoids, strongly supporting the idea that dietary carotenoids can serve to decrease oxidative stress in humans. In fact, a recent report showing a significant decreased oxidizability by high fruit and vegetable diets followed by a significant increase in oxidizability by a low fruit and vegetable diet supports the antioxidant activity of carotenoids (6). However, it should be noted that any dialdehyde in the plasma can react with the thiobarbituric acid resulting in increased MDA concentrations.

Measurements of prostaglandin F_2-like compounds (F_2-isoprostanes), which are produced in vivo by nonenzymatic free radical catalyzed peroxidation of arachidonic acid, have emerged as one of the most reliable approaches to assess oxidative stress status (26,27). It is generally accepted that F_2-isoprostanes more accurately reflect in vivo lipid peroxidation than that of thiobarbituric acid substances (28) and that F_2-isoprostane concentrations can be reduced by dietary antioxidant supplements (29). However, the complicated technique to measure isoprostanes and their unstable nature in biological samples still needs to be overcome. Recent efforts (30) to determine plasma isoprostanes using HPLC and tandem mass spectrometry (MS) may reduce the experimental error driven by sample preparation steps including derivatization by traditional gas chromatography (GC)–MS or GC-MS-MS.

Low-Density Lipoprotein Oxidation

Recently, antioxidant properties of bioactive components of food including vitamins E and C, polyphenols and carotenoids on the low-density lipoprotein (LDL) oxidation has been extensively reviewed (31). The general approach to measure antioxidant capacity in the lipid compartment of plasma is to determine the oxidizability of isolated LDL using hydrophilic (AAPH, transition metal ions) or lipophilic radical inducers such as 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN). Lipid peroxidation can be monitored by conjugated diene production

(234 nm) (32), 2',7'-dichlorodihydrofluoresein (DCFH) oxidation or diphenyl-1-pyrenylphosphine (DPPP) oxidation to produce the fluorescent product, DCF and DPPP oxide respectively (33). By using lipophilic AMVN as a radical initiator and luminol as an oxidizable substrate, the percentage contribution of the fat-soluble antioxidants to the antioxidant activity of isolated LDL has been shown to be greater than 70% (tocopherol 73%; ubiquinol-10 2.5%) (34). However, this approach is limited since it does not take into account the potential interaction between water-soluble and fat-soluble antioxidants, a synergism that greatly increases the total antioxidant activity.

When isolated LDL particles, enriched with carotenoids through dietary intervention with fruits and vegetables or by supplementation with carotenoids, are exposed to oxidants, they are reported to be protected against oxidation (35,36). Chopra et al. reported that supplementation with green vegetables does not protect LDL in either smokers or nonsmokers, whereas red vegetable supplementation was protective only in nonsmokers, and not in smokers (37). These variable results might be attributed to different lengths of time on the diets, different degrees of changes in the plasma carotenoid levels, and certainly, different study populations. In addition, when fruits and vegetables are added to the diet, not only do plasma carotenoids increase, but vitamin C and other potential antioxidants such as polyphenols and flavonoids may also increase. Therefore, it is very difficult to interpret whether the changes observed in LDL oxidizability are due to an increase in carotenoids or to other components of the fruits and vegetables. In addition, in the studies that looked at carotenoids other than β-carotene, very mixed results were reported. In some cases carotenoids such as lycopene, α-carotene, β-cryptoxanthin, zeaxanthin and lutein were effective antioxidants (38). However, in some studies in which β-carotene was effective, the addition of either lutein or lycopene actually increased LDL oxidation (39). A 12-week period of daily supplementation with either 13 mg lycopene or 112 mg β-carotene resulted in an increase in LDL carotenoids, but no change in LDL oxidizability (40).

Based on the above, the protective effect of dietary or supplemental carotenoids against LDL oxidation is still controversial.

DNA Damage

The single-cell microgel electrophoresis technique, namely the comet assay, was developed to detect DNA single or double strand breaks. The broken DNA fractions result in an increased migration in electrophoresis and form a diffuse DNA substance area which resembles a comet tail after staining (41). Endogenous strand breaks as well as DNA resistance to oxidative stress by treating lymphocytes with hydrogen peroxide (H_2O_2) can be evaluated by the comet assay. Much of the recent material relating to the effects of carotenoids on DNA damage, as well as effects on DNA synthesis and proliferation has been reviewed (42).

The most common product indicative of DNA damage that can be measured in urine and/or blood is 8-hydroxy-2'-deoxyguanosine (8-OHdG), even though

there is still some question as to the relative importance of this marker in terms of evaluating DNA damage due to the lack of baseline value standardization and reliability (43).

The relationship between the consumption of fruits and vegetables and DNA damage has been suggested by the observation of lower DNA damage in the summer than in the winter corresponding to an increased intake of dietary antioxidants (44). However, intervention trials involving increased fruit and vegetable intake have shown mixed results regarding DNA damage. Decreased oxidative DNA damage with 12 servings/day of fruits and vegetables for 14 days has been reported (45) whereas 600 grams of fruit and vegetable consumption for four weeks showed no effect on DNA damage and repair (46) determined by urinary and blood 8-OHdG. Table 1 shows the intervention studies involving carotenoid-rich diets and lymphocyte DNA damage determined by single-cell gel electrophoresis (comet assay). Short-term dietary intervention studies (21 to 26 days) using carotenoid-rich foods such as 600 mL of orange juice for 21 days (47), approximately 25 g of tomato puree for 14 days (48), or for 21 days (49) and/or 150 g of spinach for 21 days (50) resulted in reduced oxidative DNA damage in healthy women. The same was observed in a group of men and women treated with

Table 1 Studies on the Effects of Carotenoid-Rich Fruit and Vegetable Diets on Lymphocyte DNA Damage

Subjects	Intervention	Design	Duration	Outcome (DNA damage)	Reference
Female ($n = 16$, 20–27 yr)	Orange juice (600 mL) + standard diet Standard diet	Cross-over	21 days	Decreased	(47)
Male (25.4 yr) Female (26 yr) ($n = 26$)	Tomato drink (250 mL) Placebo	Cross-over Double blinded	26 days	Decreased	(51)
Female ($n = 10$, 23.1 yr)	Tomato puree 60 g/day Tomato-free diet	Cross-over	21 days	Decreased	(49)
Female ($n = 9$, 25.2 yr)	Spinach 150 g Spinach + tomato puree 25 g	Consecutive	21 days	Decreased	(50)
Male (nonsmoker) ($n = 23$, 27–40 yr)	Tomato juice 330 mL/day, Carrot juice 330 mL/day Spinach powder 10 g/day	Consecutive	14 days each	Decreased	(52)

250 mL of tomato extract drink for 26 days (51). Also, a 14 day intervention with tomato juice, carrot juice or dried spinach powder (52) was reported to be beneficial against basal DNA damage in healthy men. Assuming that the bioavailability in humans of lycopene and lutein supplements are similar to those of pureed and oil-containing tomato-based foods (53) and green leafy vegetables (54), similar biological actions of pure forms could be expected.

The effects of a single carotenoid supplement against DNA damage is still controversial. Collins et al. (55) reported that there was no effect on endogenous DNA damage following supplementation with 15 mg/day each of α/β-carotene, lutein or lycopene for 12 weeks in men and women (25 to 45 years) in a placebo controlled parallel study design. However, it is interesting to note that there was an inverse correlation between total serum carotenoids and oxidized pyrimidines in their study. On the other hand, another study using the same amount of lutein, β-carotene or lycopene supplementation as reported above, each for one week, showed significant increases in DNA repair in younger men and women (24 to 34 years) (56). A recent study reported that there was a significant decrease in basal lymphocyte DNA damage after 15 days supplementation with either 12 mg of a single carotenoid or 12 mg of a combination of carotenoids (4 mg each of lutein, β-carotene and lycopene) in elderly women (50 to 70 years). The protective effect was maintained throughout the study period of 57 days (9). However, it is not yet known whether oxidative DNA damage in blood cells reflects such damage in target tissue.

Carotenoid-rich diets and a combination of carotenoids showed a stronger protective effect against DNA damage than that of single carotenoid. Further studies on the effect of physiological doses of carotenoids in combination with other antioxidants contained in fruits and vegetables on oxidative DNA damage are needed to support the role of a diet high in fruit and vegetables in the prevention of chronic diseases such as cardiovascular diseases and cancer.

ANTIOXIDANT ACTIONS OF CAROTENOIDS

Carotenoid–Radical Interactions

Carotenoids have been reported to react with virtually any radical species likely to be encountered in a biological system. The products of such reactions are frequently short-lived radical species that can decay to more stable products. In some cases, stable adducts can be observed, but in the majority of interactions with radicals, carotenoids break down to degradation products very similar to what is seen with oxidative degradation. It is only recently that the biological activity of these breakdown products has begun to be investigated.

There are at least three possible mechanisms, radical addition, electron transfer to the radical or allylic hydrogen abstraction, for the reaction of carotenoids with radical species as shown in Figure 4. The radical addition/adduct formation was proposed by Burton and Ingold (57). It has been suggested that a

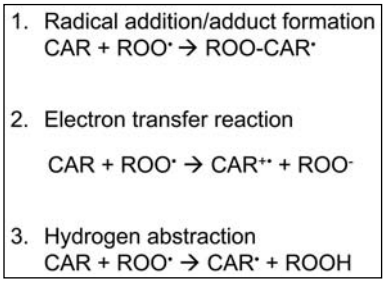

Figure 4 Possible mechanisms of carotenoid and radical interactions.

lipid peroxyl radical (ROO•) might add at any place across the carotenoid (CAR) polyene chain, resulting in the formation of a carbon-centered radical (ROO-CAR•). Since this radical would be resonance-stabilized, it would interfere with the propagating step in lipid peroxidation and would explain the many examples of the antioxidant effect of carotenoids in solution as reviewed by Palozza and Krinsky (3). Electron transfer reactions have been reported, resulting either in the formation of a carotenoid radical such as the cation radical CAR^{+}•, the anion radical CAR^{-}•, or in the formation of an alkyl radical CAR•. The carotenoid cation radical is frequently detected by very fast spectroscopic techniques such as laser flash photolysis. Hydrogen abstraction process has been suggested by Woodall et al. (58) who observed the formation of both the 4-methoxy and 4,4'-dimethoxy derivatives of β-carotene when reacted with either AIBN or AMVN in the presence of small amounts of methanol. Baker et al. reported that β-carotene that was exposed to cigarette smoke, resulted in the production of 4-nitro-β-carotene, also presumably via hydrogen abstraction at the allylic hydrogen (59).

Interactions of Carotenoids with Other Antioxidants and Among Carotenoids

Possible interactions between carotenoids in terms of competition for incorporation into micelles, carotenoid exchange between lipoproteins, and inhibition of provitamin A cleavage has been extensively reviewed by van den Berg (60). Supplementation with 24 mg/day of β-carotene for 12 weeks or an equal amount of a carotenoid mixture containing, lutein, β-carotene and lycopene, ameliorates UV-induced erythema in humans (61). In addition, a protective effect of a carotenoid mixture supplementation (β-carotene, α-carotene, lycopene, lutein, bixin and mixed paprika carotenoids) against LDL oxidation induced by fish oil, has been reported (62). Furthermore, a recent study comparing the effect of equal amounts of individual carotenoids (12 mg each of lutein, β-carotene or lycopene) vs. mixed carotenoids (4 mg each of lutein, β-carotene and lycopene) against lymphocyte DNA damage clearly indicate synergistic interaction among carotenoids in vivo (9).

Interactions of plasma antioxidant nutrients have been extensively studied in the last decade. In particular, work has focused on the interaction between hydrophilic and lipophilic antioxidants, such as ascorbic acid and α-tocopherol (63), carotenoids and ascorbic acid (64) as well as among lipophilic antioxidants (i.e., carotenoids and α-tocopherol) (65,66). It has been reported that β-carotene, which is located in the core of plasma lipoproteins, can directly interact with water-soluble antioxidants. Since β-carotene can be converted to β-carotene peroxyl radical cations by scavenging radical species in a heterogeneous micellar environment (67), the more polar β-carotene radical cation (β-C$^{\bullet+}$) can be reoriented towards the hydrophilic compartment, allowing ascorbic acid to repair the β-carotene radical, as has been discussed by El-Agamey et al. (68). These observations are in line with the work of Burke et al. (64) who showed an interaction between β-carotene radical cations and ascorbic acid.

Antioxidant Actions in Skin

When carotenoids are administered at fairly high doses, they can accumulate in the skin. This phenomenon has been the subject of many investigations to determine if this accumulation can lead to sun protection, which may or not be related to the antioxidant action. The question as to whether dietary or supplemental carotenoids can serve as antioxidants in skin has been reviewed by Stahl and Sies (69), and although there is increasing evidence that these compounds play a role in photoprotection, there is still no direct measurement of that effect. Lee et al. (70) supplemented a group of volunteers for 24 weeks with natural carotene (99% β-carotene) starting at 30 mg/day and increasing to 90 mg/day by the end of the experiment. They observed modest effects of sun protection, but did observe a significant dose-dependent inhibition in a commercial assay for serum lipid peroxidation. It is not clear whether the increased tissue concentrations of carotenoids are directly associated with antioxidant activity, though some authors suggest that the mere presence of carotenoids in the ciliary body, the retinal pigment epithelium, and the choroid of the eye are indicative of antioxidant activity by these compounds (71).

OXIDATION OF CAROTENOIDS

Photodegradation of Carotenoids

There has been very little work on the photodegradation of carotenoids in skin since the pioneering work of Roe in 1987 (72). At that time, and in a subsequent publication (73), Roe et al. demonstrated that irradiating humans with UV light (90% UV-A/10% UV-B) resulted in significant reductions in total plasma carotenoids after two weeks. It is of some interest that the carotenoid level fell much further in women than in men, which they attributed to an increased erythema in the women subjects (73).

Other studies of photodegradation have focused on model systems, such as aqueous dispersions (74) or liposomes (75). In the latter system it was demonstrated that carotenoids photodegrade more rapidly in liposomes than in solvents, and this was attributed to deceased vibrational deactivation in the rigid lipid membrane than in the solvents (75). Since carotenoids are expected to be localized to lipid membranes in the skin, one might expect fairly rapid photodegradation to occur.

One interesting aspect of photodegradation is that oxidized by-products of the carotenoids would be expected to be formed, and Rahman and Parker have investigated some of the biological activities of such products of both β-carotene and lycopene. In their first study, they demonstrated that the photodegradation products inhibited proliferation of human peripheral blood mononuclear cells (76). Their next study demonstrated that these products could inhibit the proliferation of both murine T and B-cells, without affecting cell viability (77). It will prove to be of great interest to elaborate the structures of the photodegraded products that carry out these actions.

Oxidation of β-Carotene

Carotenoids can undergo an oxidation (cleavage) process by either enzymatic reactions (78), cooxidation by lipoxygenase (79,80), autoxidation, or direct reaction with free radicals. Figure 5 shows possible cleavage processes of β-carotene.

