

**MOLECULAR AND
CELLULAR BIOLOGY OF
PROSTATE CANCER**

MOLECULAR AND CELLULAR BIOLOGY OF PROSTATE CANCER

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PREFACE

These proceedings of the 5th Prouts Neck Meeting on Prostate Cancer, held in October, 1989, highlight the many advances in the understanding of prostatic growth and function at the cellular and molecular levels which have been registered since the first Prouts Neck Meeting in 1985, a meeting which also focused on the then current concepts and basic approaches to understanding prostate cancer. Intervening Prouts Neck Meetings in 1986, 1987 and 1988 were devoted to treatment, image cytometry and clinical markers. As before, the Prouts Neck tradition of bringing together an international, multidisciplinary group of experts for 3 days to exchange ideas and new data, in the relaxed atmosphere of an old inn on the scenic Maine coast, proved to be an ideal combination for a highly successful conference. Accordingly, the Organ System Program of the National Cancer Institute plans to use the Prouts Neck model for future conferences on other solid tumors (bladder in 1990 and kidney in 1991) and will return to the prostate in 1992.

A new dimension was added to the current program through the inclusion of a poster session to recognize the research of pre- and postdoctoral investigators. The posters were judged by Drs. Collette Freeman, Frank French, Shutsung Liao, Robert Matusik and Henry Sun. The three winners, in alphabetical order, were John Fabian, Robert Getzenberg and Ming Fong-Lin.

Over the past five years, the efforts of the Prostate Cancer Working Group have brought into precise focus the priorities and specific areas for basic and clinical research. The many accomplishments which they have registered for the nation through countless hours of endeavor would not have been possible without their non-self-serving dedication to the advancement of prostate cancer research. We are honored to dedicate this book to the Prostate Cancer Working Group and to the many consultants who contributed to the remarkable success of the Organ Systems Program.

J.P. Karr
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TISSUE SPECIFICITY AND CELL DEATH ARE ASSOCIATED WITH SPECIFIC ALTERATIONS IN NUCLEAR MATRIX PROTEINS*

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INTRODUCTION

The ability of identical signals to interact with specific tissues in the same animal and result in the expression of different genes, is a fundamental question in cell regulation. A suitable model system for investigating the tissue specific regulation of gene expression is found in comparing the dihydrotestosterone (DHT) response in ventral prostate with the seminal vesicle. The rat ventral prostate and the seminal vesicle both contain the same genome, possess nuclear DHT receptors, and respond to DHT with the production of markedly different secretory products (figure 1). Upon DHT stimulation, the ventral prostate produces several specific secretory proteins. Similarly, the seminal vesicle produces its own unique tissue specific secretory proteins (figure 2). These tissue specific secretory proteins are all under control of the androgen receptor. Evidently there is another form(s) of regulation which determines what genes are activated when the cells are stimulated with androgen. We hypothesize that this regulation is brought about by alterations in the three dimensional conformation of the genome within the nucleus. It is believed that the genomic configuration within the nucleus is determined in part by the binding of DNA loop domains to the nuclear matrix proteins. If the ventral prostate and seminal vesicle each possess unique conformations of their DNA, it is possible that the androgen receptor is able to bind and activate tissue specific transcription on different locations of the DNA depending on the tissue. Since the nuclear matrix is the organizing structure of the DNA in the nucleus, tissue specificity in three dimensional DNA organization may be caused by a unique nuclear matrix composition. We propose that the nuclear matrix is tissue specific and is involved in the regulation of gene expression.

MAJOR SECRETORY PROTEINS

A major secretory protein of the ventral prostate has been identified as prostatein or prostate binding protein by a number of investigators. This protein is a steroid binding protein with a molecular weight of approximately 40,000 kD and a pI of 4.8 (Lea, O.A. et al., 1979). Prostatein is a glycoprotein which consists of four subunits. The major subunit is known as C3 and has a molecular weight of 14 kD (Viskochil, D.H., et al., 1983). When the intact prostatein protein is run under denaturing SDS/PAGE, the protein separates into two bands of 22 kD and 20 kD which further dissociate to the 14 kD C3, the 10 kD C2, and the 6 kD C1 subunits (Lea, O.A. et al., 1979). The seminal vesicle produces six major secretory proteins which are referred to as SVS I to VI (Ostrowski, M.C. et al., 1979). SVS IV, the most abundant protein secreted by the seminal vesicle, has a molecular weight of 17 kD (Wagner, C.L., and Kistler, W.S., 1987). SVS IV is the major protein that we will use as a marker of androgen regulated seminal vesicle secretory proteins and the C3 subunit of prostatein will be used as an example of a ventral prostate specific androgen regulated secretory protein (figure 2).

*Supported by HHS NIDDKD DK22000

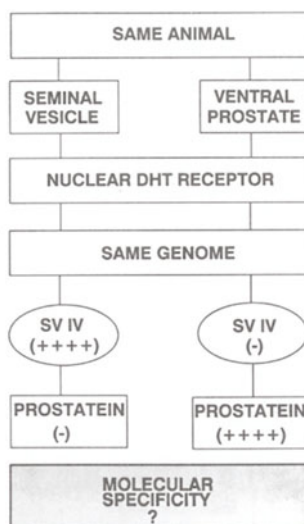


Figure 1: Statement of Problem. Why does the same signal interact with identical genomes in different tissues to produce different gene expressions? In the same animal, the ventral prostate and seminal vesicle possess nuclear DHT receptors and similar genomes. Upon DHT stimulation, each produces tissue specific secretory proteins. The major protein produced by the seminal vesicle is SV IV and the major protein produced by the ventral prostate is prostatein. This research attempts to determine what controls this tissue specificity on a molecular basis.

DNA COMPOSITION

Our hypothesis is based on the assumption that the genomes of the ventral prostate and seminal vesicle are identical. One possible exception involves the use of DNA methylation as a method of gene regulation. At present, DNA methylation does not seem to be a steroid regulatory mechanism used with these two genes (Kandala, J.C. et al., 1985; White, R. and Parker, M., 1983). Methylation of genes may be developmentally controlled (White, R. and Parker, M., 1983) but, SV IV is hypomethylated in both the ventral prostate and seminal vesicle (Kandala, J.C. et al., 1985). Rearrangements, deletions, and mutations have not been established as factors.

NUCLEAR MATRIX

The nuclear matrix is the residual element of the nucleus including the peripheral lamins and nuclear pore complexes (see Table 1). The nuclear matrix is the insoluble skeletal framework of the nucleus (Berezney, R. and Coffey, D.S., 1974). This matrix serves as the protein framework on which the DNA is organized into loop domains of approximately 60 kilobases (Pardoll, D.M. et al., 1980; Vogelstein, B. et al., 1980). The base of the loops are attached to the matrix and have been identified as the location of actively transcribed genes (Zehnbaauer, B.A. and Vogelstein, B., 1985; Robinson, S.I. et al., 1983; Small, D. et al., 1985; Small, D. and Vogelstein, B., 1985; Mirkovitch, J. et al., 1984; Buttyan, R. and Olsson, C.A., 1986). The nuclear matrix has also been identified as a site of attachment of over 80% of the heteronuclear RNA (hnRNA) (Miller, T.E. et al., 1978; Peters, K.E., and Commings, D.E., 1980; van Eekelen, C.A. and van Venrooij, W.J., 1981; Long, B.H. and Schrier, W.H., 1983; Fey, E.G. et al., 1988). Transforming proteins and oncogene products such as the *myc* protein (Eisenman, R.N., et al., 1985), the large T antigen of the SV40 virus (Staufenbiel, M. and Deppert, W., 1983; Covey, L. et al., 1984) and the transcription factor E1A from adenovirus (Sarnow, P. et al., 1982) have been found to interact with the nuclear matrix. Recent evidence also indicates that the steroid receptor is associated with the nuclear matrix (Barrack, E.R. and Coffey, D.S., 1980; Donnelly, B.J. et al., 1984; Alexander, R.B. et al., 1987; Mowszowicz, I. et al., 1988).

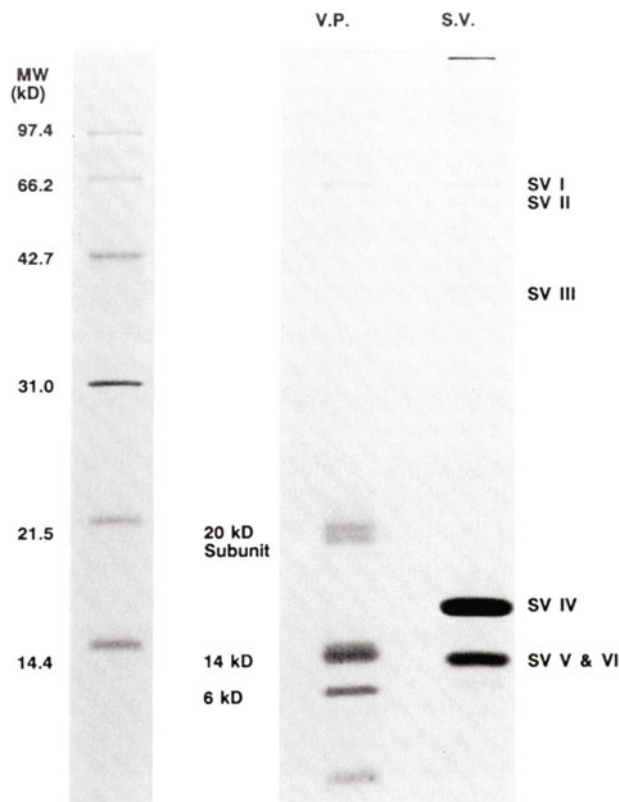


Figure 2: Major Secretory Proteins of the Seminal Vesicle(S.V.) and Ventral Prostate(V.P.) Rat seminal vesicle and ventral prostate secretory proteins reduced and separated on 15% SDS/PAGE. Major secretory proteins of the ventral prostate are similar to those identified by Lea, O.A. et al., 1979, as subunits of denatured prostatein. The 14 kD protein is the C-3 subunit. Major secretory proteins (SVS I-VI) of the seminal vesicle were identified as those described by Wagner, C.L. and Kistler, W.S., 1987.

Further evidence for the role of the nuclear matrix in the regulation of gene expression comes from studies of specific protein components of the nuclear matrix. Several studies have indicated that mitogenic stimulation (Bladon, T. et al., 1988) and induction of differentiation (Stuurman, N., et al., 1989) cause changes in the protein composition and structure of the nuclear matrix. Studies of the ovalbumin gene have provided insight into the role of the nuclear matrix in transcriptional control. Using DNA probes for different regions of the ovalbumin gene, sequences which are adjacent to and within the ovalbumin gene are associated with the nuclear matrix only in the hen oviducts and not in the nuclear matrices of other tissues in the same animal where the ovalbumin gene is not expressed (Robinson, S.I. et al., 1982; Robinson, S.I. et al., 1983; Ciejek, E.M. et al., 1983). Androgens have previously been shown to cause changes in nuclear proteins (Chung, L.W.K. and Coffey, D.S., 1971; Carmo-Fornesca, M., 1988). Using one dimensional electrophoresis, a 20 kD androgen dependent nuclear peptide was found after castration and subsequent androgen treatment (Kishimoto, R. et al., 1982; Venkatraman, J.Y. et al., 1984). The role of this 20 kD protein in regulation of gene expression is unknown. It is evident from these studies that steroid receptors may interact with the matrix and that the matrix may be important in controlling gene expression.

Table 1: Functions of the Nuclear Matrix
(For Review see Pienta, K.J., et al., 1989)

Nuclear Morphology	-Controls 3-D Structure of Nucleus and DNA
DNA Organization	-60 kD DNA Loop Domains Attached at Bases -Origins of Replication
DNA Replication	-Location of Fixed Sites of DNA Replication -Contains Replisome Complex which Includes DNA DNA Polymerase and Newly Replicated DNA
RNA Synthesis	-Site of Actively Transcribed Genes -Contains hnRNA, RNA Splicing
Cell Regulation	-Associates with Steroid Hormone Receptors -Interaction with <i>myc</i> , large T antigen, E1A -Changes in Protein Composition with: Differentiation Mitogenic Stimulation Androgens

Alterations in nuclear matrix proteins may be responsible for the differential gene expression of the seminal vesicle and the ventral prostate. Nuclear matrix proteins have recently been shown to vary significantly between different cell lines (Fey, E.G. and Penman, S., 1988). This study also found differences in nuclear matrix proteins when comparing transformed and non-transformed cell lines of similar origins. The nuclear matrix proteins in the transformed cells are currently under investigation as possible probes for the diagnosis of cancer and in determination of the tissue of origin of some cancers.

MODIFICATIONS OF NUCLEAR MATRIX PROTEINS

The components of the nuclear matrix have been found to undergo a number of protein modifications including, phosphorylation, methylation and ADP-ribosylation. The major modification studied is that of phosphorylation. Several groups have demonstrated the phosphorylation of the nuclear matrix proteins (Allen, S.L. et al., 1977; Sevaljevic, L. et al., 1982; Henry, S.M. and Hodge, L.D., 1983; Moy, B.C., and Tew, K.D., 1986). Phosphorylation of the nuclear matrix proteins has been found to be under androgenic control (Goueli, S.A. and Ahmed, K., 1984). The nuclear components themselves were demonstrated to be involved in protein phosphorylation. Recently, the nuclear matrix of rat liver was found to possess tyrosine kinase activity (Teraoka, H. et al., 1989). The role of phosphorylation and other protein modifications in the matrix has not been resolved although, it is probable that these changes, especially phosphorylation, are somehow involved in the regulation of the activity of the nuclear matrix.

NUCLEAR MATRIX ISOLATION PROCEDURES AND ANALYSIS

There are several different procedures currently used for nuclear matrix isolation. The procedure utilized in these experiments involves the use of mild salt extraction with ammonium sulfate which causes minimal disruption of the nuclear matrix proteins and structure (figure 3). The protein components of the matrix were analyzed utilizing a high resolution two dimensional electrophoresis system developed in our laboratory.

This procedure employed 10% SDS/PAGE in the second dimension and required several modifications of the original O'Farrell technique for two dimensional electrophoresis (O'Farrell, 1975). The gels were silver stained (Accurate Scientific) after enhancement techniques.

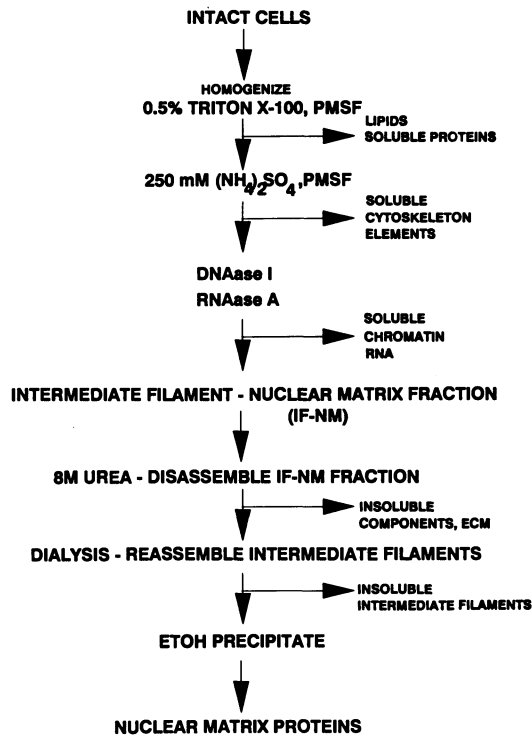


Figure 3: Nuclear Matrix Purification Procedure. Sequential isolation procedure from fresh tissue. Lipids and soluble proteins are removed with triton X-100. The soluble cytoskeletal elements are then removed with 0.25 M ammonium sulfate. DNAase I and RNAase A are then used to remove the soluble chromatin elements. The remaining intermediate filament-nuclear matrix fraction is then disassembled by solubilization in 8 M urea and 1% -mercaptoethanol at room temperature as described in Fey, E.G. et al.,1984. Removal of the urea with dialysis reassembles the intermediate filaments and the nuclear matrix proteins are removed from the supernatant by ethanol precipitation.

RESULTS

The nuclear matrix proteins of both the rat ventral prostate and seminal vesicle were compared using the two dimensional electrophoresis technique described. This technique provides high resolution of the protein components. When the gels from both the ventral prostate and seminal vesicle nuclear matrices are compared, the protein patterns of the two tissues are different. Thorough analysis reveals major proteins unique to each of the tissues which are indicated with arrows in the figures (figures 4 A & B). Numerous quantitative changes as well as minor qualitative protein changes were also noted in the gels. The major protein changes are unique for each tissue and any of these proteins can be used as a tool for tissue identification (figures 4 C & D).

To determine the effects of androgens, the protein patterns of both the intact rat ventral prostate and seminal vesicle nuclear matrices were compared with that of the 23 hour castrate ventral prostate and seminal vesicle nuclear matrices (figures 5 A & B). On visual examination these protein patterns from castrates are not markedly different from their intact counterparts. However, careful analysis of the protein patterns reveals several major proteins and numerous minor proteins which both disappear and

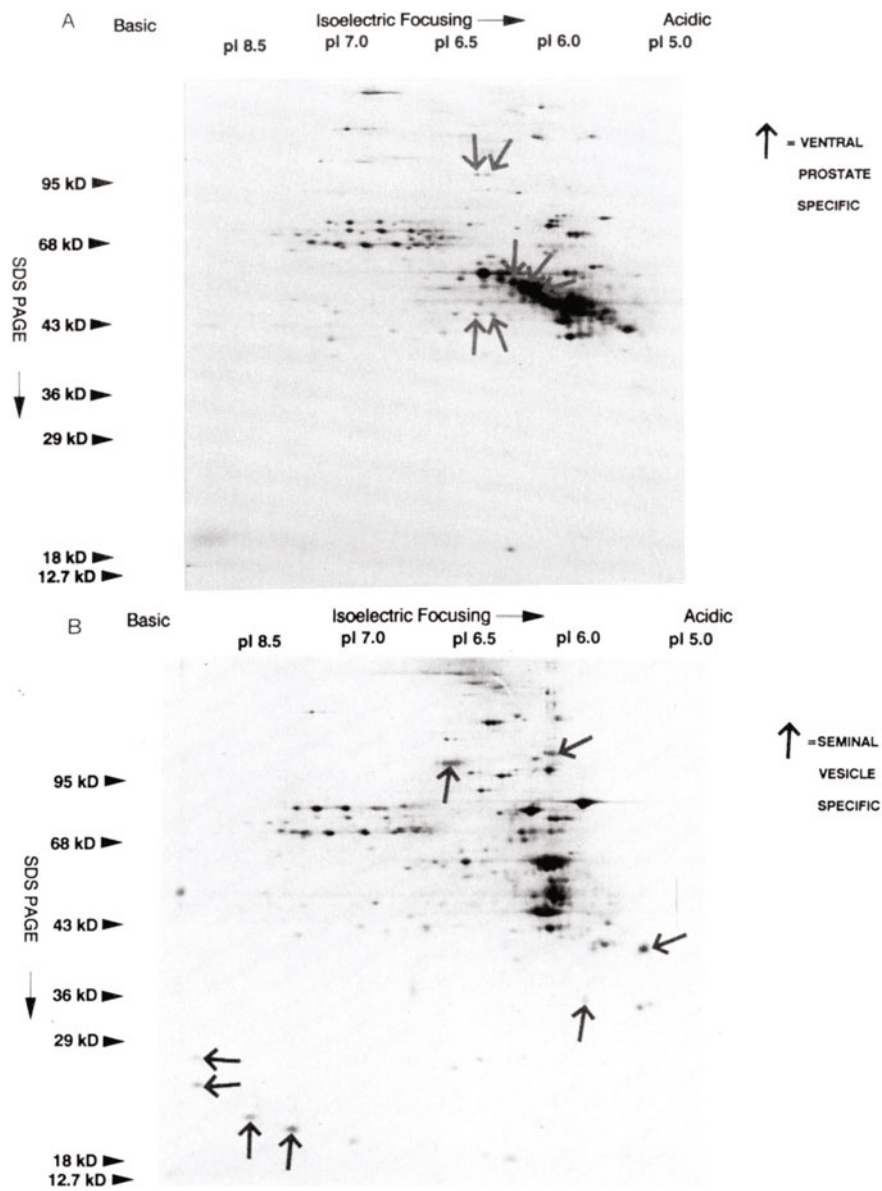
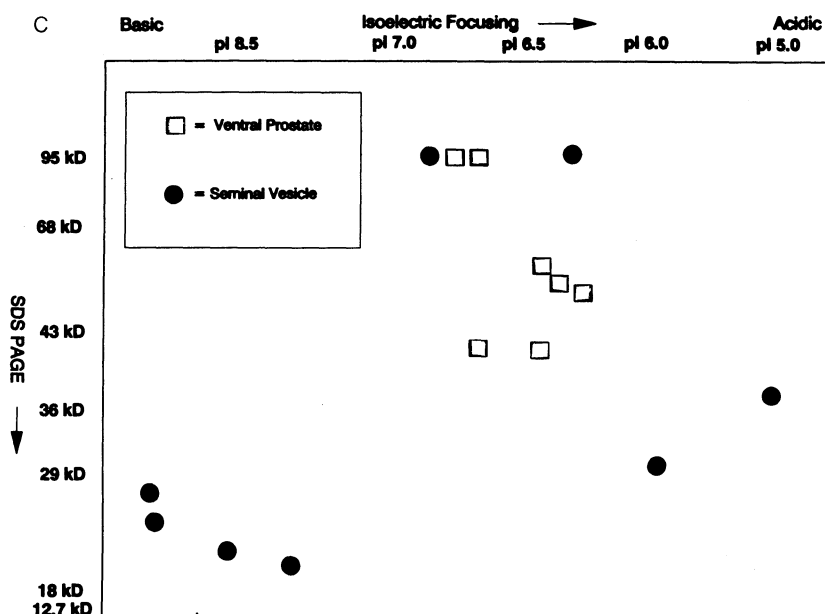


Figure 4 A & B: A. Intact - Ventral Prostate. B. Intact - Seminal Vesicle. High resolution two dimensional gel electrophoresis of rat nuclear matrix proteins was performed with modifications of O'Farrell, 1975. Second dimension electrophoresis was 10% SDS/PAGE under reducing conditions. 15ug. of protein was loaded on each gel. Gels were stained with silver stain (Accurate Chemical) after enhancement.