Radical-initiated oxidation of carotenoids can be detected by characterizing the products that are formed, which are frequently carbonyls (81) or epoxides (82). Several carbonyls were characterized by Handelman et al. (81), following the autoxidation of β-carotene, or by Stratton et al. (83) when β-carotene was treated with an azo-initiator. More recently Siems et al. proposed an interesting hypothesis on the mechanism responsible for the switching between antioxidant/

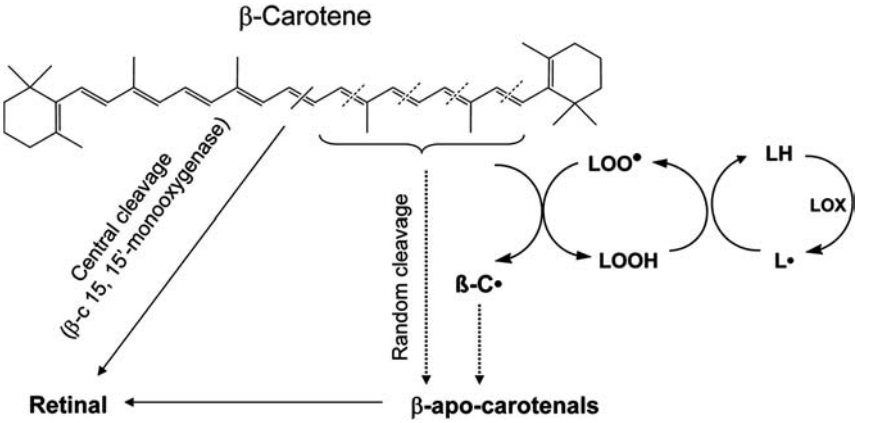

Figure 5 Central and random cleavage products of β-carotene.

pro-oxidant activity of β-carotene (84,85). Under conditions of moderate oxidative stress, the antioxidant effects of β-carotene predominate, but under heavy oxidative stress, β-carotene undergoes an oxidative cleavage leading to the formation of reactive breakdown products that are responsible for the pro-oxidant activity and/or the harmful response of β-carotene. β-Carotene breakdown products include very reactive aldehydes and epoxides such as the high-molecular weight products β-apo-8'-carotenal, β-apo-10'-carotenal, β-apo-12'-carotenal, β-apo-14'-carotenal, β-apo-15'-carotenal, as well as short-chain products, such as β-cyclocitral, β-ionone, 5,6-epoxy-β-ionone and 4-oxo-ionone (84). The chemical reactivity as well as the mechanism of reaction for most of these compounds is still unknown although it has been proposed that the reactivity as well as the biological effects can be expected to be similar to those induced by the reactive aldehydes formed from lipid peroxidation such as 4-hydroxynonenal (HNE) and MDA (84). β-Carotene breakdown products were found to exert several damaging effects such as (*i*) inhibiting state 3 respiration in isolated rat liver mitochondria, which is accompanied by increased oxidative stress in the mitochondria, as reflected by a decrease in GSH and protein SH and an increase in MDA, (*ii*) genotoxic effects (micronuclei and chromosomal aberrations) at sub-micromolar concentrations (86). The hypothesis described above is of particular value since it would explain the pro-oxidant effect induced by a series of different conditions known to induce a pro-oxidant effect of β-carotene. The conditions that induce and sustain the oxidative break-down of carotenoids include O_2 tension, UV and general oxidants, such as smoke and hypochlorous acid (HOCl).

Carotenoids also can be broken down by free radicals produced by enzymes such as lipoxygenase. Grosch et al. (87) found a series of volatile carbonyls formed when carotenoids underwent a co-oxidation with soybean lipoxygenase. Yeum et al. (88) demonstrated that both central and random cleavage of β-carotene can take place in the postmitochondrial fraction of rat intestine, but that the pathway depends on the presence or absence of the antioxidant, α-tocopherol. In their work, the presence of α-tocopherol resulted primarily in central cleavage (i.e., β-carotene was converted to retinal), whereas in the absence of α-tocopherol, both random cleavage and central cleavage took place (i.e., both retinal and β-apo-carotenals were produced).

In the recent work of Caris-Veyart et al. (89), mild oxidative cleavage of β-carotene by oxygen was induced using a ruthenium tetramesitylporphyrin catalyst. This process led to the full range of β-apo-carotenals and β-apo-carotenones indicating the involvement of free radicals to produce random cleavage products. Their observation supports earlier studies which demonstrated the formation of β-apo-carotenals from β-carotene by radical attack (57,81) or singlet oxygen (83), although in these in vitro systems the partial presence of oxygen and the concentration of β-carotene were very high (57,81).

Considering the fact that the stoichiometry of retinal production per mole of β-carotene is 1.72 to 2.00 mol (88,90), and that total amount of the β-apo-carotenoids is <5% of the retinoids formed in the intestine from β-carotene (91), it is certain that the enzymatic central cleavage of β-carotene plays the major role

in β-carotene breakdown under normal conditions when an adequate supply of antioxidants is available. However, under conditions of oxidative stress (such as smoking or diseases associated with oxidative stress such as inflammation) or when high β-carotene concentrations are present, both enzyme-related and radical-induced random cleavage can play a role in β-carotene breakdown. The species and tissue specificities of β-carotene oxidation (cleavage) and the factors controlling cleavage activity remain to be elucidated.

CLINICAL APPLICATIONS OF CAROTENOIDS

Epidemiologic evidence continues to accumulate that diets high in fruits and vegetables are associated with a reduced risk of chronic diseases such as cardiovascular disease (92–95). The evidence for a cancer protective effect is much more ambiguous (93), although part of that conclusion may be due to measurement error (96). It is probable that carotenoids act as a major group of antioxidants in fruits and vegetables thereby preventing damage from harmful reactive oxygen species, which are continuously produced in the body during normal cellular functioning as well as from exogenous sources (97). It is believed that dietary supplementation with antioxidants such as carotenoids can be a part of a protective strategy to minimize the oxidative damage in vulnerable populations, such as the elderly. Carotenoids have in vitro antioxidant activity at physiological oxygen tensions (98). However this antioxidant effect of carotenoids is still controversial and difficult to demonstrate in vivo (10,99). It should be pointed out that the metabolism and functions of carotenoids may differ in an in vivo versus an in vitro system. For example, antioxidant nutrients can interact with each other during gastrointestinal absorption and metabolism (100–103). The in vivo antioxidant system, which is finely balanced, requires possibly an optimal range of both hydrophilic and lipophilic antioxidants to be properly working in biological systems. Carotenoids, located in the core of the lipophilic compartment, may be necessary to properly maintain the antioxidant network.

The biological functions of carotenoids for eye health have been focused on recently. Both epidemiologic and laboratory data consistently indicate an association between lutein and zeaxanthin and the protection of the retina and retinal pigment epithelium from damage by light and oxygen (104,105). For example, Seddon et al. (12) found that individuals ($n = 356$ age-related macular degeneration (AMD) cases, $n = 520$ controls) in the highest quintile of carotenoid intake had a 43% lower risk for AMD compared with those in the lowest quintile, and among the specific carotenoids, lutein and zeaxanthin were most strongly associated with a reduced risk for AMD ($p = 0.001$). Laboratory studies, which had identified the macular pigments as lutein and zeaxanthin, are supportive of these epidemiologic observations (106–108). In a high fruit and vegetable diet, the intake of lutein is more than five times greater than that of zeaxanthin. It has been proposed that lutein and zeaxanthin in the blood are taken up by the retina, where some of the lutein is then converted into zeaxanthin through the intermediate carotenoid, *meso*-zeaxanthin (109,110). It is possible that zeaxanthin, which has more resistant

secondary hydroxyl groups against oxidation than the allylic hydroxyl group of lutein, may be more effective than lutein as an antioxidant in the central macula (106). Lutein circulates in the blood at higher concentrations than zeaxanthin, but the concentration of zeaxanthin in the central macula is higher than that of lutein. However, increased consumption of dietary sources of lutein and zeaxanthin have been shown to increase macular pigment in some, but not all individuals. It has been reported that there was 27% prevalence of non-responders in terms of macular pigment density after the consumption of lutein/zeaxanthin-rich foods such as spinach (111). It is not yet known precisely, which factors affect individual responses to carotenoid supplementation. Further research is required in an effort to determine the optimal way to accomplish an increase in macular pigment.

CONCLUSIONS

There are several methods described to evaluate the antioxidant capacity of carotenoids both in vitro and in vivo. The evidence of an antioxidant action of carotenoids in vitro has been observed in many systems, including homogenous solvents, aqueous dispersions, liposomes, lipoproteins and in tissues. However, the demonstration of an antioxidant action in vivo is somewhat more difficult to observe. Part of this failure to clearly demonstrate this action is due to the relatively small amounts of carotenoids present in tissues such as skin and in serum/plasma. Under all circumstances, the amounts of the hydrophilic antioxidants, ascorbic acid, uric acid, bilirubin, protein-SH, and so on, far exceeds the amounts of carotenoids present. Nevertheless, there has been indirect evidence for an in vivo antioxidant action of carotenoids.

One of the frequently overlooked issues with respect to carotenoids acting as antioxidants is that these labile compounds can undergo oxidative, or cleavage reactions, that generate a whole host of by-products. A large number of these have been identified, but very little is known about their biological activities. Several recent studies have demonstrated that carotenoid oxidation products have a negative effect on cell proliferation and function. This is an area that invites further exploration, particularly in the skin, where the carotenoids are exposed to light and oxygen, the ideal conditions for photodegradation and new product formation. Does high light exposure result in the formation of potentially harmful carotenoid metabolites in the skin? Only future explorations will be able to answer this question.

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Beta-Carotene in the Treatment of Skin Disorders

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INTRODUCTION

Beta-carotene is the major provitamin A carotenoid of nutritional relevance followed by alpha-carotene, lycopene, beta-cryptoxanthin, lutein, zeaxanthin and others from more than 600 known carotenoids taken up from fruit and vegetables. About 50 to 60 of these have provitamin A activity (1,2). The bioavailability of beta-carotene is highly variable and depends on various factors like the amount of carotenoids, the food source, dietary factors like fat content and type as well as inter-individual differences in absorption (3–5). There was no evidence of toxicity found so far (6). The uptake of carotenoids by the skin might additionally be influenced by cigarette smoking and UV-light (7,8).

When it comes to the treatment of skin disorders with beta-carotene there is not much to be found in the available literature. Apart from recommendations for the treatment of erythropoietic protoporphyria (see Chapter 19) there has been an increasing interest in the role of carotenoids in protection from ultraviolet radiation (UVR)-induced oxidative stress, which has been implicated in the processes and development of sunburn, premature photoaging of the skin, certain types of skin cancer and photosensitivity disorders (9) The main covered subjects concerning

carotenoids are various aspects of protection from UVR-induced skin changes, an influence on photoaging and the prophylaxis of photocarcinogenesis.

PHOTOPROTECTION

UVR-Induced Inflammation

UVB radiation (280–320 nm) is mostly absorbed in the epidermis, can directly damage DNA and has as such a strong mutagenic potential (10). UVA1 + 2 radiation (320–400 nm) penetrates deeper, can interact with epidermal keratinocytes and dermal fibroblasts, is not absorbed by DNA, but contributes to up to 95% of total UV exposure and is considered the most important source of oxidative stress in human skin (7,11–13). The major thereby generated reactive oxygen species are singlet molecular oxygen ($^1O_2$) and lipid peroxyl radicals leading to instability of cellular components and membranes. $^1O_2$ exerts its effects either by transferring its excitation energy or by oxidation reactions. These effects are used in the treatment of skin cancers by photodynamic therapy, where porphyrins or its precursors are topically applied as photosensitizers. The protection by carotenoids from $^1O_2$-induced damage as well as from its precursor, the sensitizer triplet state (14), depends mainly on physical quenching, which occurs at rate constants approaching diffusion control. (15–17). Additionally carotenoids are lipophilic antioxidants, which are capable of scavenging lipid peroxyl radicals in a chemical reaction, and can also protect against a wide range of other radical species (14,18). This protection might even be more efficient with supplementation of antioxidant combinations. In a membrane model a combination of beta-carotene and alpha-tocopherol inhibited lipid peroxidation significantly greater than the sum of the individual inhibitions, thus showing synergistical effect as radical-trapping antioxidants in membranes (19).

The first reaction of skin to exposure to UV light is erythema formation. There are well known differences in the UVR-induction of inflammation in relation to the skin types I to VI (according to Fitzpatrick). The erythematous skin reaction after UVR-exposure decreases from skin type I to IV while the pigmentation increases. This appears to be related to significantly increasing beta-carotene plasma levels as well as content in oral mucosal epithelium with skin type I to IV. So these differences might actually be genetically controlled (20). In a cross-sectional study, concentrations of micronutrients (seven carotenoids, two retinoids, two tocopherols) were measured in paired plasma, buccal mucosal cells and skin samples from 96 healthy volunteers. The correlations between plasma and buccal mucosal cells as well as plasma and skin concentrations of these micronutrients except retinol were significant in all subjects and even stronger in nonsmokers than in smokers. These results suggest that the status of these micronutrients in buccal mucosal cells and skin may be estimated from their plasma levels (21). Interestingly, though, carotenoid concentrations were significantly higher in buccal mucosal cells after only one week of supplementation with either beta-carotene or lycopene 30 mg/day as compared to the 15 mg/day

beta-carotene or lycopene or placebo supplemented groups. Plasma concentrations were significantly higher in all carotenoid supplemented groups than in the placebo group. Incorporation of dietary carotenoids into buccal mucosal cells appears to occur rather quickly (22). The major carotenoids in human skin are beta-carotene, alpha-carotene, lycopene, beta-cryptoxanthin, lutein and zeaxanthin with differing levels in various skin areas (23). The highest carotenoid concentration has been found by noninvasive reflection spectroscopy after 12 weeks of ingestion of 24 mg of beta-carotene daily from an algal source in the skin of the forehead, closely followed by the palms (24) and by Raman scattering spectroscopy in the palms (25).

The protection of carotenoids against sunburn reactions has been the subject of various studies. A prevention of acute photodamage was shown with reduced erythema formation after 12 days of controlled exposure to sunlight following oral supplementation of 30 mg/day beta-carotene for 10 weeks. During sun exposure cutaneous beta-carotene levels decreased significantly while the serum levels also decreased, but not significantly (26,27). The supplementation of healthy volunteers with a mixture of natural carotenoids (14.7 mg beta-carotene, 0.18 mg alpha-carotene, 0.036 mg zeaxanthin, 0.042 mg cryptoxanthin, 0.027 mg lutein in vegetable oil packaged in gelatine) increasing from 30 mg/day to 60 mg/day and then 90 mg/day in eight-week intervals led to a significant increase of the minimal erythema dose (MED) as determined by solar simulated UVR. Additionally lipid peroxidation was significantly inhibited dose-dependently during natural carotenoid supplementation. The authors conclude there may be a partial but modest protection of human skin from UVR-induced erythema with natural carotenoids. (28). In a comparison of 24 mg beta-carotene with a carotenoid mix (8 mg beta-carotene, 8 mg lutein, 8 mg lycopene) daily for 12 weeks, there was a significant increase in serum beta-carotene in the beta-carotene group as well as in the serum levels of the supplemented carotenoids in the carotenoid mix group. The increase of total carotenoids was similar in the skin. In both groups, the erythema induced by a blue-light solar simulator was comparably significantly lower than baseline after 12 weeks of supplementation. There was no difference in protection in both treatment groups (29). A presupplementation of healthy volunteers with 30 mg beta-carotene daily for 10 weeks before and two weeks during a time and intensity controlled exposure to natural sunlight in combination with a topical sunscreen was more effective in suppressing erythema than the topical sunscreen alone (30). In a study with the ingestion of tomato paste equivalent to 16 mg lycopene/day, an increase in serum lycopene levels and total carotenoids in skin was observed. Sunburn reaction induced by irradiation with a solar light simulator was significantly reduced at week 10 but not at week 4 as compared to controls (31). In skin lycopene was shown to be destroyed preferentially by UVR over beta-carotene (32).

The supplementation of 15 mg/day beta-carotene over eight weeks led to increased beta-carotene plasma levels, but no detectable skin concentrations. There was no photoprotection detectable as assessed by the MED before and after supplementation (33). When beta-carotene was taken orally either as 120 mg

single dose or 90 mg daily over 23 days there were increased skin and plasma levels of beta-carotene detectable, but no protection against a sunburn reaction induced by three times the individual MED with a solar simulator (34). In another study, healthy volunteers were orally supplemented with 150 mg carotenoids (beta-carotene and canthaxanthin 2:3) daily for four weeks. There was a marked increase in serum carotenoid levels detectable, but no reduction in UVB-, UVA- or psoralen UVA-induced erythema before and after supplementation in these volunteers (35).

Interestingly in studies showing protective effects of carotenoid supplementation against sunburn reactions the treatment period was for at least 10 weeks (36,37), in studies failing to show a protective effect the treatment period was only three to four weeks. Due to the differences in the dosage and duration of beta-carotene supplementation as well as in UV exposure, a direct comparison of the studies is very difficult. When measured serum beta-carotene levels increased with carotenoid supplementation. The comparison of beta-carotene skin concentrations is also problematic since there were different methods of analysis employed in different skin layers and areas of the body. Basically, the duration of the carotenoid supplementation appears to be an important factor for the contrasting effects of these studies. This is well in line with the observation that beta-carotene supplementation has a long time constant for accumulation in human serum (about 10 days) and skin (several weeks) (38).