D There are quantitative differences in proteins between gels, however, proteins which consistently appear as unique are described below:

Seminal Vesicle		Ventral Prostate	
M.W.	pI	M.W.	pI
96 kD	6.27	95 kD	6.76
95 kD	6.84	95 kD	6.71
38 kD	5.10	52 kD	6.51
31 kD	6.01	50 kD	6.44
27 kD	* 9.75	48 kD	6.37
24 kD	* 9.50	41 kD	6.71
22 kD	8.50	41 kD	6.51
21 kD	8.00		

* = estimated pI

Figure 4 C & D: Tissue Specific Nuclear Matrix Proteins. Schematic of major tissue specific nuclear matrix proteins of ventral prostate and seminal vesicle.

appear when the rat is castrated (figures 5C & D). Quantitative alterations in the proteins were also noted between the intact and the castrate.

The phosphorylation patterns of the nuclear matrix proteins were also studied and these patterns were compared between ventral prostate and seminal vesicle. There are a large number of phosphorylated proteins in the nuclear matrix. The role of these phosphorylated proteins in nuclear matrix function and gene regulation is currently under investigation.

The results from these experiments can be summarized as follows. 1. There is a group of nuclear matrix proteins which are tissue specific for both the rat ventral prostate and seminal vesicle. 2. There is a series of nuclear matrix proteins that can vary depending on the hormonal state of the organ (castration, hormonal withdrawal). 3. Additionally, there also is a set of nuclear matrix proteins which are common between the seminal vesicle and ventral prostate (for example the lamins).

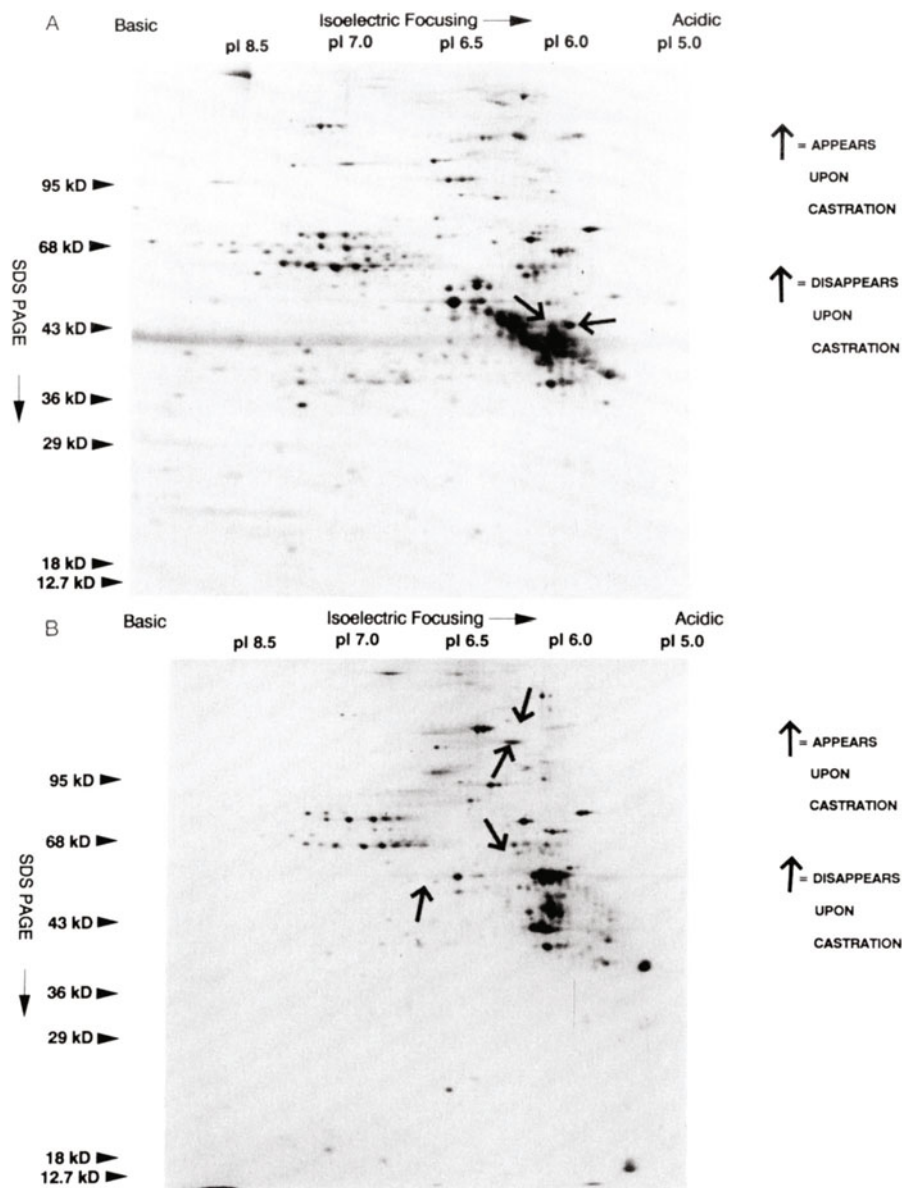
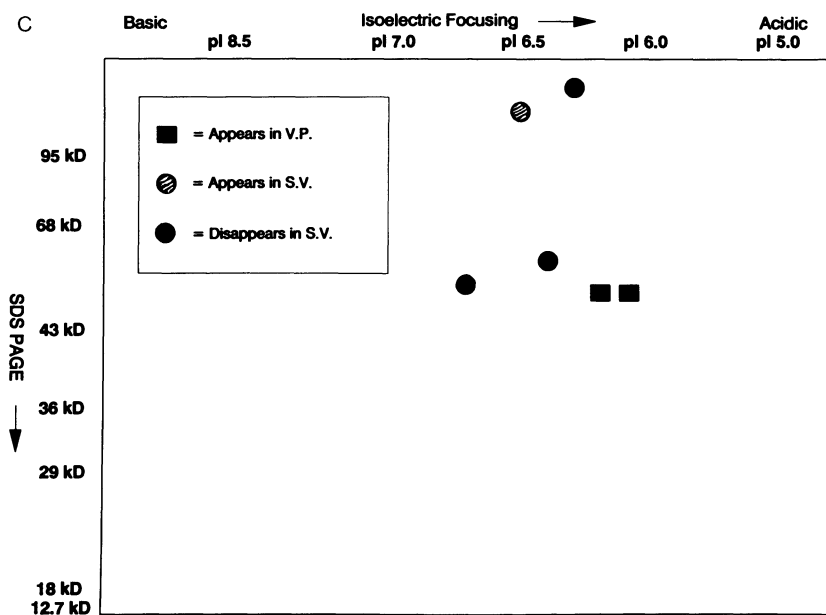


Figure 5 A & B: A. Castrate - Ventral Prostate. B. Castrate - Seminal Vesicle. High resolution two dimensional gel electrophoresis of rat nuclear matrix proteins was performed with modifications of O'Farrell, 1975. Second dimension electrophoresis was 10% SDS/PAGE under reducing conditions. 15ug. of protein was loaded on each gel. Gels were stained with silver stain (Accurate Chemical) after enhancement.



D There are quantitative differences in proteins between gels, however, proteins which consistently appear as unique are described below:

Appear Upon Castration

Ventral Prostate		Seminal Vesicle	
M.W.	pI	M.W.	pI
50 kD	6.27	105 kD	6.58
50 kD	6.14		

Disappear Upon Castration

Ventral Prostate		Seminal Vesicle	
M.W.	pI	M.W.	pI
NO MAJOR PROTEINS		110 kD	6.37
		59 kD	6.44
		52 kD	6.86

Figure 5 C & D: Casrate Induced Changes in Nuclear Matrix Proteins. Schematic of major nuclear matrix proteins which both appear and disappear in ventral prostate and seminal vesicle.

DISCUSSION

Our results demonstrate that the nuclear matrix is tissue specific. The nuclear matrix proteins which are specific for the seminal vesicle and ventral prostate may be involved in organizing the DNA into a specific three dimensional conformation which is unique for each tissue. This characteristic DNA structure can allow for the differential gene expression seen in the seminal vesicle and ventral prostate. These unique nuclear matrix proteins may be involved in binding DNA and/or interacting with the androgen receptor to permit transcription of the specific secretory products (figure 6).

The nuclear matrix undergoes characteristic alterations in protein composition upon castration. We have demonstrated that there are proteins which both appear and disappear in the ventral prostate and seminal vesicle nuclear matrices upon androgen withdrawal. These proteins may be indicative of alterations in nuclear matrix structure which occur in the seminal vesicle and ventral prostate from castrate animals. Specific alterations may be responsible for initiating the early events in programmed cell death which is known to occur in both the seminal vesicle and ventral prostate following androgen withdrawal. The exact function of those proteins which are tissue specific or appear/disappear upon castration is unknown. It is possible that these proteins are important determinants of gene expression.

FUTURE DIRECTION

The evidence presented in this chapter is the beginning of an extensive investigation into the function of the nuclear matrix in controlling gene expression. Future research will concentrate on learning

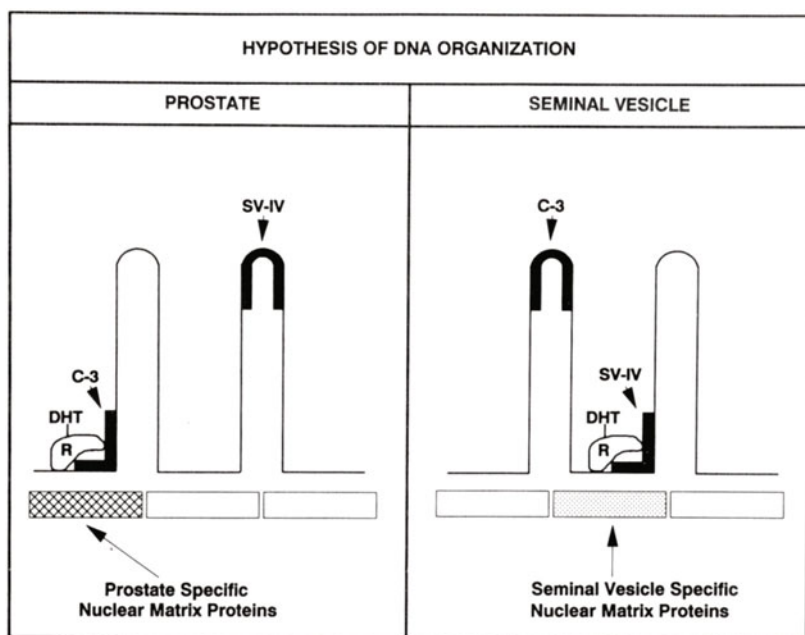


Figure 6: Model of tissue specific DNA organization. It has been reported that active genes are associated with the nuclear matrix (see text). This schematic is our hypothesis of tissue specific DNA loop organization in the ventral prostate and the seminal vesicle. Tissue specific nuclear matrix proteins are involved in the localization of specific DNA sequences and genes in proper configuration for DHT receptor to interact and allow activation of gene expression. By controlling the three dimensional conformation of the DNA, the tissue specific nuclear matrix proteins confer specificity to the protein products. The genes are on different DNA loops but not necessarily adjacent loops as is shown here for simplicity. R is the DHT receptor. C-3 and SV-IV signify gene sequences.

more about the properties of the nuclear matrix proteins which are specific for the ventral prostate and seminal vesicle and those involved in androgen action and withdrawal. Further analysis will include work on the interaction, if any, of these proteins with both the C3 gene and the SVS IV gene and any role that these proteins may serve as tissue specific transcription factors.

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DISCUSSION

Dr. French:

When a fairly well differentiated tumor cell was transformed with *ras*, what did it do to genetic instability? Does it have any relationship there?

Dr. Coffey:

We have not measured that, but that's what we would like to think happens. Now *ras* is very complicated and cells respond to that differently in different situations. Some cells actually differentiate when *ras* is activated. But I think many more elements will be attributed to this mechanical control unit. *ras* is just one.

Dr. John Isaacs:

Dr. Ichikawa in our laboratory is a cytogeneticist and we have been asking whether or not when you transfect in an activated Harvey *ras* if there is an induction of genetic instability as monitored by chromosomal changes. We've done this both in mammary as well as prostatic cancer cells. In the mammary system, elevated *ras* expression itself does not directly induce metastatic ability. It increases the likelihood of becoming metastatic, but there's no direct dose response correlation between the amount of P21 protein and metastatic ability. In that system, there is a good correlation, however, in transfects which express activated Harvey *ras* having chromosomal changes. I'm not saying that those chromosomal changes are specifically involved in gaining metastatic ability, but having *ras* activated does seem to make the cells more genetically unstable. Dr. Ichikawa is also looking at the same transfects where the motility is up with *ras* to see if that same situation occurs. I don't want to leave the impression that just having activated *ras* drives the cell's motility itself, and makes the cells metastatic.

Dr. Coffey:

When you put in *ras*, what you do is you activate G and you do get motility changes, etc. There are many steps in metastasis in which one is motility. It is required, but it is not in self sufficient to do that. Now many people have injected *ras* protein in the cell, and the protein itself will make the cells motile, and the half life of that motility changes as you mutate the protein. That's been published in Science. Dr. Bill Isaacs is first at our lab to look at some bladder cells that were transfected, and it's pretty safe to say that it does change their internal movements. Roughly how that occurs is going to take a lot more research to determine.

Concerning the cytoskeleton, at the Gordon Research Conference they were able to show in cells that the tension cable was actin, and the microtubules were the compression elements, which is the way our body works with our muscles being the tension cables.

MECHANISM OF GROWTH REGULATION OF ANDROGEN RESPONSIVE CELLS

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ABSTRACT

The DDT₁MF-2 cell line, which was derived from a leiomyosarcoma of the hamster ductus deferens, provides an excellent model for the study of androgen responsive cell growth. In this report, we describe serum-free conditions for the maintenance of DDT₁MF-2 cells and demonstrate their high sensitivity to steroids and competence factors. Subclones of DDT₁MF-2 cells exhibited a range of androgen stimulated growth from highly responsive to totally androgen insensitive. All subclones of DDT₁MF-2 cell lines which are non-androgen responsive and grow rapidly in the absence of any exogenous factors are growth inhibited by triamcinolone acetonide treatment. A plasmid containing an androgen response element linked to a chloramphenicol acetyl transferase reporter gene was activated by androgens when it was transfected into subclones that exhibited no androgen dependent growth response. This demonstrates that androgen receptors are biologically active in the non-responsive clones. The doubling time of a highly androgen responsive clone was reduced two-fold by treatment with androgens. The same increase in proliferation rate occurred when testosterone was substituted by acidic FGF, basic FGF, or by PDGF. The increased rate of cell proliferation was completely blocked by treatment with the glucocorticoid triamcinolone acetonide. The effects of androgens and competence factors was also mimicked by the addition of agents which increased intracellular cAMP, such as forskolin, cholera toxin, or beta agonists. Administration of either dibutyl or 8-bromo cAMP also stimulated cell proliferation. Overall, the DDT₁MF-2 cell growth experiments, which were carried out under serum-free defined conditions, illustrate the plasticity of tumor cells and explain how cells which are dependent upon androgens for growth can use alternative survival pathways following androgen ablation. In the absence of androgens, the cells proliferated in response to FGF's, PDGF, or any factor which increased intracellular cAMP levels. Since glucocorticoids inhibit growth of both androgen responsive and non-androgen responsive clones and block the alternative growth pathways, an understanding of this antagonism will provide important targets for the control of both hormone responsive and autonomous tumor growth.

INTRODUCTION

Tumors of the male and female reproductive tract frequently exhibit steroid-regulated growth. Indeed, stimulation of some breast cancer growth by estrogens has been well documented (Foulds, 1969; Lippman, 1980), as has androgen-regulated growth of tumors of the male reproductive tract, such as prostate cancer (Resnick and Grayhack, 1975). One approach to the therapy of these steroid-regulated tumors has been steroid ablation therapy, which is effected either by castration or administration of steroid antagonists or gonadotropin releasing hormone (GnRH) antagonists. In general, although steroid ablation therapy initially achieves diminution or inhibition of tumor growth, after a period of prolonged steroid ablation the tumors progress to a state of steroid-unresponsiveness. This phenomena has been observed both *in vivo* (Foulds, 1969) and *in vitro* (Eckert, et al., 1984; Darbre and King, 1987a,b, 1988).

Steroid hormones act in the nucleus to regulate gene expression (reviewed in Beato, 1989) and control cell proliferation of hormone responsive tumors. It is possible therefore that steroids regulate the expression of growth factors, competence factors or growth factor receptor genes in the tumor. Oncogenes which encode key regulatory molecules such as growth factors associated with cellular growth and differentiation (Bishop, 1983) might also be expressed in response to steroids. The oncogene products may then act in an autocrine or paracrine manner to stimulate tumor cell proliferation (Sporn and Todaro, 1980). With respect to the male reproductive tract, a variety of oncogenes and growth factors have been implicated in tumors of the male urogenital tract, including *c-myc*, *n-myc*, *H-ras*, *p-53*, neu, prostatic growth factor, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), prostatic growth factor, heparin binding growth factor 1 or acidic FGF (Buttyn, et al., 1987; Cooke, et al., 1988a, 1988b; Flemming et al., 1986; Fujita et al., 1985; Harris et al., 1989; Jacobs et al., 1979; Jinno et al., 1986; Katzenellenbogen et al., 1987; Matusik et al., 1987; Mydlo et al., 1988; Nag and Smith, 1989; Nishi et al., 1988; Peehl and Stamey, 1986; Peehl, et al., 1987; Phillips, et al., 1987; Rijnders et al., 1985; Siteras et al., 1988; Storey et al., 1984; Tackett et al., 1985; Viola et al., 1986).

In vitro studies on steroid regulated growth control in tumors of the male reproductive tract have been hindered due to the paucity of model cell lines available which exhibit androgen regulated growth. The studies have been further hindered by the lack of a demonstration that androgen-regulation of control of cell growth can occur in serum-free medium.

Our laboratory has used two model cell lines for experiments aimed at determining the mechanisms controlling growth and differentiated function of androgen-responsive cells. The DDT₁MF-2 cell line is stromal in nature and was derived from a leiomyosarcoma of the hamster ductus deferens (Norris and Kohler, 1977). These cells exhibit androgen responsive growth in the presence of serum, but growth is inhibited by glucocorticoids (Syms et al., 1983a, 1983b, 1984). In contrast, the R3327H-G8-A1 cell line derived from the hormone dependent rat prostate adenocarcinoma (Sestili et al., 1987) is epithelial like and is not as sensitive to androgen stimulation but in common with the DDT₁MF-2 cell line its growth is inhibited by glucocorticoids (Sestili et al., 1983; Smith et al., 1985).

Both cell lines contain androgen and glucocorticoid receptors, however, DDT₁MF-2 cells are far more sensitive to androgen stimulation than R3327H-G8-A1 cells. Several reports (Eckert et al., 1984; Voight et al., 1975) have presented data to suggest that the level of steroid hormone receptors is a major factor regulating cell sensitivity to steroids. In androgen responsive carcinomas of Copenhagen rats, the concentration of androgen receptor has been shown to be associated with the androgen sensitivity of the tumor (Voight et al., 1975). Our laboratory (Syms et al., 1985), using the DDT₁MF-2 cell line, presented evidence that androgens increase intracellular receptor concentration through stabilizing existing receptors and by increasing *de novo* receptor synthesis. Since androgen administration to this cell line normally results in a marked increase in cell proliferation, there appeared to be an association between an increase in receptor concentration and increase in the rate of cell proliferation. However, direct evidence for equating receptor concentrations with androgenic response is lacking.

Both the DDT₁MF-2 and R3327H-G8-A1 cell lines are growth arrested in Go by glucocorticoids, and glucocorticoid treatment blocks the stimulatory effect of androgens on growth and androgen mediated augmentation of androgen receptors (Smith et al., 1985; Syms et al., 1987). These observations are particularly relevant for the study of androgen regulated tumor growth. Metastatic tumors of human prostatic cancer frequently contain high concentrations of glucocorticoid receptors (Ekman et al., 1979). Glucocorticoid treatment of patients has been used clinically for treatment of prostatic cancer (Miller and Hinman, 1954). Although in general, this treatment is not ideal therapy due to systemic effects, the delineation of

growth inhibitory mechanisms induced by glucocorticoids will provide important tools for understanding how dedifferentiated cells are maintained in an arrested Go or A state; indeed, activation of this mechanism could provide the cells an opportunity to redifferentiate.

The mechanisms of growth factor signal transduction are very important in understanding autocrine growth. We recently established serum-free culture conditions to further define the mechanism of androgen-regulated growth (Smith et al., submitted). Under these conditions, in addition to testosterone stimulated proliferation, the DDT₁MF-2 cells exhibited growth stimulation by a number of growth factors and agents which increase the cAMP content of cells, (Smith et al., submitted). Furthermore, cells plated at low density exhibit increased acidic FGF mRNA expression as measured by *in situ* hybridization (Harris et al., 1989). Our studies demonstrated that one mechanism by which androgens stimulate growth is to increase growth factor (aFGF) gene expression (Harris et al., 1989). However, while cells can be stimulated to proliferate by other pathways, we demonstrate herein that in DDT₁MF-2 cells the stimuli are blocked by glucocorticoid administration.

RESULTS AND DISCUSSION

Establishment of Culture Conditions in Defined Medium and Selection of Clones Exhibiting Different Sensitivities to Testosterone

Experiments designed to maximize androgen responsivity showed that particular passage numbers of DDT₁MF-2 cells differed in both their growth rates and in their androgen sensitivity. The cells were therefore subcloned and the response of each clone to androgens was investigated. Certain clones grew extremely rapidly in the absence of androgens and surprisingly others were inhibited by androgens. To determine whether the lack of responsiveness of particular clones was explained by non-functional androgen receptors three clones were selected which differed in their response to testosterone. The doubling time of these clones in the presence or absence of testosterone is shown in Table 1. The clone with the highest growth rate was not affected by androgen treatment. All clones were maintained in the presence of an equivolume mixture of DME/F12 medium containing insulin (5 ug/ml) transferrin (5 pg/ml) and selenium (5 ng/ml). Testosterone was added at a concentration of 10 nM.

The doubling time of even the most androgen responsive clone in serum-free medium was dependent upon cell density. Although testosterone stimulated growth of cells plated at low density over eight-fold, cells plated at higher density exhibited only a two-fold increase in cell number. Over a nine-day culture period, as cells grown in the absence of testosterone increased in number the doubling time decreased and eventually reached a doubling time indistinguishable from testosterone treated cells. This increase was not due to time in culture but reflected the initial plating density. Cells were plated at different concentrations, the cells at low density maintained a slow doubling time, whereas those plated at high density progressively decreased their doubling time over the same period.

Androgen Receptor Analysis of the Slow, Medium and Fast Growing Clones Derived from the DDT₁MF-2 Cell Line. Total cellular androgen receptor content was determined using a whole cell binding assay as described previously (Syms et al., 1983a, 1983b, Smith et al.,1984). The DDT₁MF-2 cells were cultured in serum free DME as described above. The cells were washed and the medium replaced with DME contain-

Table 1: Doubling Time of Slow, Medium, and Fast Growing DDT₁MF-2 Cells in the Presence or Absence of Testosterone

Treatment	Slow	Doubling Time	
		Medium	Fast
Control	55	45	26
10 nM Testosterone	30	27	26

Kd nM (n, fmol/ng			
protein	0.22 (90)	0.11 (60)	0.10 (100)

ing 1-10 nM 3H-R1881 (86 Ci/mmol) in the presence or absence of 100 M excess of unlabeled R1881. The cells were incubated with the steroid at 37 ° C for one hour. After extensive washing with phosphate buffered saline (PBS, pH 7.4, 40) to remove free steroid, the cells were scraped from the dishes and washed by centrifugation. The cell pellet was extracted over-night with 1 ml ethanol and the radioactivity which was extracted was counted in the liquid scintillation counter after evaporating the ethanol. The radioactivity was converted from CPM to DPM by comparison with a set of internal quench standards. The cell pellets were used for protein determination. The number of binding sites and the equilibrium dissociation constants were derived using the method of Scatchard (1949).

Biological Activity of Androgen Receptors is Retained in Clones Which are not Androgen Responsive

To test the functionality of the androgen receptor in the three different DDT₁MF-2 clones a reporter plasmid was constructed. Figure 1 shows the presence of a putative glucocorticoid (pGRE) and an androgen response element (ARE) derived from the 5' flanking region of the seminal vesicle IV gene linked to the chloroamphenicol acetyl transferase (CAT) reporter gene (Harris et al., 1988). While the three different DDT₁MF-2 clones differed in their growth response to testosterone, the growth of all three was inhibited by triamcinolone acetone showing clearly that each clone possessed a biologically active glucocorticoid receptor. Because the reporter plasmid contains both a putative GRE in addition to an ARE the glucocorticoid receptor mediated response could act as an internal control for androgen receptor mediated activation of CAT.

Figure 2 shows the results of transfection of the reporter plasmid into each clone and assaying for a testosterone and glucocorticoid induced increases in CAT activity. The figure demonstrates that testosterone is effective in all three clones. These results support the notion that androgen receptors are biologically active in each clone and that the lack of an androgenic stimulatory effect on growth is not explained by the presence of disfunctional androgen receptors.

FGF and PDGF Can Mimic Testosterone in Stimulating Growth of DDT₁MF-2 Cells

The most highly androgen sensitive clone was selected for studying the effect of growth factors. Androgen effects on cell proliferation are illustrated in Figure 3. Testosterone is highly stimulatory with half maximal effects being observed at 0.1 nM (Figure 3). Similar increases in proliferation were induced by basic FGF (Figure 3) and by acidic FGF (data not shown). Maximal effects of basic FGF were observed at 0.1 ng/ml (Figure 3) and a dose response titration of the effects of PDGF on cell growth demonstrated that stimulation was observed at 0.01 ng/ml, with half-maximal effects at 0.05-0.1 ng/ml, and maximum stimulation at 1 and 10 ng/ml (data not shown). Combinations of testosterone and FGF or PDGF did not result in a greater increase in growth rates.

Figure 4 shows that both testosterone and PDGF stimulate growth of DDT₁MF-2 cells to an equivalent extent. When the two growth promotants are used in combination, no further increase in cell

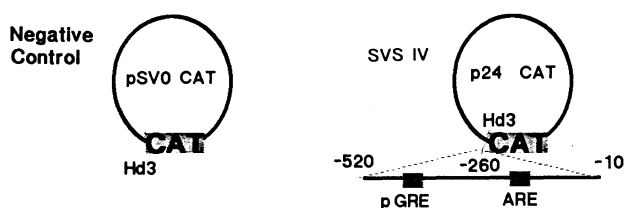


Figure 1: Construction of the Androgen Responsive Seminal Vesicle IV Reporter Plasmid. The details of the construction of the P₂₄ and pSV₀-CAT constructs are described in Harris et al., (1988). The chloramphenicol acetyl transferase (CAT) vector was a derivative of pSV/CAT (Gorman, 1985). The SV40 splice and polyadenylation signals, as well as the CAT gene were placed into the polylinker of pSP65 (Gorman et al., 1982; Parker et al., 1987). A -523 to -10 region of SVS IV gene was cloned into the Hind III site of pSV₀ CAT; the resulting plasmid is referred to as p₂₄CAT. The p₂₄CAT construct was demonstrated to contain an androgen response element and based upon sequence analysis a putative glucocorticoid response element (pGRE) was found 5' to the androgen responsive element (Harris et al., 1988). The control for the transfection of the p₂₄CAT plasmid is the pSV₀-CAT which lacks the hormone response elements.

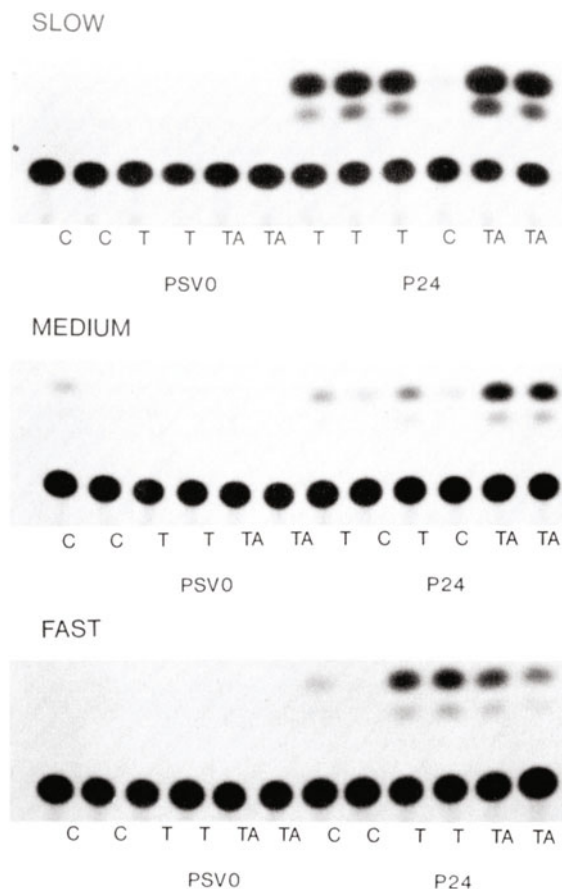


Figure 2: Androgen and Glucocorticoid Regulation of P24 CAT Expression in DDT₁MF-2 Cells. Slow, medium and fast growing subclones of DDT₁MF-Z cells were transfected with 25 ug pSV₀-CAT or 25 ug p₂₄-CAT as described below. The cells were incubated with control (vehicle, ethanol), 10 nM testosterone (T) or 10 nM T + 10 nM triamcinolone acetonide (TA) for 36 hours prior to assay for CAT. The pSV₀-CAT, p24-CAT, RSV-CAT plasmids were prepared as previously described (Harris et al., 1988). The plasmid constructs containing the reporter gene (CAT) were transfected in HB101 bacteria and isolated as described by (Davis et al., 1986). Slow, medium and fast growing DDT₁MF-2 cells were plated in DME supplemented with 5% charcoal stripped DME in 100 mm petri dishes. On day 1 of culture, the cells were transfected with 25 ug RSV-CAT (positive control) (not shown), 25 ug pSV₀-CAT (negative control), 25 ug p₂₄-CAT, or no DNA (negative control) (data not shown) using the CaCl₂ method (Gorman et al., 1982; Davis et al., 1986). After 24h of culture, each plate was incubated in the presence of 10 nM T; 10 nM TA; 10 nM T + TA; or control (vehicle, ethanol) for 36h. The cells were collected by scraping the dishes and after homogenization, the cytosol was used in a chloramphenicol acetyl transferase assay. Each condition was tested in triplicate.

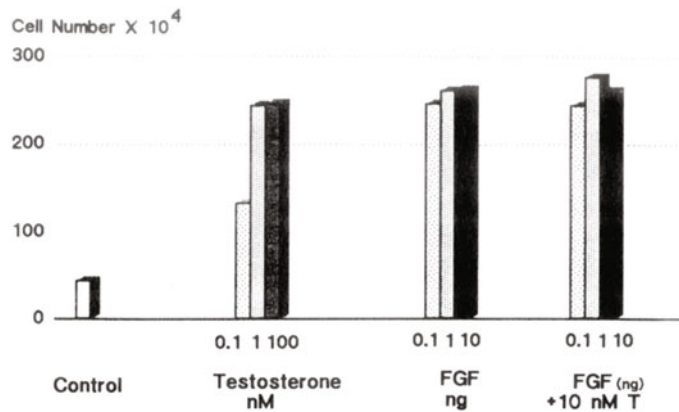


Figure 3: Androgen and FGF Stimulation of DDT₁MF-2 Cell Proliferation. DDT₁MF-2 cells were plated on collagen coated dishes at a concentration of 10,000 cells per dish in 0.1% FBS-medium. After allowing 24 hours of culture for cell attachment, the medium was replaced with serum-free medium, DME/F12 + ITS (insulin 5 ng/ml, transferrin 5 ng/ml, selenium 5 ng/ml). The cells were cultured for six additional days with medium changes every 48 hr. On day 7 of culture, the dishes were divided into 14 groups. To test the dose-dependence of androgen stimulated growth, groups of dishes were incubated in triplicate with 0, 0.1, 1.0 and 100 nM testosterone. Groups of dishes were also incubated with 0.1 ng/ml FGF, 1.0 ng/ml FGF, and 10 ng/ml FGF. To determine whether the androgen and growth factor stimulation of growth was additive, dishes were also incubated with 100 nM testosterone in the presence of FGF. Growth factors were added daily and the cells harvested and counted on day 9 of culture.

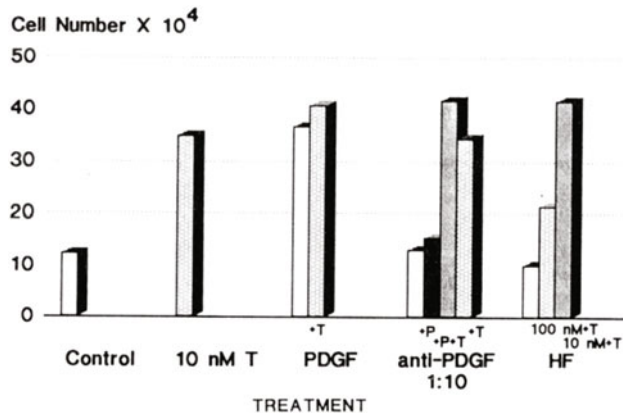


Figure 4: The Role of PDGF Anti-PDGF and Hydroxy Flutamide on Androgen Regulated Growth of DDT₁MF-2 Cells. Twenty-four hours after plating DDT₁MF-2 cells on collagen coated dishes (10,000 cell/dish) in 0.1% FBS/DME/F12/ITS, the medium was changed to serum free defined medium with medium changes every 48 hours. On day 11 of culture, the cells were incubated with control, 10 nM testosterone, 10 ng/ml PDGF in the presence or absence of 10 nM testosterone, or in combination with anti-PDGF antibody at a final dilution of 1:10, and testosterone in the absence or presence of hydroxyflutamide (HF). The growth factors were added to the cultures daily and 48h after treatment the cells were harvested from the dishes with 0.2% collagenase and trypsin EDTA and counted in the Coulter Counter.

number is induced suggesting that either each growth factor acts through a common pathway or that the cells are already maximally stimulated. The effects of anti-PDGF are also shown in Figure 4. Anti-PDGF alone has no effect on cell proliferation, while it clearly inhibits the stimulatory effects of exogenous PDGF. Importantly, testosterone stimulated cell growth cannot be inhibited by PDGF antibodies suggesting that testosterone treatment does not promote production and secretion of PDGF. Figure 4 also shows that when hydroxyflutamide is present in a ten-fold but not equimolar concentration to testosterone, testosterone mediated growth is antagonized suggesting that the testosterone effect is receptor mediated. This conclusion is also supported by the fact that the concentration required for half-maximal stimulation of growth approximates the equilibrium dissociation constant of binding (K_d) to the androgen receptor.

Glucocorticoid Inhibition of Growth

Figure 5 shows the dramatic inhibitory effect of triamcinolone acetonide on cell growth and illustrates under serum-free conditions that the presence of FGF, PDGF, and EGF is unable to overcome this inhibition. Figure 6 illustrates that $TGF\beta$ is unable to antagonize either testosterone or growth factor effects on cell proliferation. Thus the effect of triamcinolone acetonide is not caused by production and secretion of $TGF\beta$.

Recent studies showed that testosterone stimulated increases in acidic FGF mRNA when DDT₁MF-2 cells were maintained at low density (Harris et al., 1989). The time required for maximal induction suggested that the FGF gene was transcriptionally regulated in a cell cycle dependent manner. While glucocorticoid treatment had no stimulatory effect on FGF mRNA levels, in the presence of triamcinolone acetonide the testosterone induced effect was maintained between 6-72 hours. Cycloheximide also maintained the androgen mediated increase. Both observations suggested that testosterone stimulates acidic FGF expression during the G_0/G_1 phase of the cell cycle. The results also indicate that glucocorticoids do not exhibit growth by preventing expression of a FGF mRNA. Thus, glucocorticoid may act by inhibiting production of a FGF protein, by inhibiting a FGF receptor synthesis, or by interfering in the signal transduction pathway essential for cell proliferation.

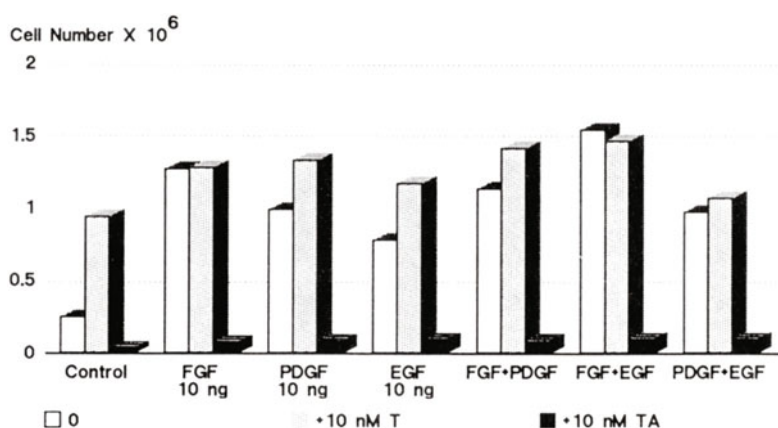


Figure 5: Growth Factor Stimulation of DDT₁MF-2 Cell Growth in Vitro is Antagonized by Triamcinolone Acetonide. DDT₁MF-2 cells were plated onto collagen-coated dishes (20,000 cells per dish) in medium containing 0.1% FBS. After 24 hours, the medium was changed to serum free defined medium with medium changes every 48 hours. On day 7 of culture, the dishes were divided into 7 groups of triplicate samples and the cells were incubated with a) control (medium only), b) 10 nM testosterone, c) 10 nM triamcinolone acetonide in the presence or absence of 10 ng/ml FGF, 10 ng/ml PDGF, 10 ng/ml EGF, 10 ng/ml FGF + 10 ng/ml PDGF, 10 ng/ml PDGF + 10 ng/ml EGF. On day 9 of culture cells were collected by treatment with 0.1% collagenase and counted in the Coulter Counter.

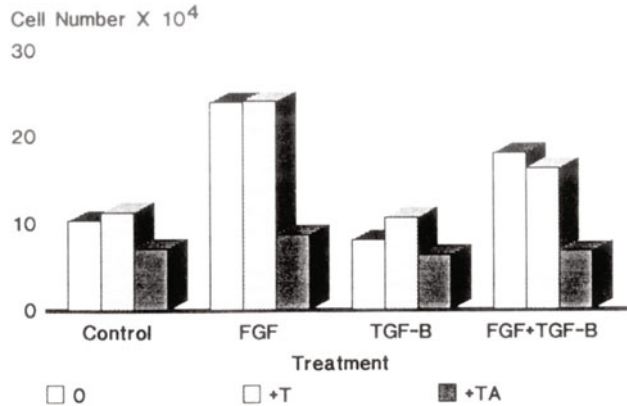


Figure 6: Effects of $TGF\beta$ on DDT_1MF-2 Cell Growth *in Vitro*. Cells were plated and were treated with hormones for 48h and the cells were counted on day 9 of culture exactly as described for Figure 5. Transforming growth factor-beta ($TGF-\beta$). Testosterone 10 nM (T), triamcinolone acetone, 10 nM (TA). Basic fibroblast growth factor 10 ng/ml (FGF).

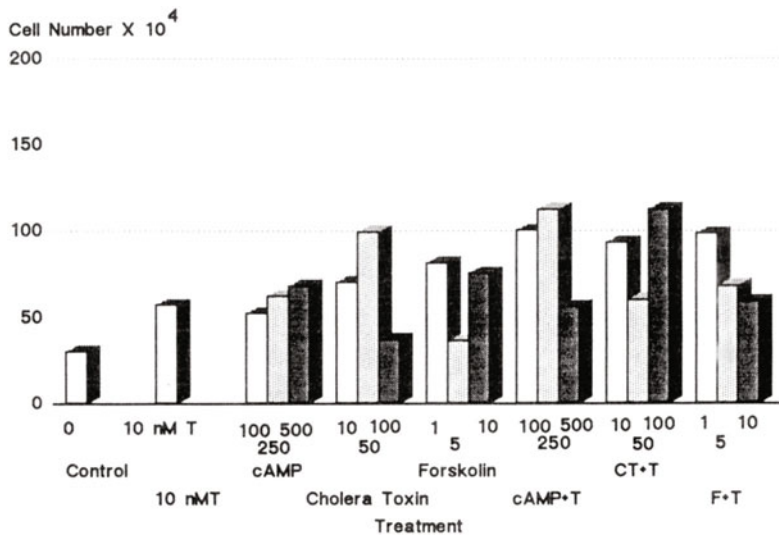


Figure 7: Effect of Agents Which Influence cAMP Levels on Growth of DDT_1MF-2 Cells. To test the effect of compounds which alter intracellular cAMP levels on cell proliferation, DDT_1MF-2 cells were plated onto collagen coated dishes as described in figure 5 at a concentration of 10,000 cells per dish. After a medium change to place the cells in defined medium (DME/F12/ITS), the cells were incubated for an additional 6 days. Groups of dishes were treated with control (DME/F12/ITS), 10 nM testosterone, 8-bromo-cAMP 100, 250, 500 pM/ml (cAMP), cholera toxin, 10, 50, 100 ng/ml (CT), and forskolin (1, 5, 10 uM) alone or in the presence of 10 nM testosterone (T). Growth factors were added daily and the cells were counted in the Counter Counter on day 9.

cAMP Stimulates Growth of DDT₁MF-2 Cells

The effects of cAMP on growth of DDT₁MF-2 cells in serum free medium was investigated to determine whether cAMP could replace testosterone, FGF and PDGF for maximal stimulation of growth. Figure 7 compares the stimulatory effects of testosterone on growth with that induced by 8-bromo-cAMP, cholera toxin and forskolin. 8-bromo-cAMP at 250 μ M, cholera toxin at 10 ng/ml and forskolin at 1 μ M are as efficacious as 10 nM testosterone in stimulating cell growth. Combinations of testosterone and 8-bromo-cAMP cholera toxin or forskolin showed some further stimulation of cell proliferation. In a dose dependent manner (50-500 μ M) dibutyl cAMP stimulated growth to the same level as that observed with PDGF and FGF (data not shown). Cholera toxin at both 10 and 100 ng/ml also stimulated growth. Interestingly, pertussis toxin at 10 and 100 ng/ml had no effect on DDT₁MF-2 cell growth but at both concentrations it inhibited testosterone and FGF stimulated growth which suggested that the growth stimulation signal transduction pathway is mediated via a G protein.