One major problem with carotenoid supplementation is an increased risk of lung cancer in smokers with higher dosages of beta-carotene that was shown in two intervention trials (39,40), that has been contributed to prooxidant effects shown in vitro (41–43). These may be related to increasing beta-carotene concentrations or due to the presence of chronic oxidative stress (44). The partial oxygen pressure may also play a role in these processes (45). Another possibility might be missing interactions with other dietary factors that are not available due to various reasons (46). One hint for the latter notion is a decreased alpha-tocopherol steady state plasma concentration in healthy volunteers by long-term oral administration of 15, 30, 45, or 60 mg oral beta-carotene daily for about nine months in all dosage groups (47). In another study, 30 mg/day beta-carotene for 60 days increased beta-carotene plasma levels and decreased leukocyte superoxide dismutase activity as well as serum glutathione peroxidase concentrations significantly, thus suggesting an alteration of antioxidant capacity by beta-carotene supplementation (48).

When healthy volunteers were supplemented with either 25 mg total carotenoids/day (23.8 mg beta-carotene, 0.75 mg alpha-carotene, 0.15 mg zeaxanthin, 0.18 mg cryptoxanthin, 0.12 mg lutein in soybean oil) or a combination of these carotenoids plus 335 mg alpha-tocopherol for 12 weeks, the serum beta-carotene and alpha-tocopherol concentrations increased with supplementation. Development of a slight yellowing of the skin especially the palms of the hands and the facial skin became detectable. Erythema induced by a blue light solar simulator was significantly diminished after week 8 and even more so with the combination of carotenoids and alpha-tocopherol. This additional protection might be associated with additive or synergistic effects of the two compounds in the scavenging

process (19,49). So the antioxidants in this study provided protection against erythema in humans and may thus be useful for diminishing sensitivity to UVR (50). Supplementation of 25 healthy volunteers with an antioxidant complex (3 mg alpha- and beta-carotene, 3 mg lycopene, 5 mg alpha-tocopherol, 37.5 µg selenium) twice daily for seven weeks reduced MED, UV-induced p53 expression and sunburn cell count as well as lipid peroxidation when compared to pretreatment values. This implies a significant improvement of the epidermal defenses against UV-induced damages (51).

In two different in vitro skin models, a membrane equivalent and a collagen equivalent, supplementation for five days with an antioxidant mixture (10 mg alpha-tocopherol, 2.4 mg beta-carotene, 60 mg ascorbic acid, 25 µg selenium, 25 mg standardized tomato extract, 25 mg standardized grape seed extract) followed by UVA irradiation resulted in an impressive dose-dependent increase in antioxidant capacity as determined in a fluoroscan assay (52). In a cell culture study with human embryonic lung fibroblasts the protection of preincubation with antioxidants beta-carotene, alpha-tocopherol and ascorbic acid or combinations against the damage against UVA or UVB light was investigated. After UVA-irradiation, there was a pronounced protective effect with beta-carotene with an about 300% increased cell survival, which increased even further in combination with the other two antioxidants, which did not show any protection on their own. A different situation was found after UVB-irradiation. Each antioxidant alone led to an about 25%, the combinations to an about 50% increased cell survival. According to the authors, there were no significant differences in the results when these experiments were done with dermal fibroblasts. The results for UVA light might be due to the different subcellular distributions of the antioxidants and possible subsequent regeneration of the generated radicals. As a conclusion a long-term radical initiated UV-damage may be mitigated by a balanced diet (49).

When lycopene was applied topically to healthy volunteers at 0.03% in a gel-emulsion followed by UV irradiation erythematous reactions were significantly more reduced than vehicle alone. The topical application of the antioxidant alpha-tocopherol (0.5%) and ascorbic acid (1%) in the same base also reduced erythematous reactions, but not significantly. None of these topical formulations showed a marked difference for hydration or skin barrier function (53). Topical application of a 5% beta-carotene solution on mouse skin 15 minutes prior to UVB or UVA exposure reduced the formation of UV-induced thiobarbituric acid reactive substances (TBARS), an indicator for lipid peroxidation in skin (54). When rat skin was treated topically with a 0.05%, 0.1%, or 0.2% beta-carotene solution one hour prior to UVA irradiation beta-carotene penetration and accumulation in the epidermal and dermal layers was detected and a significantly reduced UVA-induced lipid peroxidation observed with lower beta-carotene concentrations being more effective than higher concentrations (55). Topical treatment of ears of Institute for Cancer Research (ICR) mice with either alpha-carotene, beta-carotene or solvent followed after 30 minutes by topical application of croton oil or solvent resulted in a significant suppression of croton-oil-induced lipid peroxidation, hydroperoxide production and edema formation (56). BALB/c mice were fed a standard diet either

with or without 50 mg/day beta-carotene for three weeks, then sacrificed, the skin harvested and homogenized followed by photoirradiation with UVA-light. Beta-carotene accumulated in the skin of the supplemented animals while none was detectable in the control animals. Beta-carotene supplementation suppressed the occurrence of UVA-induced lipid peroxidation in skin by quenching of $^1O_2$ (57). In an ex vivo model with human skin homogenates lipid peroxidation was effectively diminished by addition of beta-carotene (58). Preculturing of human skin fibroblasts (foreskin) with beta-carotene, lycopene or lutein dose-dependently decreased UVB-induced formation of TBARS. Concentrations above the optimal protection dose led to pro-oxidant effects, suggesting that there are optimum levels for protection in vivo (59). Similarly was shown in cultured human skin fibroblasts (foreskin) that pretreatment with beta-carotene protected the cells from UVA-induced plasma membrane damage, which was associated with lipid peroxidation (60). A later study though did not show any protection of pretreatment with beta-carotene against UVR-induced oxidative stress in human skin fibroblasts (CCD 922SK), but rather pro-oxidative effects (43).

Irradiation of HR-1 hairless mice with UV-light for five days resulted in skin as compared to unirradiated control animals in a significant reduction in skin retinoid concentration and, as a possible response, a significant increase in beta-carotene 15,15'-mono-oxygenase (formerly known as beta-carotene 15,15'-dioxygenase) activity, an enzyme which converts beta-carotene to retinal (61). The expression of this enzyme in mouse skin had already been shown previously (62). Incubation of normal human keratinocytes and melanocytes (foreskin) with beta-carotene showed an accumulation of cellular beta-carotene concentration reaching a maximum after five days, which was five-fold higher in melanocytes than in keratinocytes. Concomitantly the cellular retinol concentration increased for up to four days, also much more so in melanocytes than in keratinocytes implying the bioconversion of beta-carotene to retinol in these cells. This result suggests that beta-carotene can function as a local supply of vitamin A in the skin with the melanocytes especially likely to store beta-carotene (63). Topical beta-carotene (2 mM solution) penetrated well into human and hairless mouse epidermis and induced a 10-fold (human) and a three-fold (mouse) increase of epidermal retinyl esters, thus demonstrating that topical beta-carotene is converted into retinyl esters by human and mouse epidermis (64). Meanwhile the expression of beta-carotene 15,15'-mono-oxygenase has also been demonstrated in keratinocytes of the squamous epithelium of human skin (65).

UVR-Sensitivity in Various Diseases

Polymorphic Light Eruption

Polymorphic light eruption (PLE) is a delayed-type hypersensitivity response to a sunlight-induced so far not defined cutaneous antigen/reactive agent. The disorder appears likely to be genetic with its actual expression depending on the degree of gene penetrance. PLE presents with many morphological variants

usually in spring following sun exposure. The most common lesions are papules, papulovesicles and plaques. Apart from the use of sunscreens in mild disease, the main treatment is UVR-hardening by prophylactic exposure to UVA or UVB. Sometimes oral corticosteroids are helpful. If none of these treatment modalities are effective or advisable beta-carotene supplementation may be tried, but the degree of improvement is quite small and was only comparable with a sun protection factor of 2 (66,67). In a randomized, double-blind cross-over study the efficacy of systemic beta-carotene has been disappointing (68,69). One study, though, did show a benefit for PLE patients. Sixty six patients received 25 mg carotenoids (10 mg beta-carotene, 15 mg canthaxanthin) three times daily for four weeks. Time-controlled sun exposure revealed a 3- to 30-fold increase in the tolerance level in 55 patients, while the remaining 11 were nonresponders. The overall results showed that different subtypes of PLE have different levels of improvement irrespective of the initial tolerance level and that patients with lower initial tolerance levels experienced a larger benefit than those with higher ones (70,71).

Hydroa Vacciniformia

Hydroa vacciniformia is a rare childhood photodermatosis characterized by recurrent papulovesicles or vesicles in sun-exposed skin areas resolving with pock like scarring, There is a case report of a five-year-old child where the skin changes stopped with the administration of beta-carotene 25 mg twice daily, avoidance of sun exposure and use of topical sunscreens (72). Previous trials showed conflicting results with the use of beta-carotene.

Vitiligo

Apart from the photoprotective effect of carotenoids in vitiligo, the carotenemic yellowish skin coloring, usually accompanied by elevated serum beta-carotene levels ranging from 250–500 µg/100 mL (73), might reduce the obvious contrast of the vitiligous lesions to normal pigmented skin and as such might have a positive effect on the often occurring psychologic distress. In a study of antioxidant nutrient intake consisting of daily 5 mg alpha-tocopherol, 30 mg ascorbic acid together with either 13 mg beta-carotene, 2 mg lycopene or 3 mg beta-carotene, 3 mg lycopene, there was carotenodermia detectable in the group with higher carotenoid intake after eight weeks. More interestingly, for both groups there was a significant increase in melanin concentrations in skin found after week 4, 5, 6, and 8. This might be due to the regulation of the tyrosinase activity in melanocytes by the cellular redox state. One important player in the intracellular scavenging of free radicals is the gluthatione cycle, which is dependent on alpha-tocopherol and carotenoids for its enzymatic activity (74). When melanocytes in a cell culture system were incubated with a tomato extract containing lycopene, a palm fruit extract rich in carotenoids, ascorbic acid and alpha-tocopherol production of melanin pigment increased. Preincubation with the same mixture also reduced DNA damage following UVA irradiation (75).

Connective Tissue Disorders

Sun sensitivity in connective tissue disorders is well known. 60% to 70% of patients with systemic lupus erythematosus (SLE) are photosensitive (76). Both UVA and UVB exposure can induce lesions in SLE patients (77). Beta-carotene supplementation in these patients has been implicated in the amelioration of photosensitivity, but apparently has never been subject of a clinical trial in that context. As beta-carotene is established in the treatment of erythropoietic porphyria to control the symptoms of acute photosensitivity (78,79) this might lead together with the above-mentioned photoprotective effects to the conclusion that beta-carotene should also be helpful in patients with photosensitivity due to connective tissue disorders. But then, there has never a convincing photoprotective effect been demonstrated in porphyria cutanea tarda (79). Also, intraperitoneal administration of beta-carotene in guinea pigs two hours or five consecutive days prior to intraperitoneal administration of a new derivative of antibacterial fluoroquinolones, which are known potent photosensitizers accumulating in the skin, had no suppressive effect on UVA- or UVB-induced erythema as compared to the fluoroquinolone derivative alone (80). This leaves it open if there is a benefit to be expected. Either the individual patient might find that out for himself by taking oral beta-carotene supplements or a photoprotective effect might be determined in appropriate clinical trials.

PHOTOAGING

UVR leads to photodamage with subsequent photoaging of the skin characterized by wrinkling, scaling, reduced elasticity, dryness, pigment abnormalities, and eventually culminating in the development of skin cancer (81,82). The main histological features are accumulation of disorganized elastin-containing fibres and reduced amounts of of type I and type III procollagens in the extracellular matrix (83). On a molecular level UVB exposure induces activation of transcription factors like activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) leading to the induction of matrix metalloproteases (MMPs), which can also be upregulated by formation of free radicals (84). MMPs can then degrade various collagens and other matrix proteins.

Forty-eight volunteers received either an antioxidant combination (10 mg alpha-tocopherol, 2.4 mg beta-carotene, 60 mg ascorbic acid, 25 μg selenium, 25 mg standardized tomato extract, 25 mg standardized grape seed extract) or placebo three times daily for 12 weeks. At weeks 11 and 12 controlled daily body skin UVB irradiation was performed. Even though an accumulation of beta-carotene could be shown in skin biopsies, there were no significant differences in UV light sensitivity or in skin levels of MMP-9 between verum and placebo group. There was a significant difference though in the MMP-1 skin levels, which increased in the placebo group and decreased in the verum group after two weeks of UVR, implying a definite role of UVR in the processes of premature skin aging (84). In an in vitro system with HaCaT keratinocytes beta-carotene accumulated

time and dose-dependently in the cells. When this was followed by UVA irradiation cellular beta-carotene content was massively reduced. Beta-carotene suppressed UVA-induction of MMP-1, MMP-3, and MMP-10, which are the three major MMPs involved in photoaging. This regulation involved $^1O_2$-dependent mechanisms as $^1O_2$-mediated induction of MMP-1 and MMP-10 was dose-dependently quenched by beta-carotene (85).

Another gene that is activated following UVA irradiation is the human heme oxygenase 1 (HO-1) gene, which is used as a marker of oxidative stress in cells. This induction occurs via $^1O_2$-production and can be suppressed by preincubation with beta-carotene concentration and UVA dose dependently in normal human dermal fibroblasts (FEK4) (86). In a study of the photoprotective potential of beta-carotene, lycopene, ascorbic acid and alpha-tocopherol in human dermal fibroblasts neither beta-carotene nor lycopene presence was able to suppress the UVA induced MMP-1 or HO-1 expression but led to a further increase. The inclusion of alpha-tocopherol completely suppressed the increase of MMP-1 expression (87). Similarly, a preincubation with beta-carotene in normal human skin fibroblasts (HFP-1) enhanced the UVA-induced HO-1 and interleukin-6 (IL-6) expression, another player in the pathogenesis of photoaging of the skin. Interestingly beta-carotene was not able to modulate levels of HO-1 and IL-6 in UVB-irradiated cells (88,89). These contrasting results might be due to the differences in the beta-carotene concentrations and, more important, in the employed carotenoid delivery vehicles in the different studies (90).

Mutations of mitochondrial DNA might also be involved in the process of photoaging as these can be induced by repetitive exposure to sublethal doses of UVA irradiation (91). Assessment in cultured normal human fibroblasts (foreskin) after beta-carotene incubation, followed by UVA irradiation indicated that beta-carotene is taken up into the cells dose-dependently, is decreased by UVA exposure and leads to reduced levels of mitochondrial DNA mutagenesis. So beta-carotene may be capable of protecting from mutations relevant in the process of photoaging (92).

Carotenoids have also the capability to induce gap junctional communication (GJC) via expression of connexin 43 (Cx43), a structural pore component of the gap junction. GJC allows exchange of low-molecular-weight molecules between cells, which have been implicated in the regulation of cell growth, differentiation, and apoptosis (93). Beta-carotene, canthaxanthin and lycopene upregulated GJC and expression of Cx43, in cultured human dermal fibroblasts (foreskin), but not in cultured human keratinocytes (foreskin) (94). This has been correlated with the inhibition of the progression of carcinogen-initiated fibroblasts to the transformed state (95).

Furthermore carotenoids can induce phase I and phase II metabolic enzymes, which play a role in detoxification processes (1). This induction may occur via a pregnan-X-receptor (PXR)-mediated mechanism. PXR is a novel nuclear receptor of the nuclear hormone-receptor superfamily, is ligand-dependently activated and

transmits its activity via DNA binding. Transcriptional regulation is partially mediated through ligand-activated heterodimers with retinoid-X receptor (2).

In human keratinocytes (HaCaT) pretreatment with beta-carotene prevented UVA-induced gene regulation, assessed by using microarrays. Hallmarks of UVA irradiation were downregulation of growth factor signaling, moderate induction of proinflammatory genes, upregulation of immediate early genes including apoptotic regulators and suppression of cell cycle genes. Of the 568 UVA-regulated genes, beta-carotene reduced the UVA effect for 143, enhanced it for 180, and did not interact with UVA for 245 genes, thus implying that there are multiple mechanisms involved in the interaction of beta-carotene with UVA light (96).

OTHERS

Psoriasis

In an Italian study on dietary factors and the risk of psoriasis there was a significant inverse relation observed for psoriasis and the intake of carrots, tomatoes, fresh fruits and the index of beta-carotene intake. This led to the conclusion that there might be a potential role of the diet in psoriasis (97).

Delayed Type Hypersensitivity

In a group of healthy older men the individual and interactive effects of UVR and beta-carotene supplementation (30 mg or placebo daily for 47 days) on the delayed-type hypersensitivity (DTH) response as measured using seven common antigens in the Multitest CMI kit (Merieux Institute, Miami, Florida, U.S.A.; tetanus, diphteria, streptococcus, tuberculin, candida, proteus, trichophyton) was examined. Suberythmogenic UVR resulted in a significantly suppressed DTH response in the placebo group, while higher beta-carotene plasma levels were significantly associated with the maintenance of the DTH response (98). Interestingly, the immunosuppressive effect of UVR was similar, while the protection of beta-carotene against the DTH response appeared more pronounced in younger men (99). Another group did not find an enhancing or suppressive effect of beta-carotene supplementation on DTH response and as such on T cell-mediated immunity measured also with the Multitest CMI kit in healthy elderly people, either after short-term supplementation of 30 mg beta-carotene daily over three weeks in women or long-term 50 mg beta-carotene every other day for 10 to 12 years in men (100).

Solar Keratoses

In a randomized controlled trial of beta-carotene supplementation and sunscreen application on the prevention of solar keratoses, which are among the strongest determinants of skin cancer, participants of subtropical Australia were assigned to one of four treatment groups: 30 mg beta-carotene daily either with or without the

daily use of a broad-spectrum sunscreen with a sun protection factor of 16 or placebo daily with or without the daily use of the same sunscreen for 4½ years. The beta-carotene supplementation had no influence on the occurrence of solar keratoses, while daily application of the sunscreen showed a 24% reduction equivalent to the prevention of about one additional solar keratoses per person over that time (101).

CONCLUSION

Carotenoids are efficient in photoprotection, quenching singlet molecular oxygen and scavenging lipid peroxyl radicals. For successful systemic photoprotection, as measured as a decrease in UV-induced erythema, treatment with carotenoids is needed for at least 10 weeks. Oral intake of a beta-carotene containing antioxidant combination may even more efficiently contribute to life-long protection against UV-induced skin damage which can eventually lead to premature skin aging and skin cancers.

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Carotenoids as Cancer Preventive Agents

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INTRODUCTION

From a biological perspective, carotenoids can be the most easily classified as those that possess provitamin A activity, and those that do not. From a chemical perspective, carotenoids are generally classified as the hydrocarbon carotenoids, of which the prototype would be β-carotene, a $C_{40}H_{56}$ hydrocarbon containing 2 beta-ionone rings and 11 conjugated double bonds, and the xanthophylls, oxygenated carotenoids such as lutein, $C_{40}H_{56}O_2$, found in all green leaves, with hydroxyl groups on each of the rings. Both compounds can be found in μM concentrations in human serum and are obtained from consumption of carotenoid-containing fruits and vegetables (1). Carotenoids can also be straight-chain molecules, such as lycopene, $C_{40}H_{56}$, the red pigment found in tomatoes. The principal structural features of carotenoids are the possession of a methyl-substituted, conjugated double-bond system, reflecting their synthesis from isoprene units in the plant (2). Until recently, carotenoids were considered to have two major biological functions: first, the possession of a β-ionone ring by the provitamin A carotenoids allows their bioconversion in mammals to retinoids—compounds with essential roles in vision and in normal growth and differentiation (3); second, the conjugated double-bond system possessed by all carotenoids allows them to be effective lipid-phase antioxidants which can protect cell organelles from free-radicals, believed to be a major source of DNA damage (4). As discussed later, there is now evidence that carotenoids can directly regulate the expression of genes protective against

carcinogenesis and inflammation. The role of carotenoids in the biosynthesis of retinal, the visual pigment in the retina is a separate and discrete function, which will not be considered further here. Instead, we will concentrate on the role of carotenoids as antioxidants and as modulators of gene expression.

ARE CAROTENOIDS PROTECTIVE AGAINST HUMAN CANCER?

Instead of reviewing all studies that have been conducted on carotenoid-inhibition of carcinogenesis, the author has chosen to focus primarily on two organs, the lung and the prostate, where the evidence for a protective effect of carotenoids appears the most strong. The author additionally focused on those studies which have a mechanistic component to their design. However, because of the focus on dermatology in this book, the potential role of carotenoids as preventive agents against skin cancer will be briefly reviewed.

Carotenoids and Skin Cancer

It is well established that carotenoids can act essentially as internal sunscreens after oral administration and deposition on the skin. This was first described in the 1970s and led to the use of beta-carotene in the hypersensitivity disease erythropoietic protoporphyria, where doses of up to 180 mg/day have been administered without obvious toxicity (5). In experimental animal studies, lutein, the major carotenoid responsible for photoprotection of plants, was also shown to suppress acute effects of UV by reducing erythema and inflammation (6). Several carotenoids have also been demonstrated to reduce the incidence of experimental skin tumors after UV irradiation or at least delay their development (7,8). The interpretation of some studies examining the ability of carotenoids to inhibit chemically-induced two-stage carcinogenesis is complicated by the feeding of a retinoid-free diet to the experimental animals making it difficult to distinguish responses to carotenoids from those of retinoid deficiency or possibly excess (9,10). The functions of carotenoids as photoprotective agents have recently been reviewed (11).

Clinical studies of carotenoids as preventive agents against skin cancer have been uniformly negative. In epidemiological studies, dietary intake of carotenoids was not found to protect against either basal cell (12,13) or squamous cell carcinoma (14), in despite of the aforementioned moderate protection against acute effects of UV exposure. Furthermore, in intervention trials of beta-carotene conducted over at least one decade, beta-carotene was not found to reduce the incidence of nonmelanoma skin cancer (15,16). In a study designed to compare the protective effects of sunscreen versus beta-carotene against UV-induced skin tumors, beta-carotene was again found to be ineffective, whereas sunscreen use protected against squamous but not basal cell carcinoma (17). It must be concluded that either the small protection afforded by carotenoids against acute effects of UV radiation is insufficient to materially reduce long-term damage or that protection against acute effects is not a good marker for protection against

carcinogenic damage, thought to be primarily the production of thymidine-thymidine photodimers in irradiated DNA. Recently technologies have been developed to noninvasively detect carotenoids in skin (18) and it is envisioned that more tightly controlled experiments may now be possible.

Carotenoids and Lung Cancer

The epidemiological literature is consistent in describing a diet rich in fruits and vegetables as protective against cancer at many sites. The availability of food-composition databases has allowed the individual components of such "healthy" foods to be assessed and in many cases the carotenoid composition of such foods appear most highly correlated with decreased risk (19). The early association of the beta-carotene content of the diet with decreased risk of lung cancer (20) led to the conduct of three large clinical trials of synthetic beta-carotene; two trials were conducted in tobacco- or tobacco and asbestos-exposed individuals, while a third was conducted in much lower risk, predominantly nonsmoking, U.S. physicians. The results were to say the least, disappointing: in studies conducted in the high-risk smokers, lung cancer rates actually increased by approximately 20% in the intervention group (21,22), while no effect was seen in the low-risk physicians (23). Needless to say, these results have severely dampened enthusiasm for other large trials in healthy individuals. However, it should be noted that supplemental levels of beta-carotene were approximately 10-fold higher than those normally consumed in a healthy diet moreover, the deleterious effects of supplementation seems restricted to the lung. In a separate study comparable high doses of beta-carotene were shown capable of protecting smokers against head and neck cancer, although again moderately increasing lung cancer rates (24).

STUDIES IN EXPERIMENTAL ANIMALS

In an attempt to explain these disturbing and confusing results, Wang, Russell et al. began studies in the ferret, an animal that, unlike rats and mice, readily absorbs dietary carotenoids. Utilizing ferrets fed low-dose and high-dose beta-carotene at levels designed to mimic dietary exposure and supplemental exposure of humans respectively, they were able to convincingly reproduce the results obtained in the human intervention studies. Not only did cigarette smoke exposed ferrets supplemented with high-dose beta-carotene develop more severe lung lesions than smoke-only exposed ferrets, but high-dose beta-carotene alone induced squamous metaplasia in the lungs of these animals. This was found to be most likely a consequence of the induction by high-dose beta-carotene of a CYP enzyme in part responsible for the catabolism of retinoic acid. This reduction in retinoic acid concentrations in turn led to elevated levels of the transcription factor AP1 and elevated levels of c-JUN and cyclin-D, which would be expected to increase the proliferation rate in affected tissue (25). In contrast, low-dose beta-carotene was able to weakly attenuate the pathological effects induced by smoke-exposure

of the ferret lung (26). More recently, this group has demonstrated that protection is not limited to beta-carotene but also extends to lycopene; here, both low-dose and high-dose lycopene were able to protect ferrets against lung metaplasia induced by cigarette smoke exposure. In this situation, protection was reported to be unrelated to retinoic acid metabolism, but instead involved the insulin-like growth factor-1 system (IGF-I) system. While IGF-I levels themselves were not modified by lycopene treatment, levels of an IGF-binding protein (IGFBP-3) were elevated by approximately 50% and smoke-induced decreases in this binding protein were abrogated by lycopene treatment (27). At present it is not known whether lycopene may have deleterious effects in the smoke-exposed lung, since the concentrations of lycopene in lung tissue attained in these experiments, even with a high oral dosage, were approximately 10-fold lower than those obtained by beta-carotene at toxic doses.

Lycopene and Prostate Cancer

Stimulated by epidemiological evidence that dietary lycopene negatively correlates with risk of prostate cancer (28,29), a number of investigators have studied the effects of this tomato-derived carotenoid in cell cultures, experimental animals and in clinical trials. While the experimental results have been encouraging, the additional epidemiological studies triggered by this original report have not always found such a relationship, particularly a study from the author's own institute (30). A recent meta-analysis reported that a review of 11 case-control studies and 10 cohort studies revealed only a modest protection limited to those individuals consuming high amounts of tomato products (31).

Human Intervention Trials

Two intervention trials have examined the effects of lycopene (30 mg/day) given for 30 days to men with biopsy-positive prostate adenocarcinoma and scheduled for radical prostatectomy. In one study, the lycopene was derived from a tomato oleoresin (32), while the second study used tomato sauce as the source of lycopene (33). Pathological examination of excised tumors after surgery both provided evidence for increased apoptosis in the intervention group. Both investigators showed a trend for lower serum prostate specific antigen (PSA) levels in the intervention groups. In patients fed tomato sauce as a supplement, prostate tissue, as well as leukocytes revealed less oxidative DNA damage. Additionally, increased connexin 43 (Cx43) expression was determined by immunoblotting by the first group of investigators. The role of Cx43 as a potential tumor suppressor gene will be discussed later in this chapter.

A third trial studied the role of lycopene as a preventive agent in prostatic intraepithelial neoplasia. Here men were treated with 4 mg/day of lycopene as tomato oleoresin and their PSA levels compared to a control group over the subsequent one-year observation period. In the intervention group, serum lycopene almost doubled and PSA levels actually fell from 6 to 3.5 ng/mL whereas PSA

levels in the control group increased to 8 ng/ml (34). A fourth study compared the effects of orchidectomy alone versus the effects of orchidectomy plus lycopene, 2 mg/day in a group of patients with metastatic prostate cancer. After two years of follow-up significant improvements in clinical status and survival were observed in the lycopene-treated group versus the orchidectomy-alone group (35). Though encouraging, these results are surprising in view of the low-dose of lycopene employed and the clinical status of the patients.

Studies in Experimental Animals

A dramatic example of the cancer preventive properties of an antioxidant mixture (lycopene, vitamin E, and selenium) was reported using a mouse transgenic model of prostate carcinogenesis. Here prostate cancer is driven by localized expression of the SV 40 large T antigen and results in up to 100% incidence of prostatic adenocarcinoma. Administration of the antioxidant mixture reduced this incidence to between 10% and 15%. Those animals tumor-free had histologically normal prostates (36). While it is clear that one cannot isolate the activity of lycopene from that of the other two experimental variables, vitamin E and selenium, nevertheless it can be argued that lycopene is the only one of these three antioxidants not present in the control diet. In a second model of prostate carcinogenesis, this time rats treated with the carcinogen *N*-methyl-*N*-nitrosourea (NMU) and testosterone, tomato powder, but not synthetic lycopene delivered in beadlet form, caused a modest but significant decrease in the rate of death from prostate cancer. This was in spite of an approximately 10-fold higher content of lycopene in the beadlet formulation than in the tomato powder. However, plasma levels of lycopene were only approximately 20% higher in the beadlet treated group (36,37). This result is reminiscent of the studies discussed below, where ethanol extracts of lycopene were as effective as lycopene itself in activating the antioxidant response element (ARE) (38), and suggests that oxidation products of lycopene, far more likely to occur in a tomato powder than in the highly stabilized beadlet formulation of lycopene, may be responsible for the observed effects. In view of the previous comments regarding the potential for carotenoids to modify carcinogen-metabolizing enzymes, it should be noted that the prostate-specific expression of the large T antigen was not modified by antioxidant treatment nor does NMU require metabolic activation. In a separate model of carcinogen induced prostate cancer in rats, neither curcumin nor lycopene reduced carcinogenesis (28). In confirmation of epidemiological data which shows no protection of lycopene consumption against the incidence of breast cancer, neither synthetic lycopene nor tomato oleoresin reduced the incidence of carcinogen induced mammary carcinoma in rats (39).

Two studies have examined the effects of lycopene supplementation in rats using gene expression arrays. The first study examined the effects in rats pretreated for four weeks with synthetic lycopene in beadlet formulation, then orthotopically injected with Dunning prostate cancer cells. No changes in tumor growth rates were observed, but lycopene treated animals had a greater incidence of tumor necrosis than the control animals. Affymetrix gene expression array of excised

tumors revealed down-regulation of genes involved in androgen signaling, including 5-alpha steroid reductase, decreased expression of insulin and IGF-I and of IL-6. Plasma levels of lycopene were equivalent to those found in humans (40). In the second study by the same authors, the same concentration of lycopene was given to normal rats for eight weeks; gene expression analysis was then performed on the excised prostate tissue. As in the previous study, genes involved in androgen signaling were down-regulated, as was IGF-I, but curiously only in the ventral lobe of the prostate, a finding confirmed by real-time polymerase chain reaction (PCR). Several proinflammatory cytokines were also decreased in expression. These effects were observed without changes in the total body weight or weight of prostates in treated animals (41).

Studies in Cultured Prostatic Cells

Treatment of normal prostatic epithelial cells with concentrations of synthetic lycopene up to 5 μM resulted in G1/G0 cell cycle arrest, which was accompanied by a decrease in cyclin D1 expression (42). Other investigators have also shown cell cycle arrest and induction of apoptosis of human prostate LNCaP adenocarcinoma cells after lycopene treatment (beadlet formulation) (43). A second study in the same cell line delivered lycopene in tetrahydrofuran (THF) solution and reported no effect on proliferation but a major increase in apoptosis associated with mitochondrial dysfunction (44).