CONCLUSIONS

The establishment of serum-free conditions for an androgen responsive tumor cell-line shows that growth rate in the absence or presence of androgens is affected by cell density. At low density cells are more responsive to androgens and under these conditions testosterone positively regulated the expression of mRNA encoding acidic FGF. The regulation appears to be cell cycle dependent and appears to be maximal in the Go/G₁ stage (Harris et al., 1989). Comparisons of the biological activity of androgen receptors in sub-clones which are less responsive to androgens and which proliferate at faster rates than highly androgen sensitive clones show that the lack of androgen responsiveness is not because of dysfunctional androgen receptors. In highly androgen responsive clones addition of PDGF, acidic FGF, basic FGF, or cAMP derivatives, or agents which increase intracellular cAMP, are all able to replace the requirement for androgen in maximally stimulating cell proliferation. Glucocorticoid administration antagonizes the effects of testosterone and growth factors but does not decrease the steady state level of mRNA encoding acidic FGF; moreover, glucocorticoids also inhibit growth of cells which grow maximally in the absence of any exogenous factors. It is concluded that tumor cells are able to change their growth requirement following androgen ablation; however, these alternative pathways, at least in DDT₁MF-2 cells, are blocked by glucocorticoid treatment. It is possible that glucocorticoid treatment inhibits production of growth factor receptors and/or inhibits activation of the signal transduction pathway required for cell proliferation. Clearly, the mechanism of growth inhibition warrants further study.

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DISCUSSION

Dr. Rowley:

As you know, glucocorticoids have been shown to inhibit a number of cell lines. Do you have any data that suggest that glucocorticoid is regulating the production of some growth inhibitory factor, such as TGF beta or something like that in these cells?

Dr. Smith:

TGF beta has no inhibitory effects on the growth of these cells. We tried that. All I can really tell you is that since it's a completely reversible phenomenon, cell death is not involved. What has been done is that there is a major protein that is produced by these cells in response to glucocorticoid, a P29 protein, which has been cloned by Dr. Jim Norris. It's one of the glutathione S transferase family. Whether or not this protein has an inhibitory effect on growth, we don't know yet. We tried some time ago to identify an inhibitory factor being secreted into the medium. We had some success with that, but as you're aware it's difficult to actually prove you've got an inhibitory factor because of all the different components that can inhibit cell growth. Dr. Steve Harris might have some comments because he's seen a particular nuclear protein induced in response to glucocorticoids by these cells. Again, its appearance is correlated with inhibition of growth.

Dr. Lee:

Here you have this cell line with an end point of cell proliferation as your gage. You have shown nicely that testosterone and the various growth factors are able to stimulate its proliferation. Do you have by any chance another end point such as a specific protein production, or anything that can be regulated by testosterone, in addition to cell proliferation?

Dr. Smith:

Under certain conditions in certain stages of the cell cycle, testosterone is able to induce acidic FGF mRNA. We have some evidence that it could also control PDGF mRNA. Now, all the early work was done on PDGF. Dr. Harris did the studies with acidic FGF. Both studies were on cells grown at relatively high density. High density was used so that enough RNA could be isolated for Northern blots. One of the problems we found was that sometimes we would see regulation, other times we wouldn't. That's what lead Dr. Harris to repeat the studies by using *in situ* hybridization, allowing the studies to be done at very low cell density. Then the problem of reproducibility was resolved. It became very clear that it's very difficult to see regulation at high density, but easy at low density. At low density, there is no question that testosterone can induce FGF and PDGF mRNA.

THE IMPACT OF ANDROGEN, EXTRACELLULAR MATRIX, AND STROMA UPON PROLIFERATION AND DIFFERENTIATION OF BENIGN AND MALIGNANT PROSTATE EPITHELIAL CELLS

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ABSTRACT

Mesenchymal-epithelial interactions are thought to play a strategic role in androgen-induced regulation of prostate epithelial cell growth and differentiation. A more thorough understanding of this process has been facilitated by recent advances permitting the separation and cultivation of stromal/epithelial elements from benign and malignant human prostate glands. Our laboratory has devised an *in vitro* system to selectively dissect those factors influencing the divergent phenotypes of proliferation and differentiation. The epithelial "targets" utilized in this study included: (1) benign prostatic epithelial cells (BPE) isolated from open prostatectomy specimens; and (2) the hormone-sensitive prostate cancer cell line, LNCaP. Growth was measured by sequential cell counts and differentiation was correlated with the ability of these epithelial cell populations to secrete prostate specific acid phosphatase (PSAP) and prostate specific antigen (PSA) into their respective conditioned medium. Major variables in our culture system included the presence/absence of dihydrotestosterone (DHT), basement membrane biomatrix (Matrigel), and/or stromal extracellular matrix (ECM). Observations relevant to BPE include: (1) the formation of distinct cell clusters when grown on Matrigel vs typical monolayer morphology when cultivated on plastic; (2) a modest increase in PSAP and PSA secretion in the presence of DHT and stromal conditioned media (SCM); and (3) maximal production of PSAP and PSA when these cells are grown on Matrigel in the presence of DHT and SCM. With respect to LNCaP cells, we observed: (1) growth on plastic in the presence of DHT was associated with marked proliferation and modest production of PSAP and PSA; and (2) when cultured on ECM derived from human prostate stromal cells, LNCaP demonstrated a reduced growth response to DHT but maximized their secretion of the two differentiation markers. Thus, this *in vitro* system may facilitate more detailed biochemical and morphological studies designed to probe the impact of stromal/epithelial interaction on the phenotypes of proliferation and differentiation involving both benign and malignant prostate systems.

INTRODUCTION

Epidemiologic observations document that benign prostatic hyperplasia (BPH) and prostate cancer are major factors impacting on the health of the male population in the United States. BPH is the most common neoplastic condition afflicting men (1). Although evidence of BPH is noted in less than 10% of men under 40 years of age, its incidence increases in each succeeding decade so that by the ninth decade, 90% of men are affected (2). Associated bladder neck obstruction and its sequela are responsible for over 290,000 operations per year with a projected annual cost in excess of one billion dollars (1). Similarly, adenocarcinoma of the prostate is the most common cancer among American men and will account for 21% (103,000 cases) of newly diagnosed cancer in 1989 (3). Similar estimates project that prostate cancer will be responsible for 11% (28,500 cases) of male cancer death.

Despite the obvious importance of these disease processes, our current perceptions of the cellular aberrations responsible for the development of BPH and prostate cancer remain surprisingly primitive. Part of this knowledge deficit is attributable to the intrinsic zonal and cellular heterogeneity of the human prostate gland. McNeal has proposed that the adult prostate gland contains discrete anatomic subdivisions, which include: peripheral zone, transition zone, periurethral gland area, and central zone (4). He further postulates that the first three areas share a common embryonic origin from the urogenital sinus, while the central zone may originate from the Wolffian duct (5,6). Adding to this complexity, each of the various regions is thought to have distinct acinar, stromal, and cellular configurations (6). This morphological heterogeneity applies equally well to the epithelial and stromal compartments within each zone. The prostatic epithelium is composed of secretory epithelial cells, basal cells, stem cells and ductal epithelial elements. Similarly, the stromal compartment contains a complex admixture of fibroblasts and smooth muscle cells (6,7).

A number of studies have suggested that the cellular interactions between these stromal and epithelial elements play a dynamic role in modulating the normal and neoplastic growth of the prostate (8,9). Until recently, it was frustratingly difficult to probe the biological nuances of glandular/stromal interaction as it exists within the different zones of the human prostate gland. Many of the methodologic obstacles have been overcome as a result of recent innovations, including: (1) the development of techniques which permit the separation of epithelial/stromal elements with a high degree of purity using Percoll gradient centrifugation (10,11,12); (2) the refinement of a serum-free medium (WJJC-404) with a low ionic calcium concentration (100 μ M) plus essential growth factors which permits the propagation and expansion of fresh prostate epithelial cells in tissue culture (13,14); (3) a delineation of those factors required for the successful and repetitive passage of cultured prostate epithelial cells, particularly the use of type 1 collagenase (11,12,14); and (4) a growing awareness of the important role played by basement membrane extracellular matrix (ECM) with respect to the maintenance of epithelial cell polarity, differentiation, and phenotypic expression (15,16,17).

These stromal-epithelial interactions are probably multifaceted events which involve cell-cell contact, the impact of ECM elements, and the production of soluble mediators (18,19,20). With respect to the latter, our laboratory has recently documented that stromal cells secrete factors which are mitogenic for prostate epithelial cells. These mitogens have been partially characterized, and include both heparin-binding growth factors and extracellular matrix components (21). Similar observations were recently reported by Kabalin et al (22).

Despite these methodologic and scientific advances, we have been puzzled by two noteworthy phenotypic aberrations that are associated with the cultivation of fresh prostate epithelial cells *in vitro*. First, both rat and human prostate epithelial cells grown on tissue culture plastic can proliferate in serum-free medium without the presence of dihydrotestosterone or testosterone (11-14). This contrasts sharply with *in vivo* events, in which androgen ablation induces a marked regression of the prostate (7). Second, prostate epithelial cells *in vivo* readily express the differentiation-associated markers, PSAP and PSA (7). Paradoxically, expression of these markers is rapidly lost following adaptation to *in vitro* growth on tissue culture plastic despite the use of a presumably "optimal" culture medium (11,12,21).

It has been suggested that the optimal growth of fresh mammalian epithelial cells can be achieved only by the cultivation of such systems on tissue-specific or conducive ECM in the presence of serum-free media supplemented with requisite growth factors, hormones, and other soluble mediators present within conditioned medium (23,24). In this study, we evaluate the impact of varying culture conditions on the proliferation/differentiation of freshly isolated BPE and the androgen-sensitive prostate cancer cell line, LNCaP.

MATERIALS AND METHODS

Isolation of Epithelial and Stromal Cells from Human Prostate Tissue

Open prostatectomy specimens served as the source of benign epithelial and stromal cells. With the cooperation and compliance of the Department of Pathology and Human Subjects Review Committee of Northwestern University Medical School, 5-10 gm of tissue from these specimens was harvested under aseptic conditions and provided to our laboratory for investigative purposes. Representative sections were submitted for histopathological analysis in order to validate the diagnosis of BPH. A component of this study involved the isolation of stromal cells from prostate cancer. In the latter instance, approximately 3 gm of tissue was obtained from a radical retropubic prostatectomy specimen ultimately assigned a pathological

stage C with a Gleason's score of 5. Again, representative portions of this specimen were submitted in order to assure the diagnosis of prostate cancer.

In all cases, the remaining tissues underwent mechanical and enzymatic dissociation as described previously (11,12). In brief, the tissue was minced into 1 cubic mm fragments and transferred to sterile flasks containing 25-50 ml of dissociation solution consisting of 200 U/ml of type 1 collagenase (Sigma Chemical, St. Louis) and 100 ug/ml of DNase type 1 (Sigma Chemical) dissolved in RPMI-1640 medium containing 10% fetal bovine serum (FBS). The tissue was dissociated for 16-18 hr at 37 °C using a magnetic stirring bar to provide gentle agitation. The resulting cell suspension was washed and suspended in Hank's balanced salt solution (HBSS). This disaggregation procedure resulted in a complete dissociation of all prostatic tissue and yielded cell suspensions with greater than 95% viability as assessed by trypan blue exclusion.

The resulting cell suspension was layered over a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient for separation as previously described (11,12). To summarize, Percoll is sold as a 23% solution. The gradients are prepared by diluting 9 ml of stock solution with 1 ml of 10X phosphate buffered saline (PBS) in order to obtain a 20% Percoll solution. Using 1X PBS, dilutions were made to establish 15%, 10%, 7.5%, and 5% Percoll solutions. These Percoll preparations were carefully layered (2 ml/layer) from 20% to 5% in conical 15 ml centrifuge tubes in order to establish the discontinuous gradient. In general, 5×10^6 - 1×10^7 cells in suspension were layered over each gradient tube and centrifuged for 1 hr at 500.9 (25 °C) using a Sorvall RT-6000 swinging bucket centrifuge. This procedure results in the formation of five bands. The upper most band (media/5%) consists primarily of stromal cells, the next two bands (5%/7.5% and 7.5%/10%) are composed of epithelial cells and the fourth and fifth bands contain erythrocytes and cellular debris (11,12). Stromal cells from established cultures (see below) were utilized as the source of stromal matrix or feeder layers. For the purposes of these experiments, epithelial cells were harvested directly from the second and third Percoll layers and directly utilized as described below.

LNCaP

This cell line was established following aspiration of a supraclavicular lymph node metastasis which developed in a patient with Stage D3 prostate cancer (25). LNCaP was obtained from the American Type Culture Collection (Rockville, MD). Numerous studies have documented that this tumor system is androgenresponsive with demonstrable androgen receptors and exhibiting an increased rate of growth and secretion of PSAP in the presence of exogenous androgen (26-28). Cells were cultured in RPMI-1640 with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 ug/ml), at 37 °C in a humidified environment containing 5% CO₂. For the studies described below, these cells were harvested from subconfluent monolayers by treatment with trypsin/EDTA (0.25%; 0.1%, Hazleton, Lenexa, KS), centrifuged, and resuspended in RPMI-1640 + 10% FBS. In all cases, viability was assessed by exclusion of 0.4% Trypan blue (GIBCO) and was greater than 95% for all cells tested.

Growth of Prostate Epithelial and Stromal Cells in Selective Media

Following their elution from the discontinuous Percoll gradient, BPH epithelial cells were plated (1×10^5 cells/well) in 24-well dishes (Corning Glassworks, Corning, NY) containing stromal matrix, Matrigel or receiving no treatment. All epithelial cells were plated using WAJC-404 medium (Irvine Scientific, Santa Anna, CA) containing 10% heat-inactivated and dialyzed FBS and allowed to adhere to their respective substrate for 16-24 hr. Cells were then transferred to serum-free WAJC-404 medium supplemented with: ITS (insulin 5 ug/ml, transferrin 5 ug/ml, and selenous acid 5 ng/ml, Collaborative Research, Bedford, MA); epidermal growth factor (10 ng/ml, Sigma); bovine pituitary extract (30 ug/ml, Collaborative Research); cholera toxin (10 mg/ml, Sigma); prolactin (3 mg/ml, Sigma); polyvinyl pyrrolidone (2 mg/ml, Behring Diagnostics, La Jolla, CA); and penicillin (100 U/ml/streptomycin (100 ug/ml). In addition, varying concentrations of DHT was added to this media formulation after solubilizing the DHT in minimal amount of ethanol (0.01%) and achieving the desired dilution with appropriate amounts of complete WAJC-404 medium.

Stromal cells derived from BPH and prostate cancer specimens were maintained in phenol red-free RPMI-1640 with 10% heat-inactivated and dialyzed FBS. Phenol red was routinely excluded from our tissue culture media since it has been reported to have estrogenic properties (29). The cells were maintained in 75 sq cm tissue culture flasks (Corning Glassworks) and incubated at 37 °C in a humidified environment containing 5% CO₂. When ready for replating (see below) subconfluent monolayers were detached with 0.25% trypsin: 0.1% EDTA (Hazleton, Lenexa, KS). In all cases viability was assessed by trypan blue exclusion and was noted to be greater than 95%.

Preparation of Matrigel Substrates

Matrigel is a basement membrane extracellular matrix derived from a poorly differentiated murine chondrosarcoma, designated the Englebreth-Holme-Swarm tumor (30). This murine tumor matrix contains laminin, type IV collagen, heparan sulfate proteoglycan, and small amounts of entactin and nidogen. Laminin represents about 90% of its chemical constitution (30,31,32).

Matrigel (Collaborative Research, Bedford, MA) is supplied as a frozen 10 ml solution. The vials were thawed at 4 °C, wiped with 70% ethanol, and allowed to air dry under a laminar flow hood. The thawed extract was removed with a sterile pipette and layered (0.2 ml/well) in 24 well plates and allowed to polymerize in a tissue culture incubator at 37 °C in 5% CO₂ in a humidified environment for approximately 60 min. At this point, the systems were ready for the addition of BPE or LNCaP cells.

Preparation of Prostate Stromal Matrix

Stromal matrix was prepared from stromal cells isolated from BPH and prostate cancer specimens. The respective stromal populations (1-5 X 10⁵ cells/well) were plated in 24 well plates (Corning) and permitted to adhere to plastic substrate and achieve confluency. The resulting monolayers were washed (3X) with HBSS and incubated (25 °C) in 0.5% of Triton X-100 (BDH Chemical Company) for 30 min. with gentle agitation. The Triton X-100 was aspirated and 0.025 N ammonium hydroxide was added to each well for 1-3 min. Each well was then washed (4X) with HBSS and stored in HBSS at 4 °C until utilized.

For the preparation of "feeder" layers, stromal cells isolated from BPH and prostate cancer specimens were plated (1-5 X 10⁵ cells/well) into 24 well plates in RPMI-1640 + 10% heat-inactivated and dialyzed FBS. These systems were incubated at 37°C in a humidified environment containing 5% CO₂. When the stromal monolayers achieved sub-confluency, they were considered suitable for the introduction of LNCaP cells (1 X 10⁵ cells/well).

Preparation of Stromal Conditioned Media (SCM)

Isolated BPH stromal cells were grown to sub-confluence in 150 sq. cm. tissue culture flasks (Corning) using phenol red-free RPMI-1640 medium supplemented with 10% FBS (Hazleton) and penicillin (100 U/ml)/streptomycin (100 ug/ml). The cultures were washed (3 X) with PBS and RPMI-1640 medium containing ITS PLUS (insulin 6 ug/ml, transferrin 6 ug/ml, selenous acid 6 ng/ml, albumin 1 mg/ml, linoleic acid 5 ug/ml, Collaborative Research) was added. Stromal cells were cultured (37 °C, 5% CO₂) for 48 hr. The SCM was harvested and cell debris removed by centrifugation (400 g for 1U min.). SCM was dialyzed against distilled water for 4 days (4 °C) using a 12-14 KD cutoff dialysis membrane (Spectrum Industries, Los Angeles, CA). The resulting SCM was lyophilized and stored at -70 °C until further utilized. RPMI-1640 medium containing ITS PLUS was also dialyzed and lyophilized to provide control data.

Transmission Electron Microscopy

BPH epithelial cells grown on basement membrane MATRIGEL in culture wells were washed with PBS and fixed with Millonig's fixative for 30 min. at 4 °C. The cells were washed with HBSS and dehydrated by successive incubations with 25-100% ethanol. The cells were embedded in epoxy resin (EPON 8-12 ARALDITE). Portions of hardened EPON were cut in thin sections (DuPont Diamond Knife) perpendicular to the dish surface using an LKB Ultratome type IV. The cut sections were collected on copper grids and stained with uranyl acetate and lead citrate. Scanning was performed with the Jeol 100 S transmission electron microscope.

Determination of PSAP and PSA in Conditioned Medium

The Tandem-R PSAP and PSA immunoradiometric assay kit (Hybritech) was used for the quantitative measurement of PSAP and PSA in conditioned medium. In brief, Tandem-R PSAP (or PSA) is a solid phase, two-site immunoradiometric assay. Samples containing PSAP (or PSA) are reacted simultaneously with a plastic bead (solid phase) coated with a monoclonal antibody directed toward a unique antigenic site on the PSAP (or PSA) molecule, and with a radiolabeled monoclonal antibody directed against a distinctly different antigenic site. Following formation of the solid phase/PSAP (or PSA)/labeled antibody sandwich, the bead is washed to remove unbound labeled antibody. The radioactivity bound to the solid phase is measured in a Gamma counter. The radioactivity is directly proportional to the concentration of on the concurrent testing of the PSAP (or PSA) calibers.

Assessment of Proliferation and Differentiation in Prostate Epithelial Cells

BPE were harvested from discontinuous Percoll gradients and plated (1×10^5 /well) in 24 well dishes coated with Matrigel or receiving no treatment. The cells were plated using WAJC-404 medium containing 10% heat-inactivated and dialyzed FBS and allowed to adhere to their respective substrates for 24 hr. At that point, the medium was changed and four experimental groups constructed by varying the medium formulation. These included: (1) WAJC-404 + standard growth factors, now designated complete WAJC-404; (2) complete WAJC-404 + 10-8 M DHT; (3) complete WAJC-404 + 20% SCM; and (4) complete WAJC-404 + 10-8 M DHT + 20% SCM. Thus, eight experimental subsets were constructed through the use of these four media variations and two growth substrates (Matrigel/plastic). The BPH epithelial cells were cultured under these conditions for 14, 16, and 18 days. Conditioned medium was harvested from the respective wells and the PSAP/PSA content was determined as described above. All groups were plated in quadruplicate and repeated twice to assure reproducibility. Additional wells were fixed with 100% methanol and stained with Giemsa for phase contrast microscopic analysis, morphological assessment, and photomicroscopy.

LNCaP cells were harvested from established cultures and plated in 24 well dishes (1×10^5 /well) containing either stromal matrix or plastic substrate using RPMI-1640 medium containing 10% FBS for 24 hr to promote adherence. At this point, the cells were transferred to RPMI-1640 medium containing 10% charcoal-stripped fetal bovine serum and varying concentrations of DHT (0-10 nM). Charcoal stripping was performed in order to minimize the impact of exogenous steroid hormones and other factors present in standard FBS. The cells were cultured and counted on days 2, 4, and 5 to evaluate proliferation by detaching the cellular monolayer with 0.25% trypsin; 0.1% EDTA and performing manual cell counts with a hemacytometer. In addition, on day 6 conditioned medium was collected and evaluated for PSAP and PSA as described previously. Finally, additional wells were fixed with 100% methanol and stained with Giemsa for similar morphological analysis and photodocumentation.

In another series of experiments, stromal cells from both BPH and prostate cancer were used to prepare "feeder" layers and matrix using the 24 well system as described above. Experimental substrates consisted of: (1) tissue culture plastic; (2) BPH stromal matrix; (3) BPH stromal "feeder" layers; (4) prostate cancer stromal matrix; and (5) prostate cancer stromal "feeder" layers. LNCaP cells (1×10^5 /well) were cultivated in these systems, using RPM-1640 + 10% charcoal-stripped FBS with/without 10-8 M DHT. Cells were cultured for 6 days and counts performed on days 2, 4, and 6 to assess proliferation in subsets 1,2. On day 6, conditioned media was harvested from each well and again evaluated for PSAP and PSA content by radioimmunoassay. Duplicate wells were prepared for a morphological analysis of subsets 1-4.

RESULTS

Morphology of BPH Stromal and Epithelial Cells Grown on Tissue Culture Plastic vs Matrigel

Stromal (SC) and epithelial (EC) cell populations were isolated from histologically confirmed BPH surgical specimens following mechanical/enzymatic dissociation and passage over a discontinuous Percoll gradient. Previous studies have documented the purity of this separation to be in excess of 95% as assessed by cell morphology, differential immunocytochemistry (EC = positive PSAP, PSA, and keratin; SC = positive vimentin), and two-dimensional gel electrophoretic patterns (11,12). These cells were established and maintained independently *in vitro* using selective medium.

When grown on tissue culture plastic, the SC exhibit a rather homogenous growth pattern characterized by a monotonous array of fusiform or spindle-shaped cells typical of *in vitro* cultivated mesenchyme (Figure 1). We have previously demonstrated that these cells can be maintained for 20-30 passages in tissue culture and that flow cytometric analysis (FCM) of such isolated prostatic SC demonstrate a diploid DNA index of 1.0 with approximately 20% of cells undergoing cell division (S phase = 10.82%; G2/M = 8.93%) (12). When grown on basement membrane biomatrix (Matrigel), the SC continue to demonstrate a predominantly fibroblastic appearance, with scattered clusters of cells exhibiting more compact perimeters (Figure 2).

In distinction, the isolated prostatic EC demonstrate more heterogenous growth on tissue culture plastic which probably represents the presence of both adluminal (secretory) and basal cell elements. Cuboidal-appearing cells are frequently seen admixed with more elongated and giant cell variants. Also easily discerned are occasional senescent epithelial cells exhibiting "ballooning" of the cytoplasm and prominent vacuolization (Figure 3). Previous studies performed in our laboratory have shown that such EC can

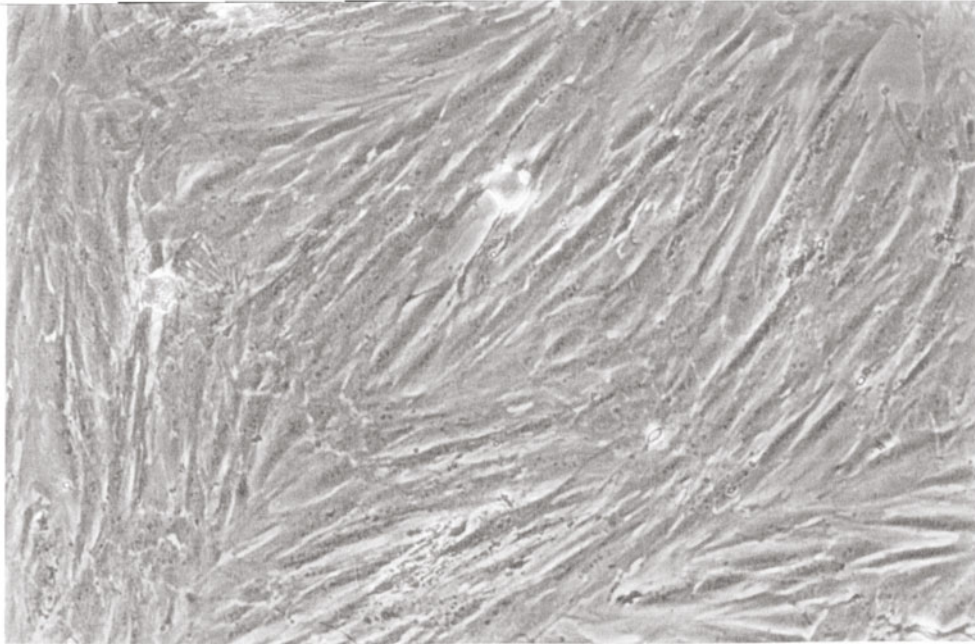


Figure 1: BPH stromal cells exhibiting confluent monolayer growth on tissue culture plastic. X 100.



Figure 2: Growth pattern exhibited by BPH stromal cells cultivated on basement membrane biomatrix (Matrigel) in serum-free WJJC-404 medium + DHT (10^{-8} M) for 7 days. X 40.

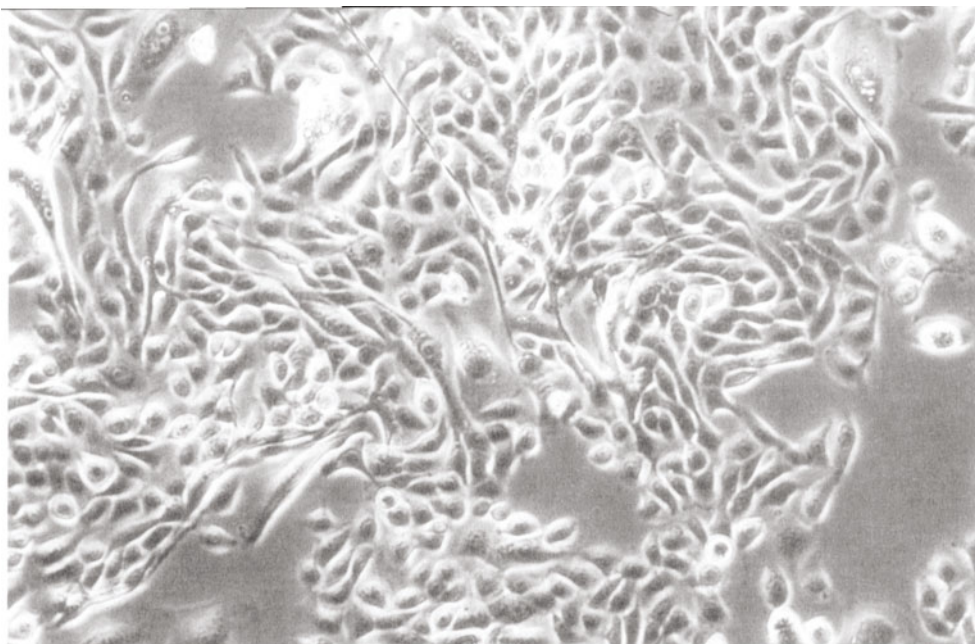


Figure 3: Sub-confluent growth of BPH epithelial cells on tissue culture plastic. These cells were eluted from the 5%/7.5% and 7.5%/10% interface of the discontinuous Percoll gradient. X 100.

be maintained in tissue culture for 4-6 passages after which time the cells begin to senesce and cease LNCaP growth as did stroma-derived matrix. Again, slowing of proliferation is evident, as is the increased tendency for LNCaP cells to aggregate (Figure 13A). The addition of DHT to this system has little impact on overall growth morphology but may induce a slight increase in cell proliferation (Figure 13B). Of interest, cancer-derived stromal "feeder" layers (with/without the addition of DHT) appears to have a similar impact on LNCaP growth morphology (Figure 14A,B).

The Effect of Growth Substrate and DHT on Proliferation/Differentiation of the LNCaP Cell Line

The proliferation of LNCaP cells was greatly facilitated by growth on tissue culture plastic in the presence of escalating doses of DHT. In fact, the latter growth environment was associated with a five-fold increase in cell numbers when compared with cultivation on BPH-derived stromal cell matrix (Figure 15). The inhibitory impact of this matrix preparation on cell turnover is nearly identical to that demonstrated on BPH epithelial cells grown on Matrigel. The addition of DHT did little to override this growth inhibition.

Despite the inhibitory effect of stromal matrix on the *in vitro* growth of LNCaP cells, this growth environment coupled with escalating concentrations of DHT maximized the secretion of PSAP/PSA. In fact, both differentiation markers were increased five-fold in the presence of stromal matrix and DHT (10-8 M) when compared to cultivation on a plastic substrate in the presence of equivalent DHT concentrations (Figure 16A,B).

For both the BPH epithelial cells and the cancer cell line LNCaP, the phenotypes of proliferation and differentiation appear to diverge under selective growth conditions. With respect to the latter, the most important features are the presence/absence of a biomatrix substrate and DHT.

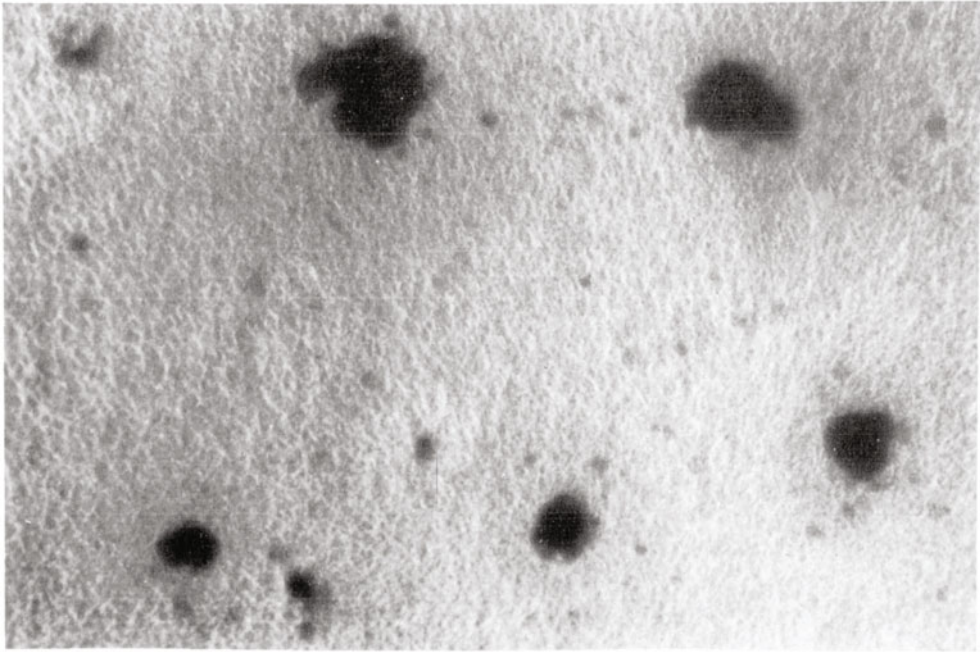


Figure 4: BPH epithelial cells demonstrating incipient cluster or "dome" formation after 2 days of *in vitro* growth on Matrigel. X 100.

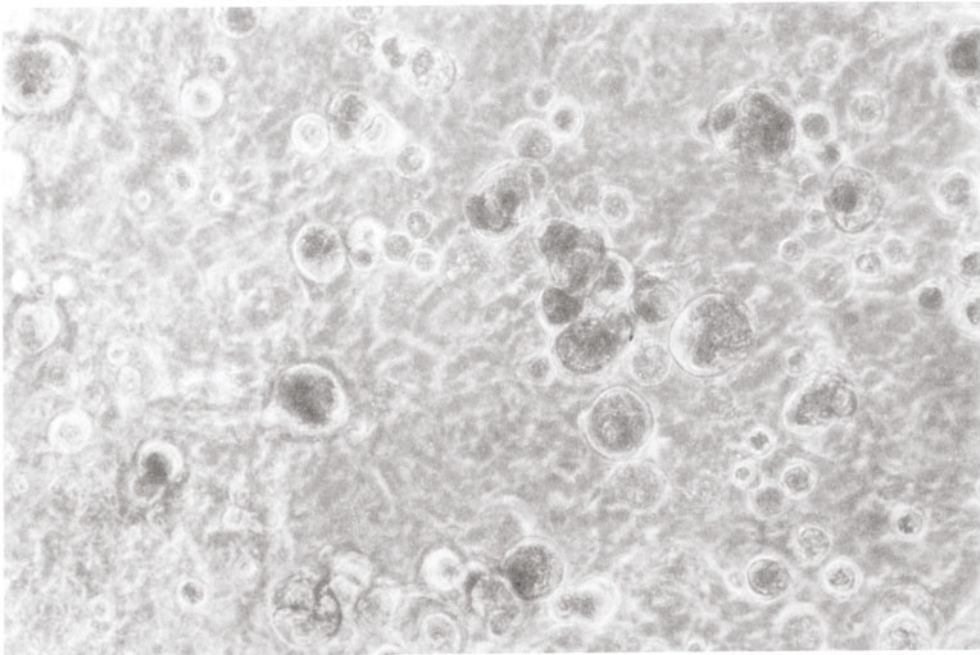


Figure 5: Clusters or "domes" of BPH epithelial cells grown on Matrigel for 6 days. X 40.

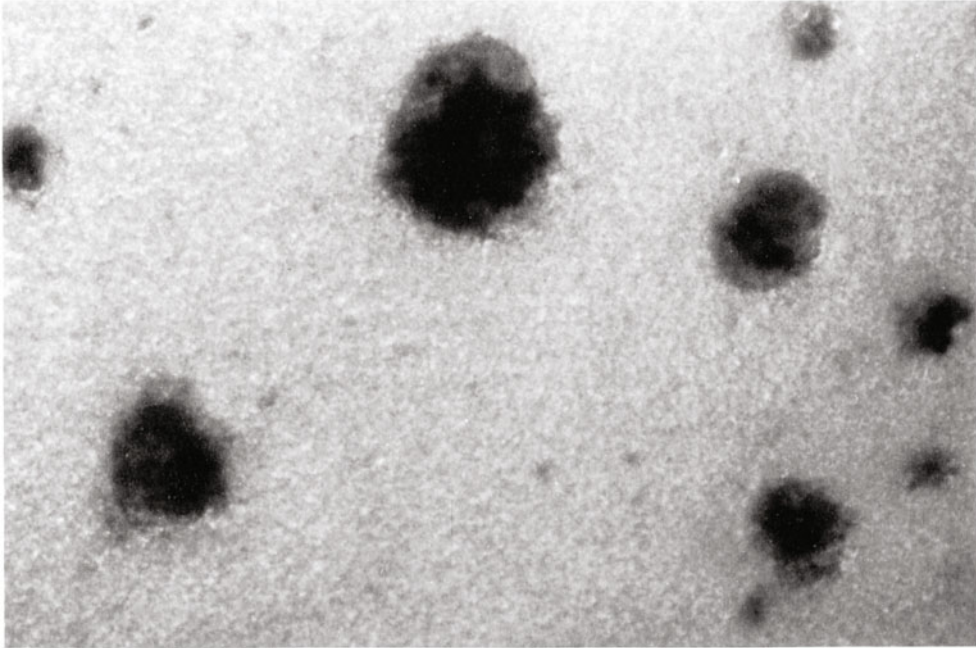


Figure 6: High power view of BPH epithelial cell clusters or "domes" cultivated on a Matrigel substrate. X 100.

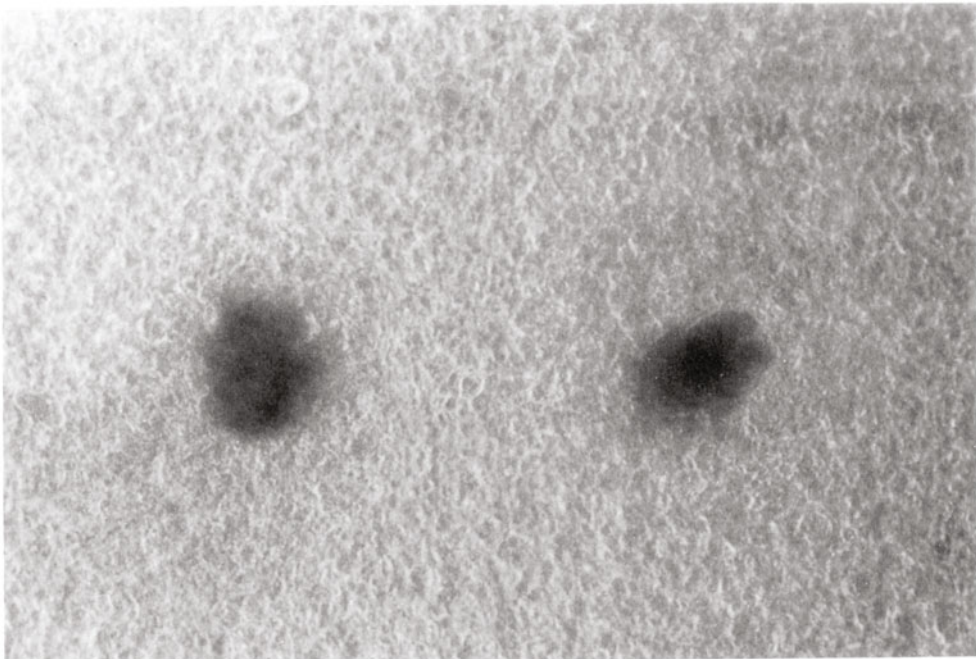


Figure 7: Appearance of BPH epithelial cell aggregates grown for 6 days in serum-free medium on a Matrigel substrate in the presence of 20% stromal conditioned medium (SCM). The addition of either SCM or DHT appears to increase the overall volume of these epithelial cell spheroids. X 40.

The Impact of Matrigel, DHT, and Stromal Conditioned Media on the Proliferation/ Differentiation of BPH Epithelial Cells

Six day *in vitro* growth curves demonstrate a distinct proliferation advantage for BPH epithelial cells cultivated on tissue culture plastic vs those grown on Matrigel. On day six, a ten-fold increase in epithelial cell number was noted for those cells cultivated on plastic. Although the addition of DHT stimulated proliferation in both groups, the substrate played an overwhelming role in dictating the magnitude of cell turnover (Figure 9). Previous studies performed in our laboratory have confirmed the mitogenic potential of stromal conditioned medium. In fact, a two-fold increase in epithelial cell mitogenesis was noted at a SCM concentration of 0.1 ug/ml. A maximal seven-fold enhancement of proliferation was observed at 100 ug/ml of SCM (21).

In distinction, the ability of benign epithelial cells to secrete PSAP and PSA was markedly inhibited by growth on tissue culture plastic. Assays performed on days 14, 16, and 18, revealed minimal secretion of these differentiation markers when EC's were cultivated on a plastic substrate in the presence of a WAJC-404 alone (Table 1). The addition of either DHT (10⁻⁸ M) or SCM (20%) was associated with a near-equivalent increase (two-three fold) of PSA secretion. Of interest, DHT had a less impressive effect on PSAP secretion than did SCM. Again, the latter was associated with a two-three fold elevation in secretion of this marker. When both DHT and SCM were added to the system, no significant changes were noted when compared to the addition of either factor separately.