Studies in Human Prostate Organ Culture

We have recently initiated a study utilizing biopsies of human prostate obtained immediately after radical prostatectomy. Biopsy cores were sliced into 1 mm thick sections and incubated in serum free medium in the presence or absence of lycopene delivered in a THF solution. Tissue samples cultured at the air/liquid interface were shown to be fully viable after eight days in culture as assessed by pathological examination and expression of cell cycle markers such as proliferating cell nuclear antigen (PCNA). In preliminary studies, we have found that lycopene induces a dose-dependent increase in apoptosis in hyperplastic regions of the gland. Remarkably, this effect was most dramatically observed in stromal tissue where apoptosis, as measured by the TUNEL assay, increased from essentially zero in control cultures to up to 70% in cultures treated with 1 μM lycopene. In glandular tissue only approximately 2% of cells were apoptotic after lycopene treatment. In a biopsy obtained from another patient, extensively occupied by adenocarcinoma cells, no increase in apoptosis was observed. Unfortunately there was little stromal tissue to be analyzed in this sample. So far we have seen little evidence for lycopene-induced decreased cell proliferation of glandular cells based on PCNA staining and BrdU uptake. (Bertram et al., unpublished).

Diverse Carotenoids Inhibit Neoplastic Transformation In Vitro

To determine if the epidemiological associations between carotenoid consumption and decreased risk of cancer could be confirmed experimentally in a highly controlled in vitro model of carcinogenesis, we began studies in transformable

C3H/10T1/2 cells (10T1/2). We had previously shown these cells to respond to chemical and physical carcinogens by the quantitative formation of neoplastically transformed foci (45), and had shown that cancer preventive retinoids could inhibit neoplastic transformation in these cells (46). Because of the problems of drug delivery of these lipophilic molecules, the first carotenoids to be tested were those available in a "beadlet" formulation, however, this limited our studies to beta-carotene and canthaxanthin (47). In order to extend studies to a more diverse series of dietary carotenoids, we developed THF as a delivery solvent. Use of THF results in the formation of a pseudo-solution of carotenoids in cell culture medium—a form which is highly bio-available (48). When carotenoids were added one week after removal of the chemical carcinogen, all carotenoids, regardless of their provitamin A activity were capable of inhibiting the development of neoplastic transformation. Moreover, just as in our earlier studies with retinoids, removal of the carotenoid led to emergence of neoplastic transformed foci some three to four weeks later (49). This indicated that we were not dealing with selective cytotoxicity, but with a reversible inhibition of the process of neoplastic transformation.

These studies demonstrated that, at least in the model cell culture system employed, dietary carotenoids had the following properties: (*i*) they inhibited neoplastic transformation in the postinitiation phase; (*ii*) their action was reversible and thus not a consequence of selective cytotoxicity; (*iii*) their action was not the result of selective growth inhibition of transformed cells; (*iv*) their action was independent of conversion to chemopreventive retinoids, since activity was observed even with straight-chain hydrocarbons such as lycopene; (*v*) their activity did not correlate with their antioxidant properties.

MOLECULAR STUDIES OF CAROTENOID ACTION

Carcinogenesis: An Overview

It is now firmly established that the induction of cancer is a consequence of the sequential acquisition of mutations, which either activate oncogenes or inactivate tumor suppressor genes. The cumulative effect of such mutations is to allow the cancer cell to evade normal homeostatic mechanisms that would seek to inhibit aberrant proliferation and to induce programmed cell death, apoptosis. Mutations themselves arise as a consequence of DNA damage, which fails to be repaired. This failure may be a consequence of mutations in genes involved in the repair process or as a consequence of damage being introduced just prior to or during DNA replication (50). Thus, strategies to reduce DNA damage and at the same time reduce cell proliferation can be expected to strongly decrease carcinogenic susceptibility (4). As will be discussed subsequently, carotenoids have the potential to inhibit both processes.

Oxidative Stress and Carcinogenesis

The role of carotenoids as antioxidants has been reviewed elsewhere in this volume, however, there is evidence that chronic oxidative stress creates conditions

highly favorable to the acquisition of carcinogenic mutations and that carotenoids can protect against such changes. There are many situations where oxidative stress has pathological consequences in humans. For example, chronic inflammation—either as a consequence of autoimmune disorders or unresolved infection—leads to the generation by immune cells of large amounts of diverse reactive oxygen species. These reactive species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) have physiological functions in being cytotoxic to invading pathogens, while the production of nitric oxide (NO) by macrophages leads to vasodilation and increased blood flow to infected tissues (51–52). In conditions of chronic inflammation, however, this production of reactive oxygen species may continue for months or years and be damaging to local tissue and surrounding stroma. For example, chronic viral hepatitis leads to progressive loss of the liver parenchyma and replacement by fibrotic lesions—a process known as cirrhosis. Similar tissue damage occurs in conditions of chronic gastritis and colitis, atherosclerosis and rheumatoid arthritis. Unfortunately, damage occurs not just to the architecture of the tissue but also to the genome. These same reactive oxygen species are known to be capable of causing DNA lesions such as 8-hydroxy deoxyguanosine and 8-oxo-guanosine, both promutagenic lesions. They also can deaminate DNA, leading again to potential mutagenic changes if unrepaired (53). The increased cell division caused by reactive hyperplasia in chronically inflamed tissues, together with the increased rate of DNA damage, almost certainly results in the increased rate of malignancy seen in these conditions (54). Indeed, chronic infection of gastric epithelium with *H. pylori*, now known to cause most cases of chronic gastritis in the West, can lead to stomach cancer in a significant proportion of cases (55).

Preliminary data from the group of Nishino in Japan has suggested that supplemental lycopene (10 mg/day) and α-tocopherol administered orally to patients with chronic hepatitis C, results in a dramatic decrease in the incidence of hepatocellular carcinoma beginning about one year after initiating treatment (56). This data is consistent with reports from the same group that liver fibrosis in rats with copper overload can be suppressed by lycopene alone (57). In view of the fact that liver cancer is the most prevalent cancer on a global scale, resulting most frequently from chronic viral infection and aflatoxin contaminated foods, this observation is of enormous potential significance. Whether the chemopreventive action of lycopene is mediated through its antioxidant properties or through more specific effects on gene regulation, as will be discussed later, remains to be determined. Nor is it clear what the contribution was of α-tocopherol to the observed protection.

Anti-inflammatory activity has also been reported with astaxanthin, a xanthophyll predominantly found in marine organisms and responsible for the pigmentation of shrimp and lobster and for the pink pigmentation of birds like the flamingo who feed on marine organisms. In studies in cell culture and in mice, astaxanthin was shown to inhibit the production of inflammatory cytokines such as tumor necrosis factor (TNF)-α, prostaglandins and NO. This activity was the

result of inhibited activation of the nuclear transcription factor NK-KB, probably a result of scavenging reactive oxygen species known to activate this inflammation pathway (58). While the concentrations of astaxanthin (50 μM) producing this effect were certainly supra-physiological, these concentrations are achievable by novel, highly bioavailable astaxanthin derivatives to be described later.

Carotenoids Modify the Expression of Phase I and Phase II Detoxifying Enzymes

The phase I and phase II detoxifying enzymes exist for the purpose of making hydrophobic xenobiotic compounds, such as the ubiquitous environmental carcinogen benzopyrene, more water-soluble so that they may be ultimately excreted by the kidneys. The phase I enzymes perform initial modifications on the xenobiotic, typically by the addition of hydroxyl groups. In contrast the phase II enzymes catalyze the addition of large hydrophilic molecules, such as glutathione or modified sugars, to xenobiotics allowing urinary excretion. Unfortunately, many chemical carcinogens become activated by phase I enzymes to their ultimate carcinogenic form. As initially reported by Astorg et al., the effects of carotenoids on phase I enzymes are complex and appear to be both carotenoid and species specific. For example, in rats the CYP11A-inducing carotenoids, beta-apo-8'-carotenal, astaxanthin and canthaxanthin, decreased in vivo the binding of aflatoxin (AFB-1) to liver DNA and plasma albumin, and increased the in vitro AFB-1 metabolism to aflatoxin M-1, a less genotoxic metabolite. However, lycopene had no effect (59). In the mouse only canthaxanthin had a strong influence on CYP1A1 induction (60). Because the multiplicity of phase I enzymes, each with defined substrate specificities, modulation of these enzymes may both activate and inactivate chemical carcinogens depending upon the site of chemical modification. It is recommended that in order to avoid these interpretational problems, experimental studies should utilize carotenoids administered after carcinogen administration or utilize carcinogens, which do not require metabolic activation. For example, in our studies with 10T1/2 cells, carotenoids were added only seven days after removal of the carcinogen or, where simultaneous treatment was required, X-irradiation was utilized as a carcinogenic stimulus (49).

The situation with phase II enzymes is somewhat more simple because induction of all enzymes in this response pathway are believed to be protective against oxidative stress and/or carcinogen damage (61). As elegantly demonstrated by the group of Talalay, in their studies of the protective role conveyed by consumption of cruciferous vegetables, it is now known that the nuclear transcription factor Nrf2 becomes activated by changes in the redox state of the cell and transcriptionally activates genes controlled by the ARE. Under normal circumstances, Nrf2 is cytoplasmic bound to the inhibitory protein Keap1. This protein contains multiple cysteine residues which act as sensors for the redox state of the cell (62). Upon modification of these residues, Nrf2 is released, translocates to the nucleus and activates genes controlled by the ARE, such glutathione S-transferase and glutathione synthesase involved in phase II enzyme protection (62).

Although there was some evidence that carotenoids could activate phase II enzymes (63), until recently their influence on genes activated by ARE had not been directly demonstrated.

Using an ARE-luciferase reporter system, lycopene in particular was shown to cause reporter activation together with the induction of the phase II enzymes NAD(P)H: quinone oxidoreductase and γ-glutamylcysteine synthetase. Lycopene additionally caused an increase in intracellular glutathione—a major intracellular antioxidant. The role of Nrf2 in mediating these responses was demonstrated using a dominant-negative Nrf2 construct (38). These results must be viewed as potentially highly significant to the protective role of carotenoids against oxidative stress; a role that does not directly depend upon their interaction with reactive oxidative species (ROS.). Interestingly, activity of lycopene could be duplicated by ethanol extraction products of lycopene, suggesting that degradation products of lycopene may be in part or totally responsible for this biological activity. Similar findings have been reported for the ability of oxidation products of lycopene to enhance gap junctional communication (GJC) (64).

The significance of stimulating the ARE system is highlighted by experiments conducted by others who have demonstrated that inhibition of this response by targeted knockout of Nrf2 enhances the susceptibility of mice to carcinogenesis (65), while conversely, the use of siRNA to down-regulate expression of Keap1, thus activating Nrf2, dramatically increased the levels of phase II protective enzymes in cultured human keratinocytes (66).

Carotenoids Increase Gap Junctional Communication

A consistent finding in studies of human or animal tumor cell lines and in studies of neoplastic transformation in vitro, is that tumor cells communicate poorly, if at all with their normal counterparts (67). These results led to the original hypothesis of growth control through junctional communication so eloquently proposed by Lowenstein (68) (Fig. 1). Gap junctions are water-filled pores called connexons that connect adjacent cells in most organs of the body. These pores allow direct cytoplasmic to cytoplasmic communication of water-soluble molecules and ions. Because of the limiting size of the pore, only molecules less than about 1000 Daltons can pass, excluding molecules, such as mRNA and protein and thus maintaining genetic identity of the cells. The existence of this network of communication creates a syncytium through which cells can exchange nutrients, waste products and signaling molecules such as cAMP, Ca^{++} and so on. (69). There is evidence that gap junctions also serve to transmit growth-inhibitory signals that can inhibit the aberrant proliferation of carcinogen-initiated and fully transformed cells (70). This was derived in part from studies in the 10T1/2 cell line and was extended by the observation that the inhibitory action of retinoids on neoplastic transformation in 10T1/2 cells was closely linked to their ability to increase GJC via increased expression of connexin43 (Cx43) at the mRNA and protein level (71) (Fig. 2). It is of interest that many classes of tumor promoters—agents that

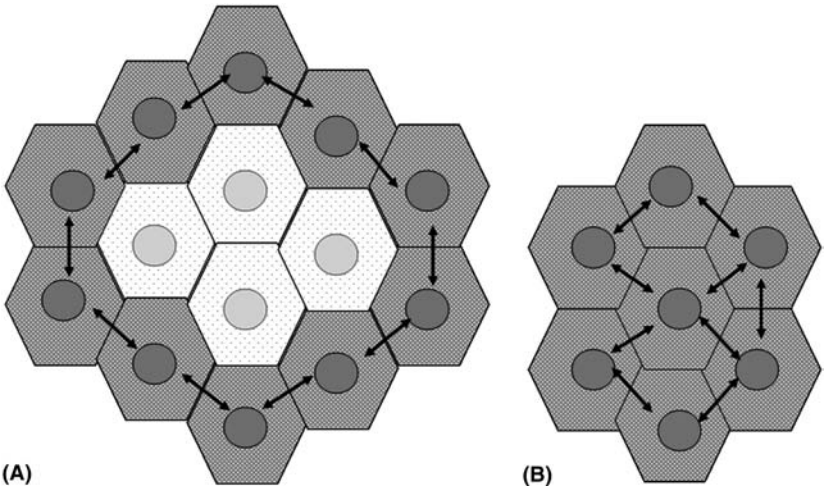

Figure 1 Illustration showing the model of growth control through junctional communication. Each hexagon represents a single cell. In the cells depicted in group A, the central cells have accumulated mutations which stimulate proliferation and reduce junctional communication. In the absence of communication with surrounding normal cells, shown by the absence of two-way arrows, proliferation is allowed and cancer progression occurs. Cells in group B have in this model being treated with retinoids or carotenoids stimulating junctional communication between themselves and surrounding normal cells. This serves to reduce aberrant proliferation and thus reduce the rate by which these cells can accumulate additional mutations leading to malignancy.

accelerate the process of carcinogenesis, but are not themselves carcinogenic—inhibit communication through gap junctions (72).

The structural element of a gap junction is a trans-membrane protein called a connexin; six of these connexin molecules are known to assemble radially to enclose the central pore. This structure can then dock with a similar structure on a contacting adjacent cell to form a complete connexon. Thus, the structural unit of the gap junction is composed of 12 connexin molecules contributed equally by each of the communicating partners (73). This arrangement is shown diagrammatically in Figure 3. Passage of molecules or ions through the central pore appears to be via passive diffusion down concentration gradients. At present, over 20 connexin family members have been recognized which are differentially expressed according to cell type and at different periods of development (74). Cx43 is the most widely expressed connexin and is the family member induced by retinoids and, as we later discovered, carotenoids.

Carotenoids induce Cx43 irrespective of their provitamin A or antioxidant properties. At the time we were characterizing the cancer chemopreventive properties of carotenoids, the only known common property shared by these

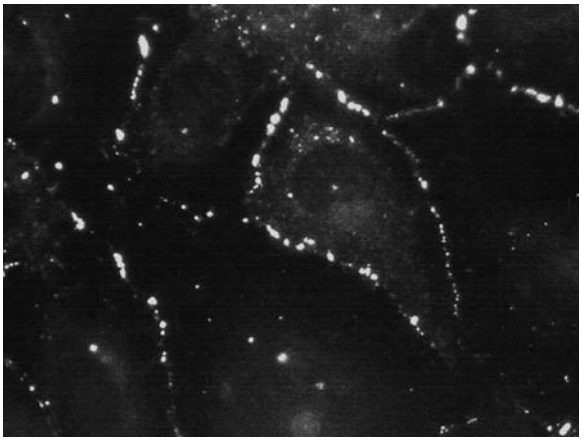

Figure 2 Immunofluorescence micrograph of confluent 10T1/2 cells treated for 96 hours with a carotenoid. Cells were fixed in acetone then labeled with a primary antibody against the C- terminal region of Connexin 43 then with a secondary fluorescent antibody. Clearly visible are the gap junctional plaques at regions of cell–cell contact. Other studies have shown that each fluorescent plaque can contain thousands of connexons.