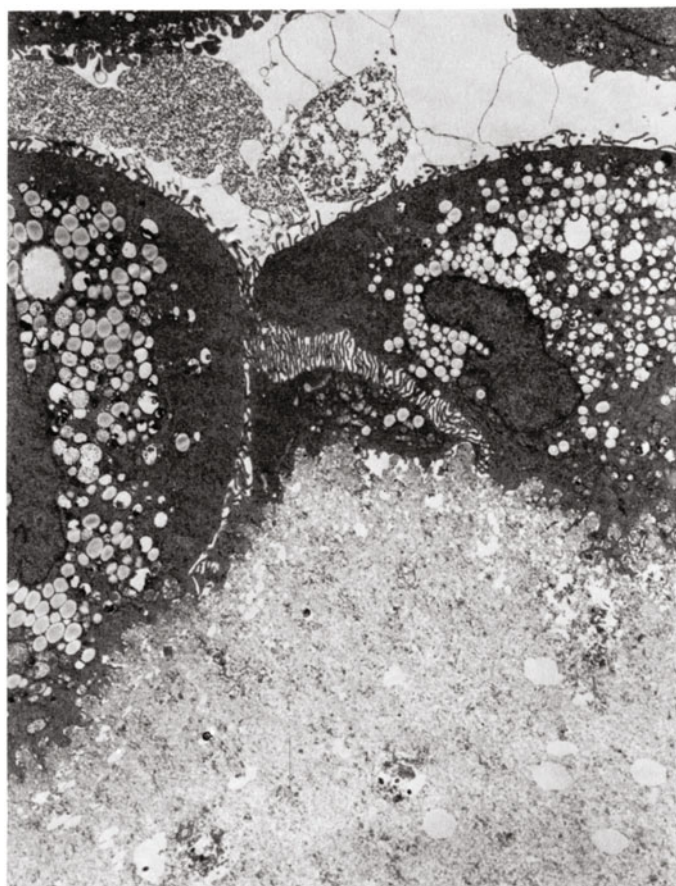


Figure 8: Transmission electron micrograph of BPH epithelial cells grown on Matrigel for 7 days. Noteworthy features include: (1) invaginations of the basal surface as it abuts the Matrigel substrate; (2) surface microvilli; and (3) numerous cytoplasmic vacuoles. X 5,000. (Photograph courtesy of Dr. H. Yokoo).

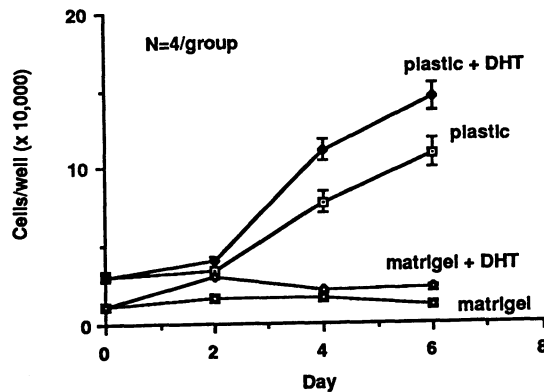


Figure 9: Growth of BPH Epithelial Cells on Plastic and Matrigel. BPH epithelial cells (1×10^5 /well) were plated in untreated 24 well plates or wells containing Matrigel and cultivated in serum-free WAJC-404 (+/- 10^{-8} M DHT). The cells were cultured for 2, 4, and 6 days at which time points cell counts were performed (in quadruplicate). Epithelial cells were eluted from plastic substrates by treatment with type 1 collagenase. Epithelial cells were dislodged from Matrigel following digestion of the latter with dispase. The results indicate (1) the inhibitory effect of Matrigel on epithelial cell proliferation; and (2) a stimulatory effect of DHT on cell turn-over.

The greatest impact on marker secretion was produced by the addition of Matrigel as the growth substrate (Table 1). The addition of Matrigel alone was responsible for an increase in PSAP and PSA secretion in excess of that produced by both DHT and SCM, alone or in combination. The growth environment most conducive to secretion of these markers was produced by the cultivation of prostatic epithelial cells on a Matrigel substrate in the presence of WAJC-404 + DHT (10^{-8} M) and SCM (20%). This growth environment was responsible for a ten-fold increase in marker secretion on day 14 when compared to growth on tissue culture plastic in the presence of WAJC-404 alone. Marker secretion was maintained through days 16 and 18 with near twenty-fold differences in secretion being noted when both groups were compared. Of interest, the inability of prostatic epithelial cells cultured on a plastic substrate to express the differentiation markers was reversed when these cells were replated on Matrigel and supplemented with this optimal, defined media (WACJ-404, DHT, SCM).

Comparative Morphology of Stromal Cells Isolated from BPH and Prostate Cancer Specimens

The BPH-derived stromal cells used in this phase of the study demonstrated the spindle-shaped morphology typical of fibroblastic cells cultivated as cellular monolayers on tissue culture plastic (Figure 10A). In distinction, stromal cells isolated from a radical prostatectomy specimen (Stage C, Gleason's score = 5) exhibited a more florid array of pallasading cells with tiny compact nuclei (Figure 10B). These latter cells adapted much more readily to growth and expansion *in vitro*. Although preliminary studies comparing these stromal populations have suggested near-equivalent proliferation as assessed by *in vitro* growth curves, the cancer-derived stroma exhibits a higher labelling index when pulsed with tritiated thymidine (data not shown).

The Impact of Growth Substrates and DHT on the Morphology of LNCaP

When cultivated on tissue culture plastic the LNCaP cell line demonstrates monolayer growth with a tendency to form cell clusters or aggregates as cell density increases (Figure 11A). With the addition of DHT (10^{-8} M) increased cell proliferation and density-dependent clustering become apparent (Figure 11B).

Table 1: Differentiation of BPH Epithelial Cells on Plastic and Matrigel

Group	Day		
	14	16	18
PAP (ng/ml/10 ⁴ cells)			
Plastic	0.46 +/- 0.03	0.13 +/- 0.01	0.14 +/- 0.01
Plastic + DHT	0.25 +/- 0.05	0.12 +/- 0.02	0.10 +/- 0.03
Plastic + SCM	1.42 +/- 0.10	0.52 +/- 0.07	0.37 +/- 0.14
Plastic + DHT + SCM	1.38 +/- 0.06	0.36 +/- 0.01	0.28 +/- 0.01
Matrigel	2.13 +/- 0.52	1.27 +/- 0.22	0.91 +/- 0.07
Matrigel + DHT	4.85 +/- 0.65	3.28 +/- 0.56	3.07 +/- 0.39
Matrigel + SCM	1.48 +/- 0.02	0.54 +/- 0.03	0.41 +/- 0.04
Matrigel + DHT + SCM	4.85 +/- 0.13	3.28 +/- 0.07	3.07 +/- 0.09
PSA (ng/ml/10 ⁴ cells)			
Plastic	0.69 +/- 0.05	0.28 +/- 0.03	0.45 +/- 0.10
Plastic + DHT	1.55 +/- 0.09	0.93 +/- 0.11	0.94 +/- 0.07
Plastic + SCM	1.60 +/- 0.22	0.94 +/- 0.08	0.60 +/- 0.09
Plastic + DHT + SCM	2.62 +/- 0.19	1.15 +/- 0.08	1.03 +/- 0.16
Matrigel	3.73 +/- 0.23	3.05 +/- 0.51	2.84 +/- 0.03
Matrigel + DHT	5.58 +/- 0.63	4.86 +/- 0.33	4.73 +/- 0.48
Matrigel + SCM	3.23 +/- 0.45	2.15 +/- 0.13	1.90 +/- 0.36
Matrigel + DHT + SCM	9.91 +/- 1.03	8.67 +/- 0.52	7.37 +/- 0.31

Following mechanical/enzymatic dissociation and Percoll gradient separation, BPH epithelial cells (1 X 10⁵/well) were plated in untreated 24 well plates or wells containing Matrigel and allowed to adhere overnight. The Media was then changed to serum-free WJJC 404 (+/- DHT, SCM) and cell were cultured for 14, 16, and 18 days. Conditioned medium was harvested from the respective wells and PAP/PSA content was determined by radioimmunoassay. All groups were plated in quadruplicate and repeated twice to assure reproducibility.

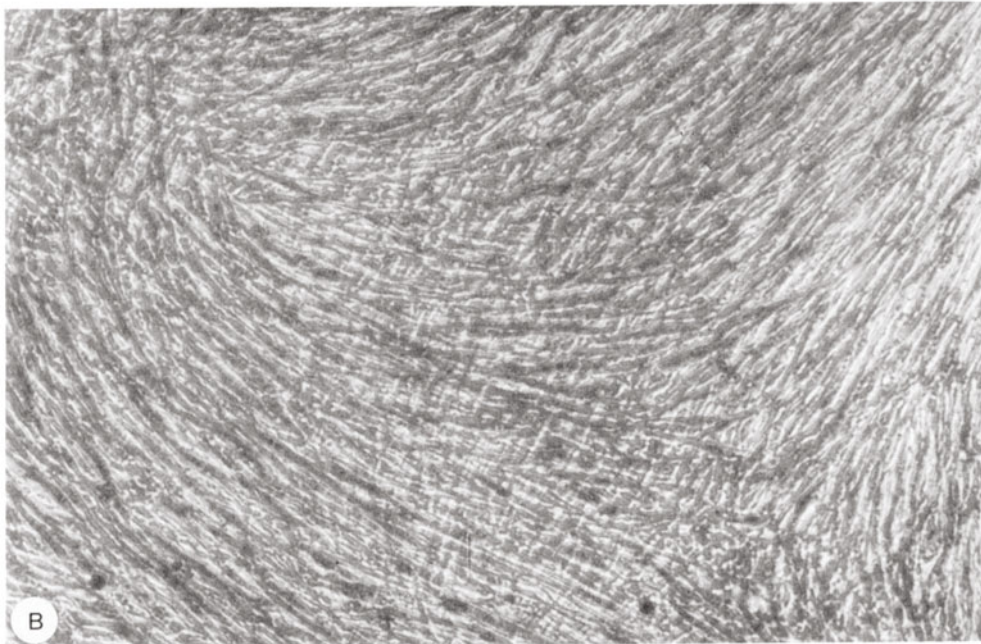
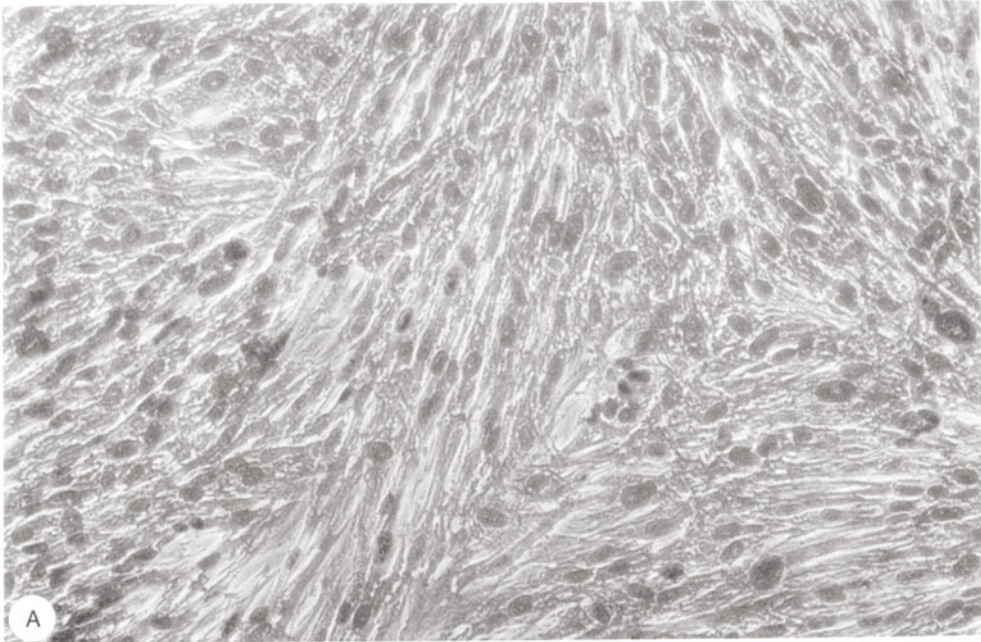


Figure 10 A & B: A. Monolayer growth of BPH stromal cells on tissue culture plastic. X 100. B. Demonstrates confluent monolayer growth of cancer-derived stromal cells cultivated under similar *in vitro* conditions. X 100. See Results section for comparative descriptions.

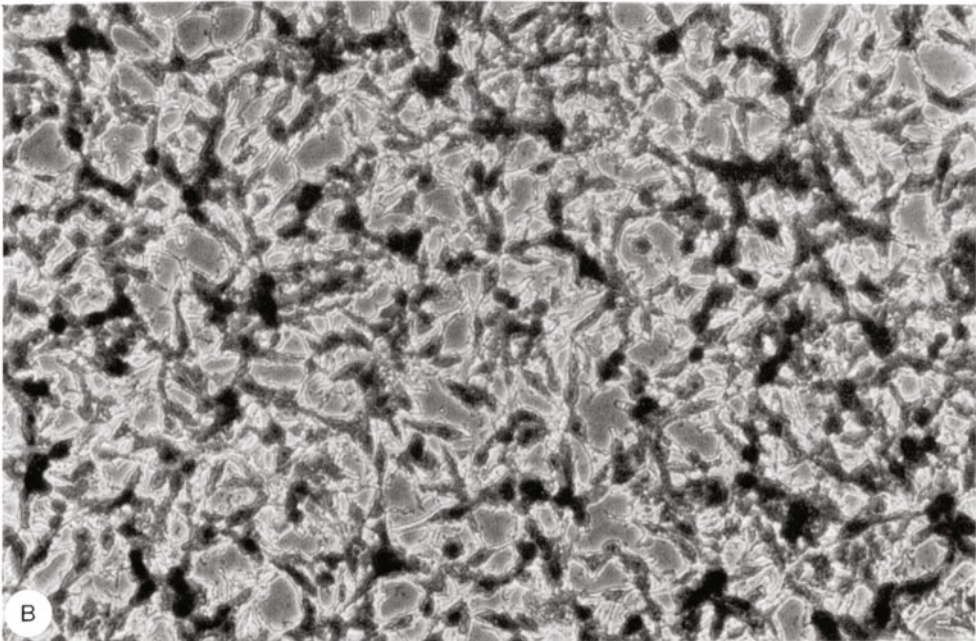
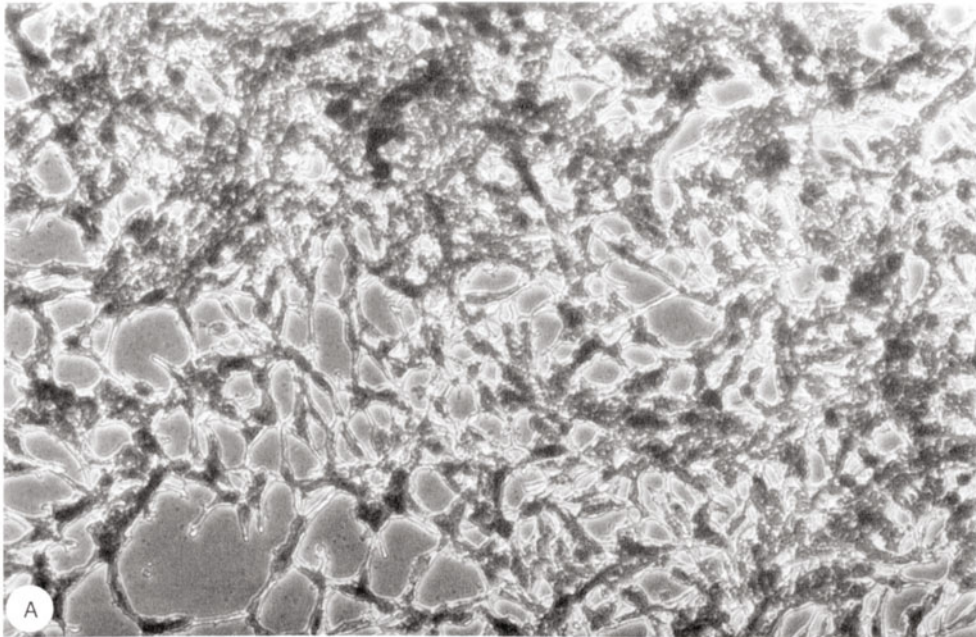


Figure 11 A & B: A. Typical *in vitro* growth of LNCaP cells on tissue culture plastic. X 40. B. The impact of DHT (10^{-8} M) on the growth morphology of LNCaP cells cultivated under similar *in vitro* conditions. Note the suggestion of increased cell numbers and more pronounced cell aggregates or clusters. X 50.

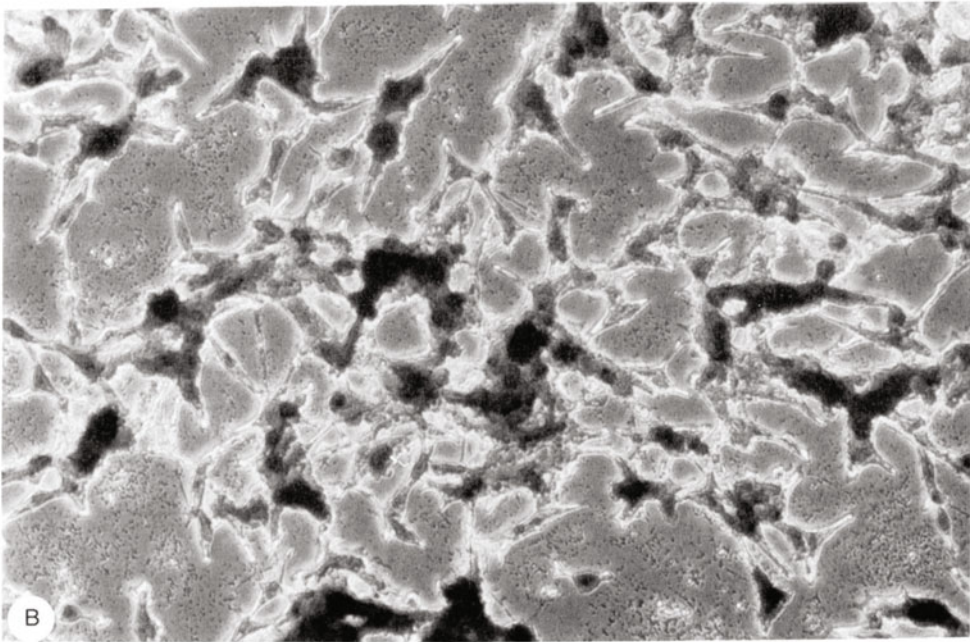
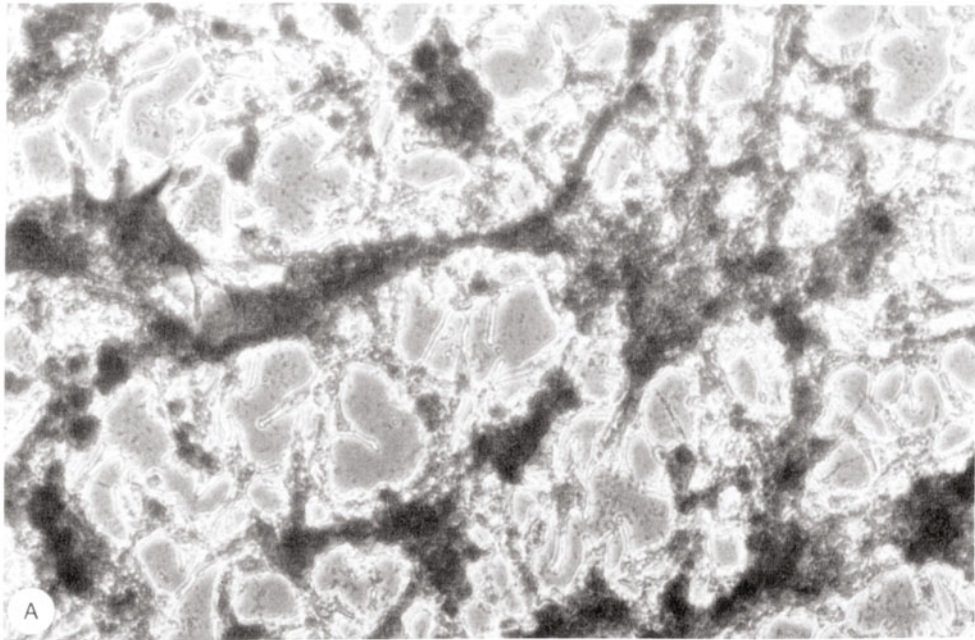


Figure 12 A & B: A. Appearance of LNCaP cells grown on BPH-derived stromal matrix. X 40. B. High lights the effect on DHT to this culture system. X 40. In both 12A,B there appears to be a rather striking inhibition of cell growth coupled with the increased formation of multicellular aggregates.