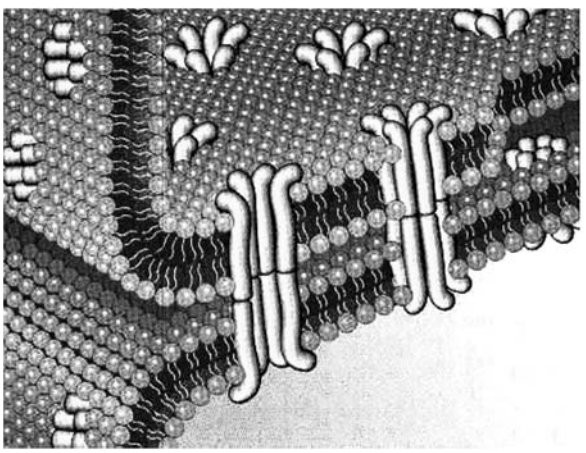

Figure 3 Organization of connexin 43 molecules into the plasma membrane. The lipid bilayers of two cells are shown in apposition; bridging this lipid barrier and at right angles to the membrane are shown six connexins donated by each of the participating cells, organized to radially enclose a water-filled pore with a diameter of approximately 16 Å. This pore directly connects the cytoplasm of one cell with that of the other and allows the transfer of ions and water-soluble molecules up to about 1000 Daltons in size.

carotenoids was the ability to act as lipid-phase antioxidants—as discussed previously, a plausible mechanism for prevention. However, when we examined the antioxidant activities of diverse carotenoids in 10T1/2 cell cultures, we discovered that whereas all carotenoids indeed did prevent oxidative damage, as indicated by a decrease in the formation of thiobarbituric acid reactive-substances (TBARS), this ability did not correlate with their activities as inhibitors of transformation. Furthermore, in this assay the most potent antioxidant was α-tocopherol, which was found to be a very poor inhibitor of neoplastic transformation even at 10^{-4}M, 10- fold higher than the concentration of many carotenoids found to totally inhibit transformation. Moreover it failed to induce Cx43 expression (75). Thus, within the limits of this particular assay system for lipid-phase oxidative damage, it appeared that while all carotenoids were, as expected, antioxidants this did not appear to be the major factor responsible for their activity as inhibitors of transformation.

Neither did activity correlate with the provitamin A activity of active carotenoids as may be expected from the similar actions of retinoids and carotenoids on Cx43 expression. Indeed recent studies have shown that these two classes of chemopreventive agents induce Cx43 expression by different mechanisms. Thus, induction of Cx43 at the level of protein and mRNA by retinoids but not carotenoids is inhibited by pharmacological antagonists of the nuclear retinoic acid receptors (RARs). In contrast, induction by nonprovitamin A carotenoids is inhibited by antagonists of the peroxisome proliferator activated nuclear receptors (PPAR). Curiously, RAR and PPAR inhibitors partially blocked the ability of β-carotene to induce Cx43 expression, suggesting that some conversion to retinoids is occurring in cell culture. Supporting this separate action of retinoids and carotenoids on the Cx43 promoter, we have recently shown that simultaneous treatment of 10T1/2 cells with carotenoids and retinoids led to supra-additive upregulation of Cx43 expression (76,77). These results suggest that combination therapy with each agent may well result in enhanced cancer preventive activity without the associated toxicity currently observed with retinoid therapy.

A water-dispersible astaxanthin derivative, Cardax, also upregulates Cx43 expression in 10T1/2 cells. There are many problems associated with delivery of carotenoids to cell cultures, experimental animals or clinically. Beadlet formulations work well but only β-carotene, canthaxanthin and lycopene have been so formulated. We developed THF as a solvent for cultures, which allows efficient delivery of all tested carotenoids (48), however, it is not acceptable for in vivo use. With a view to overcoming some of these problems, Hawaii Biotech Inc., has developed a novel carotenoid derivative, the disodium disuccinate derivative of astaxanthin, Cardax™. This compound forms a pseudo-solution in water at concentrations of up to 8 mg/mL (approximately 10 mM). Dispersibility is achieved in aqueous solution secondary to self-assembly of disodium disuccinate astaxanthin monomers into supra-molecular complexes. Monomeric solutions of compound can be achieved by inclusion of ethanol at concentrations up to 50%, thereby disrupting this self-assembly but preserving aqueous solubility (78). To

determine if this disuccinate astaxanthin derivative had activity in addition to its antioxidant properties, we treated 10T1/2 cells with various formulations of Cardax. To enhance both solubility and bioavailability, several EtOH/water formulations were tested for aggregation with UV/vis spectroscopy. The "solubility" of the derivatives was significantly enhanced by the use of 1:1 (50% EtOH) and 1:2 (33% EtOH) EtOH/water formulations. Induction levels of Cx43, as determined by immuno-blotting, were higher with the EtOH formulation at 10^{-5} M than for formulations in sterile water alone, demonstrating enhanced biological availability using EtOH as a cosolvent, as suggested by the previous physicochemical studies. The mixture of stereoisomers of Cardax in pure aqueous formulation was able to upregulate Cx43 expression with equivalent or greater potency than that previously observed for other carotenoids in organic vehicle (75,79). Importantly, treated cells were found to assemble Cx43 into immunoreactive plaques in regions of cell/cell contact, consistent with formation of gap junctions. This was confirmed by functional studies, utilizing a dye-microinjection technique, which demonstrate that treated cells were more extensively coupled than solvent-alone control treated cells (80). Unfortunately, when Cardax was tested for the ability to inhibit carcinogen-induced neoplastic transformation, the prolonged treatment times resulted in morphological changes, which invalidated the assay. Reformulation as the diphosphate, as opposed to the disuccinate derivative, overcame this problem. This racemic mixture, known as pAST, was found to be the most potent of the carotenoids tested, allowing complete inhibition of transformation at concentrations of 10^{-6} M; concentrations which also increased Cx43 expression and GJC. While some conversion to free astaxanthin was detected, these concentrations were much lower than the concentration of astaxanthin itself (delivered in THF as solvent) required to cause similar inhibition of transformation (81). Thus it would appear that the phosphate derivative of astaxanthin has enhanced biological activity compared to the parent molecule. Interestingly, it has recently been reported that astaxanthin inhibits GJC in this same cell line (82). The reasons for this discrepancy are not understood.

Forced expression of Cx43 in human carcinoma cells reduces markers of malignancy. The studies discussed previously, relating connexin-mediated functional GJC with growth control, relied on correlations to prove an association. However, these correlations do not prove a cause and effect relationship. For example, the actions of carotenoids on Cx43 gene expression may go hand-in-hand with actions of carotenoids on growth control but be functionally unrelated to these actions. To more firmly establish the role of up-regulated Cx43 expression and enhanced junctional communication as central to the role of retinoids and carotenoids as antiproliferative and cancer preventive agents, we embarked upon the development of cells in which Cx43 was inducible, not by carotenoids or retinoids, but by using a bacterial-promoter system in which activity of the artificially introduced gene is controllable by pg amounts of doxycycline, which at these concentrations is not known to produce other effects in mammalian cells. The

major advantage of this approach is that cells in the noninduced situation can serve as their own controls and the effects of Cx43 expression can be determined in the absence of concomitant exposure to carotenoids. Unfortunately, because of technical difficulties these studies have been limited to the genetic engineering of established human tumor cell lines. Three such lines have now been created; one from a cervical carcinoma (83), one from a fibrosarcoma (84) and one from a breast adenocarcinoma (Chen and Bertram, in preparation). In all cell lines, Cx43 has been shown to be rapidly inducible from the bacterial promoter, to be integrated into the plasma membrane and to form functional gap junctions with adjacent induced tumor cells. The consequences of this induction has also been consistent: in all three engineered cell lines anchorage-independent growth—that is growth as spheroids suspended in a semi-solid medium—is dramatically decreased in Cx43-induced cells. The significance of this observation is that growth in suspension has been shown to tightly correlate with the malignant potential of human tumor cells (85). Thus, Cx43 expression strongly inhibits a major in vitro marker of malignancy. A more direct test for malignancy is the ability to grow as a tumor when injected as a xenograft into immuno-compromised mice. In mice administered doxycycline in the drinking water in order to induce Cx43 expression in the injected cells, subcutaneous tumors grew much more slowly than in control animals (83). Thus, both in vivo and in vitro, Cx43 expression reduces indices of neoplasia in human carcinoma cells.

In monolayer culture, where cells grow as a two-dimensional layer on plastic, Cx43 induction did not cause changes in growth rate or saturation density. This was in contrast to the results obtained with nontransformed 10T1/2 cells, where increased junctional communication after retinoid or carotenoid treatment led to decreased proliferation. The lack of response of cells growing in monolayer culture to Cx43 induction may be attributed to the fact that these cells, derived from human tumors and which had been extensively passaged in vitro, had lost the ability to either transmit or respond to junctionally mediated cell signaling. Although none of these cell lines could be induced to junctionally communicate with growth-inhibited 10T1/2 cells, induced human breast carcinoma cells formed functional junctions with a growth-inhibited rat kidney cell line, which constitutively expressed Cx43. Under these conditions cancer cell proliferation was inhibited. The implication of these studies is that the breast carcinoma cells can no longer generate growth inhibitory signals but can still respond to signals supplied by the growth inhibited rat cells (Chen and Bertram in preparation). We have shown that down-regulated expression of Cx43 in both the human cervix and oral epithelium is an early event, observed even in dysplasia, a pathology known to predispose to malignancy but is not yet malignant (83). Our data demonstrating that up regulated expression of Cx43 achieved by pharmacological or molecular means, results in decreased proliferation of normal and malignant cells, suggests that if the observed down regulated expression of Cx43 in dysplasia can be corrected, progression to malignancy may be delayed. Indeed, in clinical intervention studies, retinoids in the case of cervical dysplasia (86) and oral leukoplakia (87),

and carotenoids in the case of oral leukoplakia (88) have been shown to significantly retard carcinogenic progression. The role of GJC in these responses to carotenoids and retinoids has not been investigated, however, we have shown in clinical trials of topical retinoic acid that dramatic up-regulation of Cx43 protein expression occurs in the suprabasal epidermis (89). Similar changes in Cx43 expression occurred after carotenoid-treatment of organotypic cultures of human keratinocytes (90).

Mechanistic studies of Cx43 induction by carotenoids. To further examine the similarities and differences between carotenoid versus retinoid induction of Cx43 at the molecular level, we have recently conducted studies utilizing the Cx43 promoter fused to a luciferase reporter gene. While others have suggested that gene regulation may be exhibited at the level of translation or as a consequence of altered mRNA stability, we have reached an opposite conclusion: that the increased expression of this gene is a direct consequence of transcriptional activation. We arrived at this conclusion as a result of the following experiments: in the 10T1/2 model system, upregulated Cx43 mRNA expression was induced by treatment with retinoids or carotenoids; additional mRNA synthesis was then blocked by treatment with actinomycin D. In such cultures, total cellular abundance of Cx43 mRNA decreased at the same rate in treated cells as in cells treated only with solvent control. The half-life of this message was approximately 5.5 hours, which is consistent with the short half-life of this message reported in other systems. Unfortunately, we were unable to get a specific mRNA signal utilizing the nuclear run-off technique. In a second series of experiments, both retinoids and carotenoids were shown to increase the activity of a luciferase reporter gene construct. Taken together, the increased abundance of Cx43 mRNA, its similar stability in treated and control conditions and the responsiveness of a luciferase reporter, all provide strong evidence for direct transcriptional activation of this gene. To further evaluate, which regions of the promoter conveyed retinoid and/or carotenoid responsiveness, we examined the promoter sequence for binding sites to know transcriptional proteins. As has been previously reported, the promoter region contains no canonical binding sites for nuclear receptors such as RAR, PPAR, or VDR. It does however contain a number of Sp1 binding sites, one of which has been previously associated with transcriptional repression. Using an electro-mobility gel shift assay system we were able to show that this sequence was capable of binding both Sp1 and Sp3 isolated from nuclei of treated 10T1/2 cells, however, we were not able to detect quantitative differences in binding between treated and control cells. To further probe the significance of this sequence to the regulation of Cx43 mRNA expression, we performed site-directed mutagenesis of this Sp1 site and ran luciferase reporter gene assays using this mutated versus wild-type sequence. The mutated sequence lost responsiveness to both retinoids and carotenoids. However, the mutated sequence had higher promoter activity than did the wild-type sequence, suggesting binding of a transcriptional repressor (Sp3?) that can be removed by retinoids or carotenoids (77). In summary, we have evidence that both retinoids and carotenoids increase GJC by increasing

transcription of the Cx43 promoter; this is supported by two lines of evidence: first, both retinoid and carotenoid treatment results in upregulated expression of luciferase Cx43 promoter constructs in F9 cells; second, that the increased steady-steady levels of Cx43 mRNA as a result of retinoid- or carotenoid-treatment are not a consequence of an increased half-life of Cx43 mRNA in 10T1/2 cells.

Lycopene may inhibit carcinogenesis by down-regulating the insulin-like growth factor pathway. The IGF growth factor system is a complex mix of two polypeptide soluble hormones with sequence similarity with insulin itself; a series of tyrosine-kinase membrane receptors with various affinities for the IGF ligands and for insulin itself; and at least six IGF binding proteins that limit the availability and thus the activity of IGF. This complex interplay of ligands, receptors and binding proteins are vital to normal growth, proliferation, differentiation and control of apoptosis, and is an attractive target for cancer prevention (91). There is accumulating evidence that perturbations in this system are responsible for carcinogenesis in several organ systems, indeed, it is an interesting hypothesis that the frequent link between obesity and cancer risk, in particular risk of breast, prostate and colon cancer, may be in part a consequence of altered signaling and interactions between insulin, IGF and downstream signaling pathways (92). A recent review of the association between levels of circulating IGF-I, reported a fairly consistent correlation between elevated serum levels and risk, with odds ratios approaching four and five for breast and prostate cancer (93). This is potentially of interest since in comparisons of cancer rates in many ethnic groups, for example, Asians versus Caucasians, rates of the two cancers are strongly correlated within the groups (94). Experimental studies have shown that both energy and protein deprivation lead to markedly lower IGF-I levels thus again potentially linking obesity with cancer risk (95). There is also evidence that such caloric restriction can increase levels of the IGF binding proteins, thus effectively decreasing receptor tyrosine kinase cell signaling.

Interest in the role of the IGF system in the potential cancer preventive activity of carotenoids stems principally from the Israeli group of Levi and Sharoni. They have reported that the influence of lycopene on this system may be mediated both by decreases in IGF-I and increases in binding proteins. Treatment of human mammary carcinoma cell lines revealed that lycopene inhibited the mitogenic activity of IGF-I via noncytotoxic mechanisms. This was shown to be a consequence of increased expression of membrane-associated IGF binding proteins, thus effectively inhibiting down-stream signaling (96). In subsequent studies, lycopene was demonstrated to inhibit cell cycle progression via reduction of the cyclin D level and retention of p27 in the cyclin E-cdk2 complex (97). More recently, because of the interest in the potential role of lycopene as a chemopreventive agent in prostate cancer, several studies have examined lycopene's effect on prostate cells, both in vivo and in cell cultures. These studies have consistently demonstrated inhibitory effects of IGF-I signaling in this organ. In normal rats, gene array expression analysis has demonstrated that expression of IGF-I in the left frontal lobe of the prostate was decreased whereas that in the dorsal lobe was

not modulated. It is unclear if the expression arrays included the IGF binding proteins. In similar studies, the same group examined expression in rat prostate injected with Dunning carcinoma cells. Expression of IGF-1 and of insulin precursors was decreased by lycopene in comparison with control treated rats. However, neither in the normal prostate, nor in rats with an implanted tumor, did lycopene decrease the mass of the organ or tumor respectively. It should be noted that multiple other genes were also modulated by lycopene treatment, most significant amongst these was the down-regulation of genes involved in androgen signaling and expression of genes such as IL-6 involved in inflammation. In view of the possible interactions between carotenoid breakdown products and retinoic acid receptors, it should be noted that retinoids have been demonstrated to increase expression of IGFBP-3 in prostate adenocarcinoma cells, which correlated with decreased proliferation of these cells. However, addition of the free protein to the culture medium was not growth inhibitory suggesting that secreted binding protein is not responsible for the observed growth inhibition (98).

Lycopene has also been demonstrated to influence the IGF system in the ferret lung cancer system developed by Wang et al. In these experiments, ferrets were treated with low-dose lycopene (1.1 mg/kg/body weight/day) or high-dose lycopene (4.3 mg/kg/body weight/day) with or without exposure to cigarette smoke. At both doses, lycopene was shown to strongly inhibit the induction of squamous metaplasia in the lung. This was accompanied by significantly increased IGFBP-3 serum levels, although levels of IGF-I were not significantly modified by lycopene treatment. Cigarette smoke alone caused significantly lower IGFBP-3 levels, which were elevated above control levels by lycopene treatment (27). In view of data from other investigators that a decrease in IGF-I signaling is responsible for induction of apoptosis, the ability of carotenoids to counteract actions of cigarette smoke on the IGF system may be responsible for the suppression of squamous metaplasia observed after lycopene treatment in the ferret lung cancer model.

It must be mentioned on a cautionary note, that while the above studies were conducted with purified lycopene from synthetic or tomato origin, tomatoes and cells contain other bioactive compounds. For example, the polyphenol quercetin found in tomatoes, and related compounds, such as genestein found in soy products, have both been shown to influence the IGF-I signaling system leading to increase apoptosis in cultured prostate carcinoma cells (99).

CONCLUSIONS

The epidemiological studies, limited clinical studies, and studies in experimental animals and in human and animal cells in culture, all support an inhibitory effect of carotenoids on carcinogenesis. Carotenoids also influence aberrant proliferation, with actions on apoptosis as well as mitosis. It is difficult to describe a single mechanism to these effects; all those discussed earlier could contribute to the epidemiological findings of an inverse association between dietary carotenoids and cancer risk and these mechanisms are not mutually exclusive. Indeed, they have

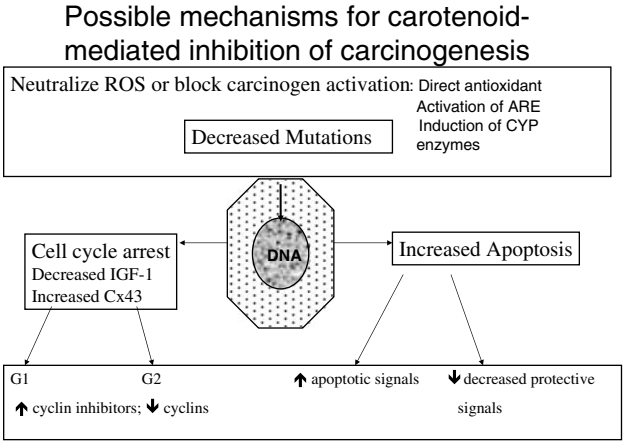

Figure 4 Representation of pathways which may contribute towards the ability of carotenoids to prevent or at least delay the onset of malignancy. These pathways serve to decrease the extent of DNA damage and the production of mutations, or, if mutations have already occurred, serve to decrease the proliferative advantage of mutated cells by inducing proliferative arrest or increased apoptosis.

the potential to synergize, thus small changes in each parameter could produce a strong overall action on carcinogenesis (Fig. 4). Induction of detoxifying enzymes could clearly reduce DNA damage by tobacco carcinogens as well as by yet unknown prostate carcinogens; the observed reduction in oxidative damage to DNA would also contribute to reduction in mutations; induction of ARE-responsive genes would also be expected to reduce cellular damage from ROS. However, additional mechanisms must contribute to the effects on proliferation and apoptosis determined in experimental settings: a reduction in IGF-I signaling has in other settings been shown to reduce susceptibility to carcinogenesis in experimental animals and to induce apoptosis in cell culture; while a reduction in androgen signaling, as detected in the gene expression arrays in the prostate, would also be expected to reduce proliferation and induce apoptosis. Finally, increased Cx43 expression, by opposing the reductions in GJC that occur during carcinogenesis, can be expected to cause both decreased mitosis and increased apoptosis. It is still somewhat uncertain whether some or all of the actions are exerted by degradation products of carotenoids—a question which is difficult to resolve because of the very unstable nature of these molecules in biological systems.

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Beta-Carotene in Erythropoietic Protoporphyria

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INTRODUCTION

Eythropoietic protoporphyria (EPP) is a rare genetic disease characterized by a defect in the enzyme ferrochelatase, which inserts iron into protoporphyrin to make heme. The genetic defect causes the accumulation of protoporphyrin in erythrocytes and plasma, resulting in painful photosensitization of any skin exposed to sunlight, and also in some cases, to strong artificial light. One of the functions of the carotenoid pigments present in all green plants and photosynthetic microorganisms is to protect these organisms against photosensitization by their own chlorophyll; the chlorophyll molecule consists of protoporphyrin and an atom of magnesium. The author relied upon this protective function of carotenoids to develop a treatment for the photosensitivity associated with EPP. The author will describe here the development of this treatment, and the use of carotenoids in the treatment of EPP and certain other photosensitivity diseases of the skin.

CAROTENOIDS AND PROTECTION AGAINST PHOTOSENSITIZATION IN BACTERIA

Sistrom, Griffiths, and Stanier (1) were the first to demonstrate that carotenoid pigments played a role in protecting photosynthetic bacteria against photodamage

induced by their own chlorophyll. They found that when the wild-type of the photosynthetic bacterium *Rhodospirillum spheroides*, and a mutant of this bacterium which lacked colored carotenoids were grown in the presence of light and air, the mutant was killed, but the wild-type, with its normal component of carotenoid pigments, survived. They showed that the cells' bacteriochlorophyll was responsible for the lethal photosensitization of the mutant, that both oxygen and light were necessary for the destructive reaction to occur, and that the carotenoid pigments were functioning as protective agents against this lethal photosensitization. Their finding that carotenoids can protect against chlorophyll photosensitization has been confirmed in other strains of photosynthetic bacteria, as well as in algae and green plants (2).

Kunisawa and Stanier (3) showed that a colorless mutant of a nonphotosynthetic bacterium that contains carotenoids, *Corynebacterium poinsettiae*, is killed in the presence of light, air, and the exogenous photosensitizer toluidine blue, whereas the wild-type (which contains colored carotenoids) was not affected by this exposure. They could not demonstrate the presence of an endogenous photosensitizer in this organism. Sistrom and the author (4,5) also showed that the carotenoids of *Sarcina lutea,* another nonphotosnythetic carotenoid-containing bacterium, could also protect this organism against lethal photosensitization by toluidine blue. Sistrom and the author further demonstrated that nonphotosynthetic carotenoid-containing bacteria do indeed contain endogenous photosensitizers. When wild-type and a colorless mutant of *S. lutea* were exposed to natural sunlight in air for four hours, it was found that, at these high light intensities with no added photosensitizer, the mutant was killed and the wild type was not; here also, oxygen was needed for killing to occur. Other workers have since confirmed the protective function of carotenoid pigments in nonphotosynthetic bacteria (6).

CLINICAL APPLICATION

The fact that carotenoid pigments can prevent photosensitization by porphyrins suggested to the author that the administration of carotenoid pigments could prevent photosensitization in patients with diseases in which the photosensitizer had some resemblance to the endogenous photosensitizer in plants, such as those porphyrias characterized by symptoms of light sensitivity. In EPP, for example, the excess porphyrins produced by the genetic defect are similar to the porphyrin group of chlorophyll. Preliminary studies in porphyria patients, which the author did in the summer of 1961 with Dr. L.C. Harber at New York University School of Medicine, suggested that the onset of erythema in response to artificial light could be delayed by the oral administration of beta-carotene. A search of the literature up to that time revealed that Kesten (7) used beta-carotene to delay the onset of erythema in a patient with urticaria solare, a disease whose photosensitizer is unknown.

STUDIES IN ANIMALS

The author developed an animal model to test more thoroughly her hypothesis that carotenoids could protect against photosensitization. Eighteen to twenty-four hours before light exposure, a suspension of 3 mg of beta-carotene in Tween-80 (8) was administered intraperitoneally to one group of 27 mice. Just prior to light exposure, each mouse in that group and each mouse in a second group of 27 mice that had not received any beta-carotene were given 1 mg of hematoporphyrin derivative (9) intraperitoneally. The author found that significantly more of the first group of 27 mice, those that had received the beta-carotene, survived the treatment with hematoporphyrin and light than did the mice in the second group of 27 mice that had not received the beta-carotene (10). Control groups indicated that Tween-80-saline suspensions are nontoxic, and afford no protection against photosensitization, and that hematoporphyrin is nontoxic to mice kept in the dark (10). Thus, beta-carotene was effective in mice in preventing the lethal photosensitivity induced by injection of hematoporphyrin and exposure to visible light.

TREATMENT OF ERYTHROPOIETIC PROTOPORPHYRIA

The successful photoprotection studies in animals suggested that the author should determine if the administration of beta-carotene could prevent or lessen photosensitivity in human patients. The disease chosen for study was EPP, in which ferrochelatase, the enzyme that inserts iron into protoporphyrin to make heme, is defective, resulting in the accumulation of protoporphyrin in blood and other tissues. Leakage of protoporphyrin from blood cells into the plasma leads to a cascade of reactions resulting in itching, burning, and ulceration of skin on exposure to visible light (11).

The first patient with EPP who was treated, a 10-year-old girl, could tolerate only brief exposures to sunlight. Exposure to a carbon arc lamp (340-640 nm) produced erythema in two minutes. In June of 1968, she was given a preparation of concentrated carrot oil in doses approximately equivalent to 30 mg beta-carotene per day. After a month of carrot oil ingestion, she could tolerate at least 30 minutes of carbon arc light and more than one hour of natural sunlight. By the middle of the summer, she could play outdoors in the afternoon without experiencing any symptoms of photosensitivity. In the summer of 1969, she and two other patients were given beta-carotene in the form of 10% beta-carotene "beadlets" (Hoffman-La Roche, Nutley, New Jersey). Here also, all three were found to have improved tolerance to sunlight exposure (12,13). In 1970, the author set up a collaborative study to include all the patients of Dr. Harber and those of other physicians who had contacted the author concerning the use of beta-carotene since the publication of their first three cases. By the summer of 1975, they had treated 133 patients suffering from EPP with beta-carotene using a standard

protocol (14,15). In July 1975, the U.S. Food and Drug Administration approved the use of beta-carotene for the treatment of EPP, and the collaborative study was terminated.

In the collaborative study, the following starting dosage schedule for beta-carotene was used (14,15) and the author still recommends it today:

1–4 years of age—60–90 mg/day;
5–8 years of age—90–120 mg/day;
9–12 years of age—120–140 mg/day;
13–15 years of age—150–180 mg/day;
16 years and older—180 mg/day.

The average dose for the patient's age should be administered for four to six weeks, and the patient should be instructed not to increase sun exposure either for four weeks or until some yellow discoloration of the skin, especially of the palms of the hands, is noted. Exposure can then be increased cautiously and gradually until the patient determines the limits of exposure to light that can be tolerated without the development of symptoms. If the degree of protection is not sufficient, the daily dose of carotene should be increased by 30–60 mg/day for children under 16, and up to a total of 300 mg/day for those over 16 years of age. If after three months of therapy at these higher doses (blood carotene levels should reach at least 800 μg/dL) tolerance to sunlight exposure has not increased significantly, beta-carotene therapy will likely not be effective for that patient, and the medication should be discontinued. The author found that 84% of the patients increased their ability to tolerate sunlight exposure at least three-fold without the development of symptoms (14,15). On the average, it took between one and two months for the patients who received benefit from carotene therapy to notice increased tolerance to sun exposure. The majority of patients reported that with beta-carotene therapy, they could engage in outdoor activities that were impossible before therapy. Children who could not play outdoors to any great extent before carotene therapy, could now spend hours outside with their friends. Many patients stated that they were able to develop a suntan for the first time in their lives, some feeling that the acquisition of the tan, plus the beta-carotene, added to their protection from the sun's effects. The majority of the patients noted that while they took beta-carotene, reactions to the sun that do occur are less severe in intensity and duration than before therapy, and that they also developed fewer cutaneous lesions during their increased exposure.

The author found that the patients' blood and stool porphyrin levels were not affected by the ingestion of large amounts of beta-carotene. Thus, treatment with beta-carotene ameliorates photosensitivity in EPP, but has no effect on the biochemical lesion in this disease.

Because of the difficulties in the subjective evaluation of therapeutic effect and of setting up a controlled double-blind study of beta-carotene and EPP, due to the fact that high-dose carotenoid ingestion can lead to some orange pigmentation of skin, the author decided to use phototesting with polychromatic light from

380 to 560 nm (as opposed to monochromatic light) as an objective measure of clinical improvement. Under the protocol we have developed (14,16), tolerance to xenon arc light exposure increased in those patients who reported benefit from beta-carotene therapy. No increased tolerance has been found in patients reporting no improvement. Other workers have also noted increased tolerance to polychromatic xenon arc light after treatment with beta-carotene (17–18). Thus, phototesting with polychromatic xenon arc light can serve as an objective method of determining improvement in tolerance to light.

CONFIRMATION OF OUR RESULTS

In previous reviews (19–23) the author listed 63 studies, and here lists three additional studies (24–26) reporting increases in tolerance to sunlight in a majority of patients with EPP treated with high doses of beta-carotene, either alone or in combination with canthaxanthin. In some of these series, as well as in other reports (27–31), some patients do not benefit from carotenoid therapy in spite of adequate dosage; this is most likely due to either poor absorption of pigment, use of a beta-carotene preparation with low bioavailability, or the patient having markedly elevated blood porphyrin levels. One controlled study of beta-carotene therapy in EPP reported little or no improvement in the subjects' photosensitivity (32): unfortunately, these workers used a lower dosage of beta-carotene than we had recommended as effective. Later, some of the patients from this unsuccessfully treated study were given higher doses of beta-carotene by another investigator and noted some increased tolerance to sun while taking the higher dose (33). These results emphasize the importance of individualizing dosage and increasing the dose until the patient reports some improvement. The problem of individualizing treatment is especially important to the conduct of a controlled trial and makes double-blinded designs almost impossible. At a minimum, such trials should use a dose of beta-carotene large enough to ameliorate symptoms in the majority of patients (a minimum period of three months' treatment at doses giving blood levels of at least 800 μg/dL—for adults at least 180 mg/day of beta-carotene). Another important factor is the form of beta-carotene used; we recommend Roche 10% beta-carotene beadlets (Lumitene, Tishcon), which has the high bioavailability necessary to produce the elevated blood levels of beta-carotene needed for effective photoprotection. To summarize, in spite of the occasional treatment failure, from the author's results, and those of the other workers listed above, it can be concluded that beta-carotene, administered in sufficiently high doses, and in bioavailable form, can be an effective therapy for ameliorating photosensitivity in most patients with EPP.

CAROTENOIDS IN OTHER PHOTOSENSITIVITY DISEASES

Since beta-carotene seemed effective in preventing photosensitivity in EPP, it was logical to see if it could be an effective treatment for other photosensitivity

diseases. It was reported previously (23) that there have been nine reports of some success in carotenoid treatment of patients suffering from congenital porphyria (Gunther's disease). New lesions were significantly decreased in number and severity, and the patients were able to increase their sun exposure to some degree. In most cases, other treatments such as transfusions and meticulous treatment of skin infections must continue.

As previously reported (23), several groups, including the author's, have used carotenoids to reduce photosensitivity in polymorphic light eruption (PMLE). Reports of improvement range from one-third to two-thirds of the patients being able to tolerate light exposure without the development of new lesions. Usually, sunscreens must also be used to get a beneficial effect from carotenoid intake; sunscreens alone did not provide relief.

Several studies reported that carotenoid treatment was effective in the photosensitivity diseases porphyria cutanea tarda, porphyria variegata, actinic reticuloid, solar urticaria, and hydroa aestivale, but other studies found carotenoids ineffective (23). It is recommended that beta-carotene be given in these conditions only after the first-line treatment modalities usually used have failed, or to enhance their effectiveness.

HOW DO CAROTENOIDS PREVENT PHOTOSENSITIZATION?