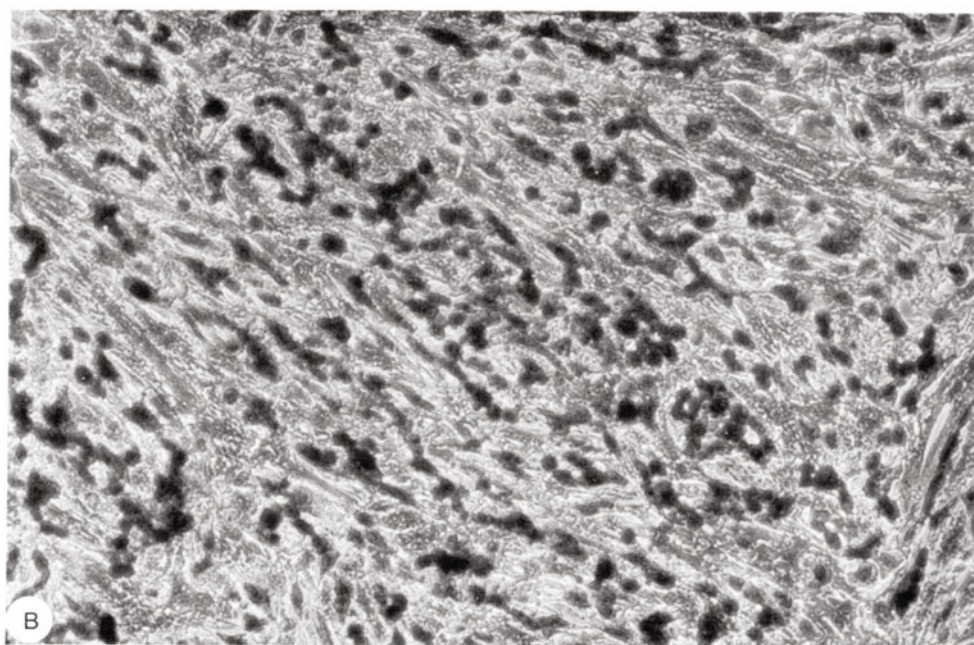
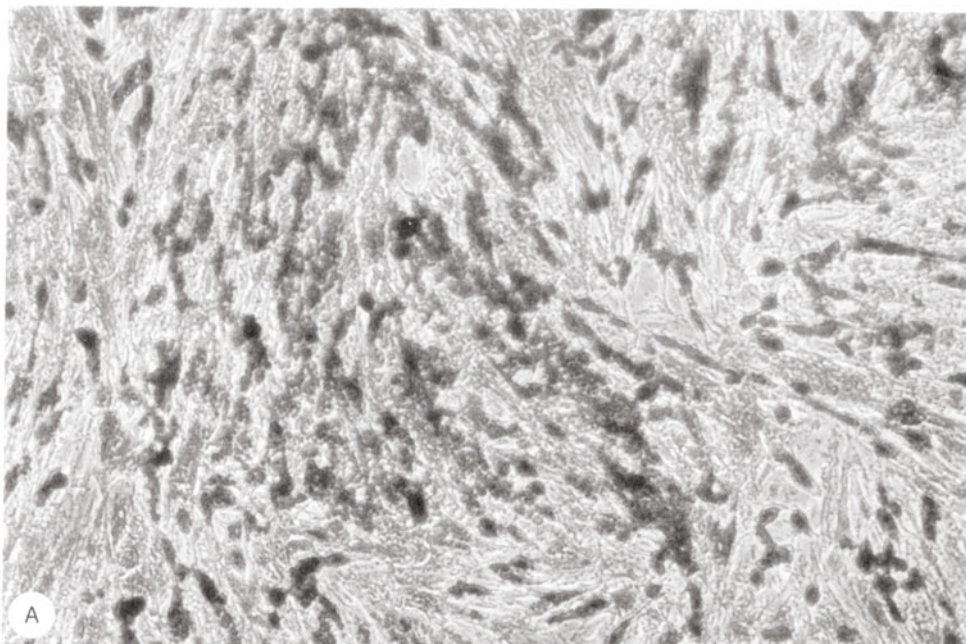


Figure 13 A & B: A. The impact of "feeder" layers of BPH stromal cells on the growth morphology of LNCaP. X 40. B. Again demonstrates the impact of 10^{-8} M DHT on similarly cultivated LNCaP cells. Although these photographs suggest relative growth inhibition, the addition of DHT may be associated with increased cellularity within the isolated cell clusters. X 40.

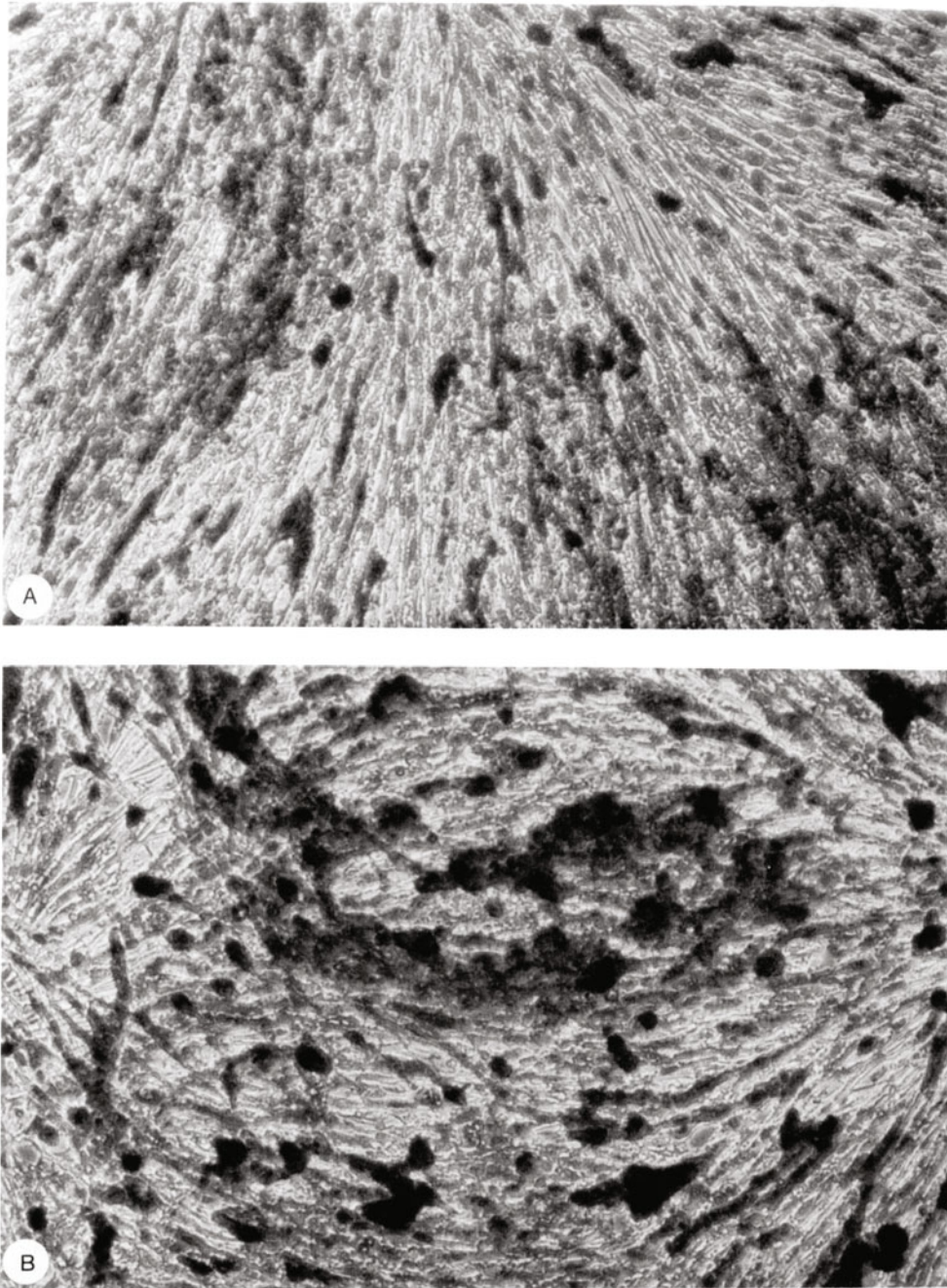


Figure 14 A & B: A. The impact of "feeder" layers of cancer-derived stromal cells on the *in vitro* growth of LNCaP. X 40. B. Represents an identical culture environment except for the addition of 10^{-8} M DHT. Again noted is the overall perception of growth inhibition. However, the addition of DHT may be associated with increased cellularity within distinctly formed aggregates. X 40.

DISCUSSION

There are certain disadvantages associated with the use of conventional tissue culture systems for the evaluation of growth/differentiation as applied to BPH and prostate cancer. For example, prostate epithelial cells cultured on plastic surfaces are capable of undergoing only 4-6 rounds of cell division (11,14) and rapidly lose those phenotypic characteristics pathognomic for prostate "epitheliality," namely expression of PSAP and PSA (11,12,21). It would appear that those cells adapted to such *in vitro* growth environments have been highly selected with respect to their proliferation phenotype and prevented from achieving their normal state of differentiation. Moreover, standard *in vitro* approaches preclude a sophisticated analysis of many biologically important events, including the impact of stromal cells and their soluble mediators, ECM, and androgen upon prostate epithelial cell function.

Despite these handicaps, cell culture is an ideal system for the evaluation of cell function and gene regulation since repetitive analyses can be performed in a stringently controlled environmental setting. With respect to BPH and prostate cancer, the *in vitro* environment should permit the evaluation of the zonal/cellular heterogeneity intrinsic to the prostate gland and establish a growth milieu more closely approximating the *in vivo* state. To this end, organ culture has been suggested as a method to maintain 3-dimensional growth *in vitro*. By preserving normal tissue organization and structure, cellular heterogeneity, differentiated function, and the ability to proliferate can be maintained (32,33). Unfortunately, such an approach does not readily permit an indepth analysis and dissection of those morphological and biochemical events taking place within the tissue microcosm.

We have previously described our approach to the separation and independent cultivation of highly purified populations of human prostate stromal and epithelial cells (11,12,21). This approach, coupled with the use of ECM, hormones, and growth factors, permits a step-wise "reconstitution" of the *in vivo* condition. In this report, we have demonstrated the utility of such an approach to selectively induce human prostate epithelial cell proliferation or differentiation by altering culture conditions. BPH epithelial cells were induced to proliferate when grown on a plastic substrate in the presence of both DHT and SCM. This growth environment, however, was not conducive to the secretion of PSAP nor PSA. In distinction, growth of BPE on basement membrane biomatrix (Matrigel) was associated with marked diminution in cell growth but enhanced production of the prostate differentiation products, PSAP and PSA. Both DHT and SCM are mitogenic for BPH epithelial cells cultivated on tissue culture plastic (21). Although neither substance is able to override the MATRIGEL + induced growth inhibition of BPE, their addition to this system maximizes secretion of both PSAP and PSA. LNCaP cells can be similarly manipulated by adjusting the *in vitro* environment. Maximal proliferation is associated with growth on tissue culture plastic in the presence of DHT. Again, the growth of these cancer cells on a substrate of stromal matrix inhibits cell turnover but maximizes expression of PSAP and PSA. From these studies, it would appear that extracellular matrix, soluble mediators of stromal origin, and androgen play an important role in modulating the phenotypes of proliferation and differentiation in both BPE and LNCaP cells.

The presence or absence of extracellular matrix appears to be the most strategic variable in our culture system. ECM is a complex mixture of collagen isotypes, attachment glycoproteins, and glycosaminoglycans (34). With respect to the prostate, collagen types I and III constitute the major components of adult skin fibroblasts, human fetal fibroblasts, and mouse 3T3 cells suggesting that the stromal mitogens are neither organ nor species-specific. Neither fixed fibroblast monolayers nor extracellular matrix prepared from such cultures were capable of stimulating prostate epithelial growth, reinforcing that the growth factors are a soluble product secreted by living cells.

Thus, available evidence suggests that prostate stromal cells secrete heparin-binding and non-heparin-binding proteins which are mitogenic for prostate epithelial cells. However, the majority of these studies were conducted using a admittedly artificial culture environment, namely the cultivation of cells on tissue culture plastic. In this study, we chose to evaluate the impact of stromal secretory products on prostate epithelial cells in the presence/absence of a biological substrate (Matrigel, stromal matrix) in order to assess the role of such factors on epithelial differentiation. We demonstrated that BPE grown on a Matrigel substrate in the presence of DHT and SCM maximize their secretion of both PSAP and PSA. This suggests that soluble stromal mediators can play a role in modulating BPE differentiation when the latter are cultivated on a biologically conducive substrate.

Dihydrotestosterone appears to play a strategic role in the maintenance, differentiation, and proliferation of human prostate tissues *in vivo* (55,56,57, 58,59). In distinction, most reports have failed to establish its role as an essential mitogen *in vitro* (11,13), although some studies suggest a modest and

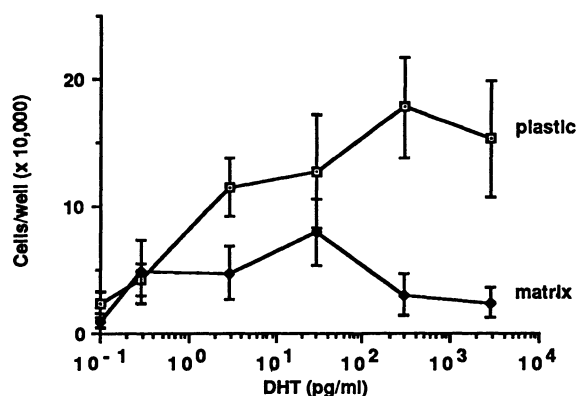


Figure 15: Growth of LNCaP on plastic and stromal ECM. LNCaP cells (1×10^5 /well) were plated in untreated 24 well plates or wells containing matrix derived from BPH stromal cells. After adherence, the cells were grown in medium containing RPMI-1640 with 10% charcoal stripped fetal bovine serum and varying concentrations of DHT. Cells were cultured and counted on days 2, 4, and 6. The results suggest inhibition in cell proliferation by stromal matrix despite the mitogenic input of DHT.

transient benefit to its presence in the tissue culture media (60). We have found that BPE can be maintained and expanded through 4-6 passages in WAJC-404 plus essential growth factors in the absence of DHT (11,12,21). However, these experiments were performed with tissue culture plastic as the cell substrate. Undoubtedly, aberrations induced by this culture environment may contribute to this biological paradox. In distinction, the study of de Launoit et al demonstrated the utility of establishing an *in vivo* - like environment through the use of organ culture in assessing the impact of steroid hormones on human hyperplastic prostatic tissue (61). Under their environmental conditions, BPH specimens demonstrated significant increases in thymidine-labelling index after a 36 hour incubation period in the presence of DHT. These observations, coupled with the inability of DHT to stimulate prostate epithelial proliferation *in vitro*, have suggested that androgen may not be a direct mitogen in isolated epithelial cells. It is conceivable, that the mitogenic effects of DHT may be mediated through the modulation of other hormones, growth factors, or their receptors. For example, the study of Traish and Wotis demonstrated that treatment of 7-day interstitial collagen, while types IV and V are found primarily within basement membrane (35). In addition to its unique collagen substrates, basement membrane contains the attachment glycoprotein laminin (with lesser amounts of entactin and nidogen) which mediates the attachment of epithelial cells to type IV collagen (36). Finally, basement membrane contains predominantly heparan sulfate proteoglycan (35,36). In addition to the latter glycosaminoglycan (GAG) other GAG's have been located within normal and hyperplastic human prostates. Of these, dermatan sulfate predominates (40%) followed by heparin (20%) chondroitin (16%) and hyaluronic acid (20%) (37). These proteoglycans are large negatively charged polymers which may play a critical role in the signaling of extracellular matrix events, including their affinity for heparin-binding growth factors (38). Finally, fibronectin is secreted by prostatic fibroblasts and functions as an important anchorage protein which binds mesenchymal and epithelial cells to various isotypes of collagen and proteoglycans (35). The ECM is synthesized by mesenchymal and epithelial cells through a process which requires dynamic cell-cell interaction (35,36). It appears that ECM represents one of three major matrix systems (extracellular matrix, cytoplasmic matrix, and nuclear matrix) that interact and constitute the "tissue matrix system" (39,40). It is theorized that phase shifts and communication through these structural matrix elements plays a central role in controlling prostate development and function (39). Important roles attributed to ECM include: (1) a dynamic role in epithelial differentiation during embryogenesis (41); (2) the induction and maintenance of a polarized epithelium during adult life (42,43,44,45); (3) modulating the impact of various growth and serum factors on epithelial cell targets (43,45); (4) the control of gene expression (46,47); and (5) a dynamic influence on cell morphology and function as a result of cytoskeletal redistribution (48).

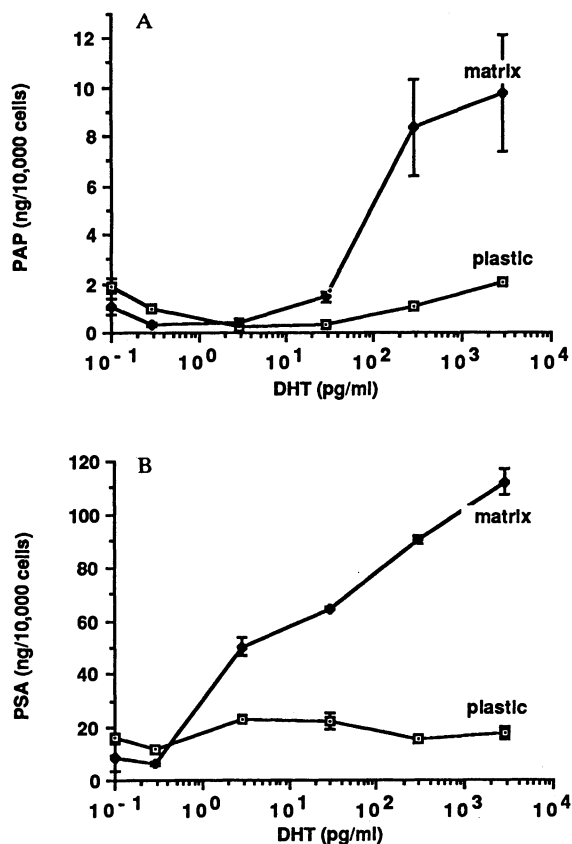


Figure 16: The impact of stromal matrix and DHT on the secretion of PAP by LNCaP (A) and PSA secretion by LNCaP cells (B). The cancer cells (1×10^5 /well) were plated in untreated 24 well plates or wells containing matrix derived from BPH stromal cells. Following adherence overnight, the media was changed to RPMI-1640 + 10% charcoal stripped fetal bovine serum containing varying concentrations of DHT. The cells were detached from the culture surface with 0.25% trypsin and counted with a hemacytometer. On day 6 conditioned medium was collected and evaluated for PAP and PSA content by radioimmunoassay. The results demonstrate a five-fold increase in secretion of both differentiation markers in the presence of matrix and DHT.

Although there is evidence to suggest that ECM is tissue specific and influences tissue-specific gene expression, Reid has emphasized two important exceptions, which include: (1) biomatrix prepared from embryonic tissues such as amnionic membranes or placenta; and (2) biomatrix produced by certain tumor systems such as the Englebreth-Holme-Swarm tumor (EHS) (47). As stated previously, the EHS tumor is capable of producing voluminous amounts of basement membrane biomatrix with laminin representing 90% of its chemical constitution (30,31). In a series of experiments described by Reid, rat ventral prostate epithelial cells were dependent upon the presence of matrix substrates in order to secrete prostate binding protein, as well as, other androgen-dependent responses (47). In this study, the Matrigel substrate inhibited the proliferation of both BPE and LNCaP cells. Moreover, BPE cultivated on Matrigel showed evidence of ultrastructural rearrangement suggestive of near-normal cell polarity. Finally, despite its inhibitory role on cell proliferation, the presence of Matrigel enhanced the ability of both BPE and LNCaP cells to secrete the differentiation proteins, PSAP and PSA.

A number of studies have been performed on crude extracts of human and rat prostate tissue which have demonstrated the presence of both high and low affinity heparin-binding growth factors, including basic fibroblast growth factor (49,50,51,52,53,54). Recent studies performed in our laboratory have demonstrated that the co-incubation of BPH epithelial cells with lyophilized stromal conditioned medium (SCM) at concentrations of 0.1-1,000 ug/ml resulted in the enhancement of BPE proliferation (21). Again, analysis of heparin-binding affinity showed that both heparin-binding and non-heparin-binding epithelial cell mitogens were present in SCM. These findings are consistent with recently reported observations demonstrating the presence of prostate epithelial cell mitogens present in the conditioned medium of sub-confluent fibroblast cultures (22). Of interest, the study by Kabalin et al showed that normal, hyperplastic, and malignant prostate epithelial cells were stimulated by SCM preparations derived from either adult prostatic fibroblasts, castrated rats with DHT consistently down-regulated epidermal growth factor (EGF) receptors (62).

In this study, BPH-derived epithelial cells cultivated in the presence of DHT exhibited no significant growth advantage when compared to control populations of BPE cultivated in identical serum-free media but without the addition of exogenous androgen. With respect to LNCaP cell line, our observations are in concert with previously reported studies suggesting the androgen sensitivity of this system as manifested by increased cell proliferation (25-28). Of interest, cultivation of either epithelial population on a natural biological substrate inhibited cell turnover and negated much of the apparent mitogenic benefit of DHT. These studies also suggest that under the experimental conditions outlined, the phenotypes of proliferation and differentiation appear divergent for both BPE and LNCaP cells. Those growth conditions which induce the greatest inhibition in cell proliferation maximize the ability of each cell population to secrete the differentiation proteins, PSAP and PSA.

As stated previously, the ability of fibroblasts to produce soluble mediators capable of stimulating the proliferation of prostate epithelial cells is most likely organ and species non-specific (22). Very little information is available concerning the morphological and biochemical differences which may exist in mesenchymal populations isolated from BPH and prostate cancer specimens. The pursuit of this issue is undoubtedly important since a number of studies suggest that the stroma surrounding tumors is inherently different from normal organ stroma and may participate in the dynamics of malignant growth (63,64,65). Indeed, recent studies by Chiquet-Ehrismann et al have shown that the stroma associated with malignant mammary tumors is capable of producing a unique extracellular matrix protein, Tenascin, not expressed in the stroma associated with benign mammary tumors (63). This protein may play an important role in altering the topology of malignant cells and facilitating their invasion of the surrounding interstitium. In this study, we report our preliminary observations on distinct differences between stromal cells isolated from BPH and a prostate cancer specimen. The growth morphology on tissue culture plastic permits obvious distinctions. Moreover, preliminary observations suggest an increased thymidine-labelling index in the tumor-derived stromal cells (data not shown). A number of additional studies are ongoing to further define the morphologic, biochemical, and genomic differences between these stromal populations.