Krinsky (2) suggested four possible means by which carotenoid pigments could exert their protective functions: (*i*) a filter system in the cell envelope to filter out potentially harmful light, (*ii*) systems that can interact with and quench photosensitizer triplet states, (*iii*) systems that can serve as preferred substrates for photosensitized oxidations, and (*iv*) systems that can stabilize membranes or repair damaged membranes. Since Krinsky's work, additional evidence has indicated that only the second suggested mechanism, now extended to include quenching of singlet oxygen by carotenoids, seems to be significantly associated with the pigments' protective function (6). Mechanism (*i*) may have some function in certain plants, although it may not be the sole explanation for the pigments' protective effects in these organisms. This mechanism is also unlikely to be involved in carotenoid protection in humans, as the amounts deposited in skin are not sufficient to act as a physical sunscreen (34,35). In addition, the photoprotective effect of the carotenoids seems to be independent of their absorption spectrum. The findings in photosynthetic organisms that led to the suggestion of mechanism (*ii*) by several groups of workers are thought to be connected with reactions involved with photosynthesis rather than with the protective function of carotenoids (6). The current author and Krinsky showed that the presence of carotenoids does not seem to be involved with membrane stability in Sarcina lutea (36), thus suggesting that mechanism (*iii*) may not be of wide significance in the protective function of carotenoids.

Thus it would seem that the quenching of excited species is the most widely applicable mechanism for the carotenoids' protective effects. Since the first demonstration of the ability of carotenoids to quench the triplet state of chlorophyll

(37) and to quench singlet oxygen (38), the author and many workers have confirmed that porphyrins form these excited species when illuminated, and that carotenoids indeed can quench them.

Other studies have examined the photoprotective action of carotenoids at the cellular level. Using a method of detecting photochemical reactions in epidermis, the author has shown that carotenoids in the skin of mice made porphyric by the ingestion of collidine and receiving supplementation of either beta-carotene or canthaxanthin, can quench photochemical reactions occurring in isolated epidermis (39). In nonporphyric mice supplemented with either of these carotenoids, or with the colorless carotenoid phytoene, the pigments also quenched excited species formed in skin exposed to UV-B (290–320 nm) radiation (40). Although more work needs to be done to determine the actual molecular mechanisms of photosensitization and photoprotection in humans, it is conceivable that carotenoids prevent the porphyrin-induced peroxidation and lipid oxidation of cellular components of endothelial and immune system cells, and thereby prevent the release of mediators that give rise to the symptoms associated with photosensitization in EPP and possibly other photosensitivity diseases.

LACK OF TOXICITY OF BETA-CAROTENE IN ERYTHROPOIETIC PROTOPORPHYRIA

The most predominant side-effect of carotenoid therapy is carotenodermia, which is most obvious on the palms of the hands and the soles of the feet of people ingesting large amounts of carotenoids, and only occasionally seen on other parts of the body. This condition disappears in a few weeks after beta-carotene intake stops.

It was found that no abnormalities in blood chemistry or hematology tests could be attributed to beta-carotene intake, nor have such abnormalities been reported by other workers. A review of the literature revealed only one instance of biochemical abnormality from beta-carotene use in EPP. Warren and George (41) report that a patient who, starting at age 11, was treated with beta-carotene alone, then with a mixture of beta-carotene and canthaxanthin, and then again with beta-carotene alone, was found on routine liver function tests after about nine years of carotenoid treatment to have an elevated aspartate transaminase (AST) test, but the patient's bilirubin level and other enzymes remained within normal limits. Withdrawal of beta-carotene brought the AST back to normal; readministration again caused the AST elevation. Unfortunately, the authors did not state which brand of beta-carotene was used at the time the abnormality was found, or whether there had been a change in brand during beta-carotene administration, or even if the patient had started taking any other medications, or possibly drinking alcohol, around the time that the abnormality was discovered. Apparently, the beta-carotene caused no serious damage, as all liver chemistries including AST remained within normal limits for at least the five years of follow-up covered by this paper.

Some patients report gastrointestinal disturbances when they first start taking carotenoids. Usually, these clear up spontaneously, but some patients require that the dose be lowered. On very rare occasions, a patient may have to stop beta-carotene intake to obtain relief.

Carotenoids, including beta-carotene, have been investigated for anticancer activity. During the course of two placebo-controlled intervention trials of beta-carotene and lung cancer prevention in high-risk individuals (heavy smokers, some of whom were also asbestos workers and heavy drinkers), the group randomized to beta-carotene intake had an increase in lung cancer rate as compared to placebo (42,43). However, in another large intervention trial studying beta-carotene prevention of all cancers, no increase in any kind of cancer was found in the group taking beta-carotene (44). It is possible that alcohol or cigarette smoke may have been a factor in generating some kind of toxic product. Studies by Lieber's group (45) suggest that ethanol reacts with beta-carotene and vitamin A (which was also given in the cancer studies but not by us), and that smoking can exacerbate this, which may explain the increase in lung cancers in the smokers' study. As we and other workers have found in EPP patients, beta-carotene intake does not seem to pose a risk to people who do not smoke and drink in excess. In fact, people with EPP are warned not to drink or take any medication, which causes cholestasis, as such intake can increase their risk of developing potentially fatal liver disease caused by the accumulation of excess protoporphyrin in their livers.

Canthaxanthin, which has been used in conjunction with beta-carotene in Europe, is also effective in preventing photosensitivity, but ingestion of large doses leads to the deposition of pigmented granules in the retinas of some patients, which occasionally may have some effect on night vision (46). The granules have been found to disappear several months after cessation of canthaxanthin ingestion, with return of any visual changes to normal (46). No such granules seem to form from beta-carotene ingestion (47). It should be noted that canthaxanthin, although approved by the U.S. Food and Drug Administration as a food coloring agent, has not yet been approved for use as a drug.

SUMMARY

Studies in bacteria, animals, and humans have demonstrated that carotenoid pigments can prevent or lessen skin photosensitivity by endogenous photosensitizers, such as chlorophyll or porphyrins, as well as by exogenous photosensitizers such as dyes (e.g., toluidine blue) or porphyrin derivatives. The carotenoids beta-carotene and canthaxanthin have been found to be very effective in the treatment of the photosensitivity associated with EPP and to a lesser extent in the treatment of certain other photosensitivity diseases. No serious toxicity has been reported, although the use of canthaxanthin is not recommended because of its propensity to form retinal granules. The pigments most likely perform their protective function by quenching excited species formed by the interaction of porphyrins or other

photosensitizers, light and air, thereby preventing the cellular damage that leads to the symptoms of photosensitivity.

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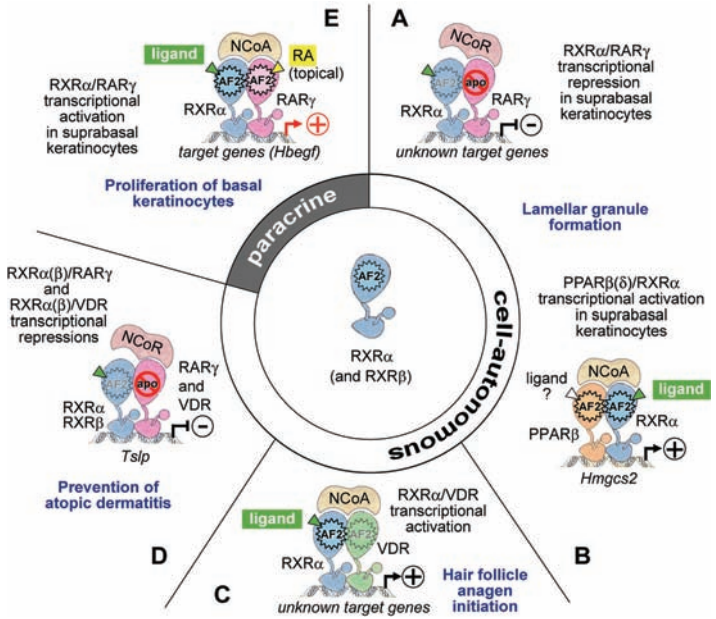

Figure 2.1 Germline and conditional mutagenesis approaches, combined with pharmacological approaches, have revealed some of the physiological roles of the retinoid nuclear receptors in homeostasis of mouse skin epidermis. (*See p. 35*)

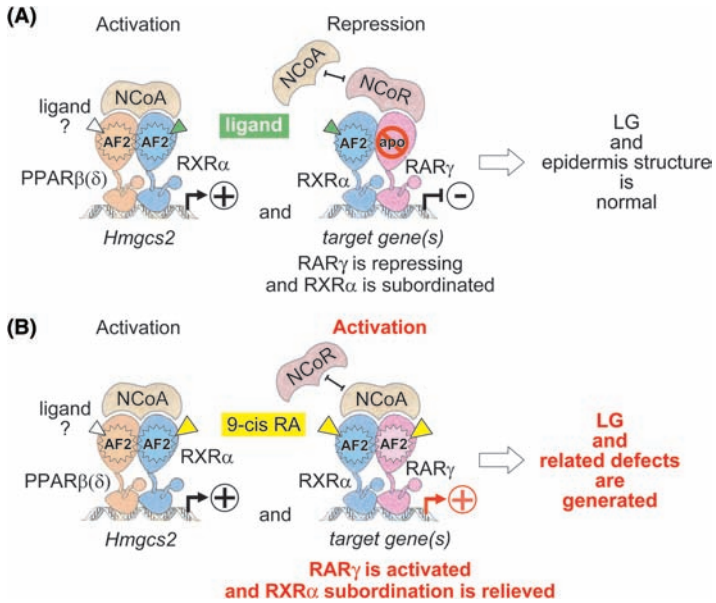

Figure 2.2 The concomitant occurrence of RAR/RXR-mediated repression and RXR/PPAR$\beta(\delta)$-mediated activation events observed in mouse epidermis suprabasal keratinocytes rules out the possibility that the ligand activating RXR AF-2 could be 9-*cis* RA. (*See p. 38*)

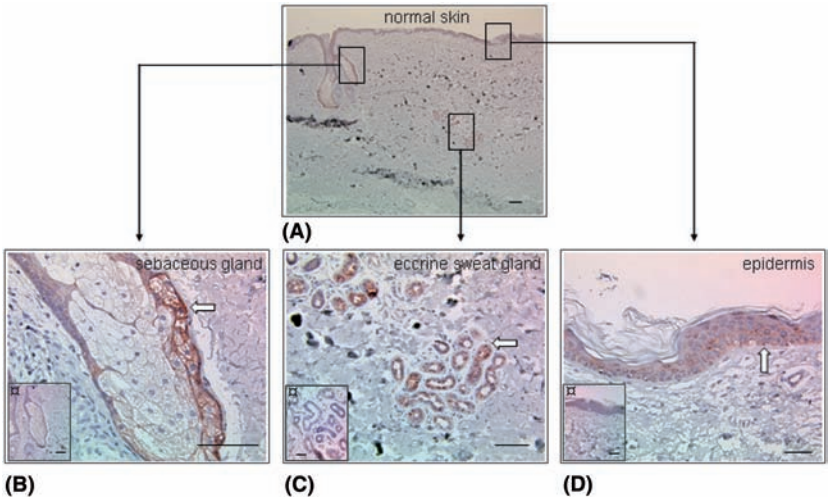

Figure 4.2 Immunohistochemical analysis of the expression of the retinoid acid metabolizing CYP26AI in normal human skin. (*See p. 72*)

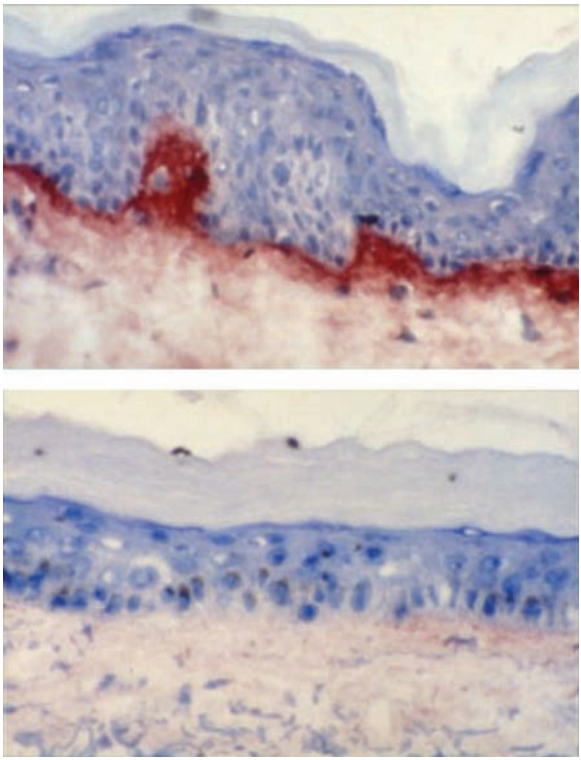

Figure 5.2 Photomicrograph of sun-protected (*upper panel*) and sun-exposed (*lower panel*) skin stained for procollagen I. (*See p. 80*)

Figure 5.5 Significant lightening of facial actinic lentigines following 10 months' treatment with 0.1% tretinoin cream. (*See p. 87*)

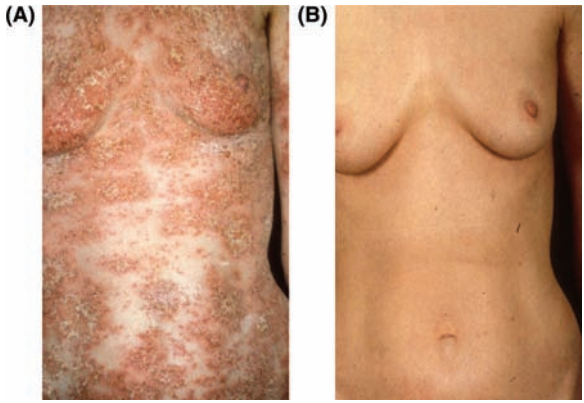

Figure 7.1 Clinical response to acitretin (1 mg/kg/day) in a patient with generalized pustular psoriasis. (*See p. 134*)

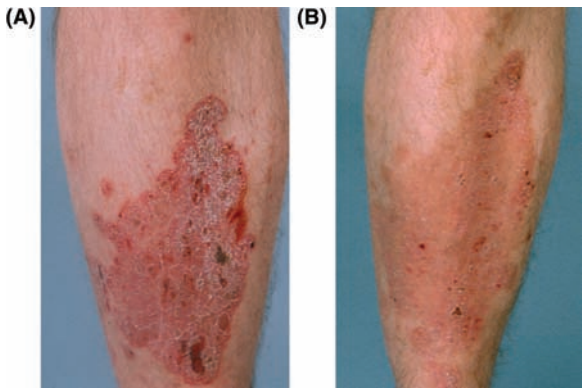

Figure 7.3 Clinical response to Rambazole™ (1 mg/ day) in a patient with moderate to severe plaque type psoriasis. (*See p. 144*)

Figure 9.1 Lamellar ichthyosis before and after one month treatment with oral etretinate 1.5 mg/kg/day. (*See p. 173*)

Figure 9.2 Epidermolytic hyperkeratosis before and after two months of oral retinoid therapy 0.5 mg/kg/day. (*See p. 176*)

Figure 9.3 Palmo-plantar keratoderma before and after one month of oral retinoid therapy 1 mg/kg/day. (*See p. 176*)

Figure 9.4 Erythrokeratoderma progressiva symmetrica before and three weeks after oral retinoid therapy. (*See p. 179*)

Dermatology

about the book...

This up-to-date reference describes how retinoids and carotenoids function in the skin and how they can be utilized to prevent and treat a wide variety of skin diseases, as well as advance biomedical research in relation to cancer treatment and immunology. Providing an in-depth update on the pharmacology, pharmacodynamics, and new applications of retinoid therapy, this source also addresses topics outside of dermatology, such as vitamin A nutrition, and the role of antioxidants in aging, metabolic activation, and cellular signaling. With chapters by internationally recognized authorities in specialties ranging from biochemistry and nutrition to molecular biology and clinical science, this source will stand as the only up-to-date source on the topic.

Summarizing the latest research concerning the therapeutic utility of these compounds, this source covers new dermatological indications for retinoid therapy, such as the use of bexarotene in lymphoma and chronic eczema...discusses the mechanisms of retinoid and carotenoid function in the skin...provides a clear understanding of the possible impact of dietary carotenoids and retinoids on metabolic and genetic pathways that lead to human carcinogenesis...studies the side-effects and adverse reactions related to retinoid therapy... and presents a current overview of the metabolism and molecular actions of retinoids for the potential design of new approaches to manage a variety of dermatological diseases.

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