CONCLUSION

Our results suggest that the phenotypes of proliferation and differentiation as applied to BPE and LNCaP cells may be divergent. An *in vitro* environment which maximizes proliferation appears to down-regulate the ability of these epithelial populations to express differentiation markers, PSAP and PSA. Conversely, the growth inhibition induced by the presence of a biological substrate (Matrigel, stromal matrix) enhances the ability of both epithelial populations to respond to exogenous growth/differentiation factors (DHT and SCM). Under these circumstances, marker expression appears to be maximized. We intend to utilize the *in vitro* systems described in this paper to investigate the dynamics of stromal-epithelial interaction within benign and malignant human prostate glands.

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THERAPEUTIC APPROACHES TO ACTIVATING PROGRAMMED CELL DEATH OF ANDROGEN-INDEPENDENT PROSTATIC CANCER CELLS

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INTRODUCTION - MAGNITUDE OF THE PROBLEM

During this year there will be approximately 30 thousand deaths due to prostatic cancer in the United States (1). This mortality rate makes prostatic cancer the second commonest fatal tumor in males of all ages in America, and the leading cause of cancer death in men over the age of 55 (1). Besides a high annual mortality rate, prostatic cancer is now the most commonly diagnosed malignancy in males of all ages in the United States (1). These high annual incidence rates translate into the human reality that one of every 12 American white males will eventually develop clinical prostatic cancer during their lifetimes (2). Rates for American black males are even higher such that the lifetime risk for cancer is one out of every 10 (3). In addition, the annual incidence rate of clinical prostatic cancer has increased steadily since 1930 to the present time (4).

Most prostatic cancers retain an androgen responsiveness for stimulation of their growth. Prostatic cancer is thus often highly responsive to androgen ablation therapy. Nearly all men with metastatic prostatic cancer treated with androgen ablation therapy have an initial, often dramatic, beneficial response to such androgen withdrawal therapy (5). While this initial response is of substantial palliative value, essentially all treated patients eventually relapse to an androgen-insensitive state in which additional forms of antiandrogen therapy are ineffective no matter how aggressively given (5-8). Because of this nearly universal relapse phenomenon, the annual death rate from prostatic cancer has not decreased at all over the subsequent 40 years since androgen withdrawal has become standard therapy (9). Over the last 40 years, the superficially benign nature of androgen withdrawal therapy has tended to disguise the fact that metastatic prostatic cancer is still a fatal disease for which no therapy is available which effectively increases survival (10, 11).

Therapeutic Importance of Androgen-Independent Prostatic Cancer Cells

Studies by a series of laboratories have demonstrated that a major reason for this universal relapse of metastatic prostatic cancer to androgen ablation is that prostatic cancer within an individual patient is heterogeneously composed of clones of both androgen-dependent and -independent cancer cells even before hormone therapy is begun (12-15). Development of such tumor cell heterogeneity can occur by a variety of mechanisms [e.g. multifocal origin of the tumor, adaptation, or genetic instability (16)]. Regardless of the mechanism of development of such cellular heterogeneity, once androgen-independent cancer cells are present within individual prostatic cancer patients, the patient is no longer curable by androgen withdrawal therapy alone, no matter how complete, since this therapy kills only the androgen-dependent cells without eliminating pre-existing androgen-independent prostatic cancer cells. To effect all the heterogeneous prostatic cancer cell populations within an individual cancer, effective chemotherapy, specifically targeted against the pre-existing androgen-independent cancer cell must be simultaneously combined with androgen

ablation to effect the androgen- dependent cells. The validity of each of these points has been demonstrated by a series of animal (15, 17-20) and human studies (21). The animal studies demonstrated that only by giving such a combined chemo-hormonal treatment, is it possible to produce any reproducible level of cures in animals bearing prostatic cancers (22). In order to produce cures however, treatment must be started early in the course of the disease, the chemotherapy must have definitive efficiency against androgen-independent cells, it must be given for a critical period, and it must be begun simultaneously with not sequential to androgen ablation. The cure rate however, even under these ideal conditions in animals are not high, i.e. 25% (22). While the concept of early combinational chemo-hormonal therapy for prostatic cancer is valid, for such an approach to be therapeutically effective in humans, a chemotherapeutic agent which can effectively control the growth of the preexisting androgen-independent prostatic cancer cells must be available. There are presently no highly effective chemotherapeutic agents which can control the growth of androgen-independent prostatic cancer cells (11).

New Approaches to Control the Growth of Androgen-Independent Prostatic Cancer Cells

The inability to control androgen-independent prostate cancer cells in human and rodent tumors by standard chemotherapeutic methods, has lead to a search for new approaches. Growth of a cancer is determined by the relationship between the rate of cell proliferation and the rate of cell death. Only when the rate of cell proliferation is greater than cell death does tumor growth continue. If the rate of cell proliferation is lower than the rate of cell death, then involution of the cancer occurs. Therefore a successful treatment of a cancer can be obtained by either lowering the rate of proliferation and/or by raising the rate of cell death so that the rate of cell proliferation is lower than the rate of cell death.

Most of the presently available chemotherapeutic agents are targeted at proliferating cancer cells. It is therefore not surprising that there is a good correlation in a large variety of cancers between the effectiveness of these agents and the respective cancer's rate of cell proliferation (23, 24). These previous studies have demonstrated that in cancers with high cell proliferation rates, chemotherapy can disturb the relationship between cell proliferation and death such that the rate of cancer cell death is now greater than production and involution of the cancer is induced, thus producing complete clinical responses. In contrast in cancers with a low cell proliferation rate, similar chemotherapy is unable to shift the relationship between proliferation and death into a negative balance thus producing, at best, only partial responses. The strong correlation between a high cell proliferation rate and chemotherapeutic sensitivity in human cancer is demonstrated from the studies of Tubiana and Malaise (24). These data demonstrate that in order for chemotherapy to be effective, not only the rate of cell proliferation, but also the rate of cell death, must be high.

For example, in an average embryonal cancer, approximately 44% of the cells are proliferating per day and 41% of the cells are dying per day (24). If this embryonal cancer is treated with a chemotherapeutic agent which produces a decrease in the cell proliferation rate of as little as 10% (i.e., changing it from 44 to 40% per day with no effect on the daily cell death rate) then the cancer will eventually involute totally. The host will be cured if this effect can be maintained since the rate of cell death, i.e., 41% per day, is now greater than the rate of cell proliferation, i.e., 40% per day. In contrast in a typical adenocarcinoma, approximately 2.9% of the cells are proliferating per day and 2% are dying per day (24). If this adenocarcinoma is treated with a chemotherapeutic agent which reduces the daily cell proliferation rate by a similar 10%, (i.e. changing it from 2.9 to 2.6% per day) with no effect on the daily cell death rate, then the cancer will not involute but will continue to grow since the rate of cell proliferation, i.e. 2.6% per day is still greater than the rate of cell death, i.e., 2% per day. These examples demonstrate, that if the daily cell death rate of a cancer is high enough, then anti-proliferative chemotherapy which induces even a small, (e.g. 10% reduction in daily cell proliferation rate) can produce tumor regression resulting in a high rate of complete durable response. In contrast, if the daily cell death rate is too low (e.g. slow growing cancers), then anti-proliferative chemotherapy must induce a much larger reduction in the daily cell proliferation rate. Unfortunately, the reduction in cell proliferation achievable at the maximum tolerated therapeutic intensity using presently available agents in slow growing cancers are not high enough to produce high complete response rates or durable complete responses (23).

These examples demonstrate that a sufficient death rate is critically important in allowing even small differences in cell proliferation rates induced by chemotherapy, to be clinically useful. While the exact magnitude of either the cell proliferation rate or cell death rate has not been precisely determined for many human prostatic cancers, available data on the thymidine labelling index, suggest that it has both a low cell proliferation rate and a low cell death rate (25,26). Successful treatment of slow growing prostatic cancers will probably require simultaneous anti-proliferative chemotherapy targeted at the small number of dividing

cancer cells and some type of additional therapy targeted at increasing the low cell death rate of the majority of androgen-independent cancer cells not proliferating within the prostatic cancer.

There are a large variety of effective anti-proliferative chemotherapy presently available which can lower the rate of cell proliferation without increasing the rate of cell death (i.e. cytostatic agents) or agents which lower the rate of cell proliferation and also increase the rate of cell death (i.e. cytotoxic agents). Unfortunately, the cytotoxic agent presently available only lead to death of cancer cells if they subsequently undergoes cell proliferation. Therefore cancer cells not in cycle (i.e. cell in G_0 of the cell cycle) at the time of exposure or not undergoing cell division soon enough after exposure to the cytotoxic chemotherapeutic agent can repair the damage induced by cytotoxic agent and are thus not killed by the therapy. Therefore what is needed is some type of cytotoxic therapy which lead to the death of cancer cells not requiring the cell to undergo proliferation in order to be killed.

Is it possible to induce the death of cells without requiring them to attempt to divide? The answer to this question is yes, as demonstrated by the rapid involution of the normal prostate following androgen ablation. Only $\approx 2\%$ of the cells in the normal adult prostate of intact males rats are undergoing cell proliferation on any day (27). Androgen ablation (i.e. castration) of the male rat leads to a decrease in cell proliferation and to an increase in the rate of cell death such that 20% of the cells present per day die within the prostate between 2-7 days following castration (27). By 7 days following castration $> 70\%$ of the total number of cells in the rat prostate have died (27). Thus the vast majority of prostatic cells which die following castration did not undergo cell proliferation (i.e. the cells are in G_0 when they die).

Programmed Cell Death in Normal Rat Prostate Following Androgen Ablation

Studies from a variety of investigators have demonstrated that the death of a cell can occur via one of two major pathways (28). The first type of cell death is termed necrotic cell death. Necrotic death is a response to pathological changes initiated outside of the cell and can be elicited by any of a large series of rather non specific factors which produce a hostile microenvironment for the cells (i.e. freezing and thawing, osmotic shock, ischemia, solubilizing agents, membranes ATPase inhibition, etc.). In necrotic cell death, the cell has a passive role in initiating the process of cell death (i.e. the cell is "murdered" by its hostile microenvironment). In addition to this necrotic type of cell death, there is a second type of cell death termed programmed cell death. In contrast to necrotic cell death which is a pathological process, programmed cell death is a physiological process whereby a cell is activated by specific signals to undergo an energy-dependent process of cell death (i.e. the cell is induced to commit suicide by specific signals in an otherwise normal microenvironment) (28, 29). Programmed cell death is a widespread phenomenon occurring normally at different stages of morphogenesis, growth and development of metazoans (30). It also occurs in adult tissues (30). Programmed cell death is initiated in specific cell types by tissue specific extra cellular agents, generally hormones or locally diffusing chemicals. The activation of this programmed cell death can occur either due to the positive presence of a tissue specific inducer [e.g. glucocorticoid induce death of small thymocytes (31)] or due to the negative lack of a tissue specific repressor [e.g. decrease in serum ACTH results in cell death in the zona reticularis of the adrenal (32)]. Once initiated, either by the positive presence of inducer or the negative lack of a repressor, programmed cell death leads to a cascade of biochemical and morphological events which results in the irreversible degradation of the genomic DNA and fragmentation of the cell (31, 33-36). The morphological pathway for programmed cell death is rather stereotypic and has been given the name apoptosis to distinguish this process from necrotic cell death.

Apoptosis was originally defined by Kerr et al (29) as the orderly and characteristic sequence of structured changes resulting in the programmed death of the cell. The temporal sequences of events of apoptosis comprise chromatin aggregation, the nuclear and cytoplasmic condensation, and the eventual fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies), which often contain morphological intact organelles. For example, in apoptosis (as opposed to necrotic death), mitochondria do not swell and lose their function as an early event in the process. Instead, functionally active mitochondria are often contained in apoptotic bodies. These apoptotic bodies are rapidly recognized, phagocytosed and digested by either macrophages or adjacent epithelial cells.

In an intact adult male, the supply of androgen is normally sufficient to maintain a balance between prostatic cell death and proliferation such that neither involution nor overgrowth of the gland occurs (27). Biochemical and morphological studies have demonstrated that the involution of the normal prostate following castration is not due to necrotic cell death but is an active process brought about by the initiation of a series of specific biochemical steps which lead to the program death (apoptosis) of the androgen-dependent glandular epithelial cells within the prostate (27, 37-44). In the androgen maintained ventral

prostate of an intact adult male rat, the rate of cell death is very low, approximately 2% per day and this low rate is balanced by an equally low rate of cell proliferation, also 2% per day (27). If animals are castrated, the serum testosterone levels drop to less than 10% of the intact control value within two hours (37). By 6 h post-castration the serum testosterone level is only 1.2% of intact control (37). By 12-24 h following castration the prostatic dihydrotestosterone (DHT) levels (i.e. the active intracellular androgen in prostatic cells) are only 5% of intact control values) (37). This lowering of prostatic DHT leads to changes in nuclear androgen receptor function (i.e. by 12 h after castration, androgen receptors are no longer retained in biochemically isolated ventral prostatic nuclei) (37). These nuclear receptor changes results in the synthesis of a series of proteins normally not present in the intact prostate (45, 46), due to the novel expression of genes normally repressed in the intact prostate. The most notable of these are the TRPM 2 gene [i.e. testosterone repressed prostate message-2] (47), the Ca^{+2} responsive *c-fos* gene (48), heat shock 70 kilodalton gene (48) and the *TGF β* gene (49).

Based upon *in vivo* data using calcium channel blockers (38, 50), these changes and/or others in gene expression probably result in an increase in the intracellular free Ca^{+2} levels. This increase in intracellular free Ca^{+2} would explain what activates the Ca^{+2} Mg^{+2} dependent endonuclease activity within the nucleus to begin to fragment the genomic DNA into low molecular weight [<1000 base pairs (bp)] nucleosomal oligomers which lack intranucleosomal break points and which contain non-degraded histones (37). This genomic DNA fragmentation process begins in some cells within the first day post-castration. Once the genomic DNA fragmentation process is initiated within an individual androgen-dependent prostatic cell, the cell fragments all of its genome into low molecular wt (i.e. <1000 BP) pieces (37).

The amount of the Ca^{+2} Mg^{+2} dependent endonuclease responsible for this DNA fragmentation increases 2-fold within the first day following castration and reaches its maximally increased 4-fold value on day 4 following castration (38). Using methods described by Orrenius' group (51, 52), isolated prostatic epithelial cell nucleus from intact male rats have been analysis for this Ca^{+2} Mg^{+2} dependent endonuclease and these studies demonstrated that the enzyme is not active at the physiological levels of intracellular free Ca^{+2} (i.e. 25 nM). Only when the free Ca^{+2} levels in the incubation medium are raised to above 150 nM and then only when at least 100 nM ATP is also present, is the genomic DNA in the isolated nuclei fragmented. Under these conditions, the DNA is fragmented into a nucleosomal ladder identical to that produced *in vivo* in the prostate following castration. These data support the presence of a ATP dependent Ca^{+2} uptake system within the nuclei of prostatic cells, as has been already demonstrated in rat liver cells (51, 52).

The glandular secretory epithelial cells constitute $\approx 80\%$, the basal epithelial cells $\approx 2\%$, and the stromal cells (i.e. fibroblasts, smooth muscle cells, endothelial cells) $\approx 18\%$ of the total cell number within the prostate of an intact adult male rat (39). Following castration, only the glandular epithelial cells die, there is no loss of any other cell type within the gland [i.e. no evidence of any necrotic or apoptotic death within any other cell type (39)]. This demonstrated that the androgen-dependent cells within the prostate are composed entirely of the glandular epithelial cells alone. Morphological analysis demonstrated that the death of these glandular cells is entirely due to apoptosis and not necrosis (39). Since the quantitations of the total Ca^{+2} Mg^{+2} -dependent endonuclease activity, TRPM 2 m-RNA expression, and DNA fragments are derived from biochemical assays of whole glands, it is possible that the demonstrated changes might not be induced by the glandular epithelial cells of the ventral prostate themselves, but instead be mediated by an influx of lymphocytes or other circulating cells into the prostate following castration. These cells might be summoned by a chemotactic mechanism to the effected glandular epithelial cells where they would act to degrade these cells by a process involving elevated endonuclease activity. Consequently, these infiltrating cell types were quantitated and the results demonstrated that the number of lymphocytes, mast cells and macrophages do not change appreciably during the earliest times post castration (i.e. ≤ 3 days) (39). This demonstrates that the observed elevations in total Ca^{+2} Mg^{+2} -dependent endonuclease activity, TRPM-2 m-RNA expression, and DNA fragmentation are initiated in the first days after castration within the existing prostatic glandular cells themselves and this occurs before any increase in infiltrating cells is observed.

While the process of DNA fragmentation is completed in a portion of the androgen-dependent glandular epithelial cells in the prostate as early as 1 day following castration, the first morphological signs of apoptotic bodies formation occurs during the second day following castration (37, 40, 43). This demonstrates that the fragmentation of the genomic DNA does not occur after the cells are dead but instead occurs as an irreversible commitment step for viable cells to die. During the next several days (i.e. day 2-7 following castration), the level of the Ca^{+2} - Mg^{+2} dependent endonuclease (37-39), TRPM 2 gene expression (47) *TGF- β* (49), and series a of other proteins (45) continues to increase with the maximal levels obtained on day 4 post castration. The fragmentation of the genomic DNA of the androgen-dependent glandular epithelial cells likewise continues, as does the production of apoptotic bodies (37-39) and the decrease in m-

RNA for the secretory proteins (53). By day 10 following castration, the androgen-dependent glandular epithelial cells have all died and there is no longer any indication of either DNA fragmentation, TRPM-2 expression, or apoptotic bodies.

These temporal studies demonstrate that DNA fragmentation is an important irreversible commitment step in the process of the programmed cell death of the androgen-dependent glandular epithelial cells in the prostate following castration. The size of these DNA fragments, as revealed by electrophoretic analysis are discrete multiples of approximately 180-bp subunit (i.e. a mononucleosome) (37). This ladder pattern of DNA degradation into nucleosomal oligomers (not detectable in the intact animal) is clearly evident 1 day after castration and is detectable during the subsequent 4 days, with the mononucleosome band progressively becoming more prominent. By day 4 after castration the mononucleosome band begins to smear, indicating complete digestion of these distinct fragments into component nucleotides. This suggests that the nucleosomal DNA is being cleaved at additional intranucleosomal sites, thus generating a heterogeneous population of even lower mol wt DNA fragments [$\sim$ 150bp] (37).

The *in vivo* DNA fragmentation occurring after castration is not a manifestation of hydrolytic degradation induced by the nonspecific release of lysosomal enzymes (i.e. proteases and nucleases) in cells already dead. This is demonstrated by the fact that the histones and nonhistone proteins extracted from prostate nuclei from rats castrated from 1, 2, 3, and 4 day are not degraded (37). This is in marked contrast with the extensive degradation of histone and nonhistone nuclear protein occurring even in prostatic tissue from intact rats following *in vitro* detergent induced lysis of lysosomal membranes (37). These latter studies also demonstrated that the DNA fragmentation pattern generated during the *in vitro* autolytic activation of lysosomal enzymes is not equivalent to the pattern produced during the *in vivo* fragmentation of DNA after castration [i.e. DNA degraded into a continuous size distribution of fragments with no induction of a discontinuous nucleosomal ladder] (37). These results demonstrate that the *in vivo* DNA fragmentation in the rat ventral prostate after castration is a nuclear specific, nuclease-induced event that irreversibly commits the cells to death, a process which precedes participation of lysosomal proteases and nucleases.

The DNA fragmentation observed in the rat ventral prostate following castration is not due to a change in the chromatin conformation increasing its sensitivity/accessibility to nucleases since the rate of *in vitro* DNA fragmentation by exogenous treatment of nuclei isolated from ventral prostates of animals castrated 4 days previously or intact rats with either DNase I or micrococcal nuclease is identical (38). The fact that castration induces an 2-4 fold increase in the Ca^{2+} - Mg^{2+} -dependent endonuclease raises the possibility that the enzyme may be inactive or highly suppressed in the ventral prostate of intact rats, due to a limited intranuclear availability of a suitable form of calcium. To investigate the relationship between perturbation of intracellular calcium homeostasis and prostatic cell death, ventral prostates of intact rats were implanted locally with pellets of the calcium ionophore, A23187. Unfortunately, these studies were severely hindered by the fact that local administration of the calcium ionophore in the ventral prostate consistently resulted in a dramatic enlargement (i.e. >5-fold) of the gland within 1 week due to non bacterial prostatitis (38). This effect is due to the ionophores ability to act as a chemotactic factor for lymphocytes.

The involvement of an increase in intracellular free calcium in castration-induced prostatic cell death was inferred, however, from studies in which rats were castrated and immediately implanted in the prostate with either a placebo or a time release pellet containing the calcium channel blocker, nifedipine. The temporal pattern of castration-induced prostatic involution is significantly slowed in nifedipine-treated compared to the placebo-treated castrated group. This nifedipine-induced delay in prostatic cell death is evident between days 3 and 7 post castration (38, 50). Histological examination of ventral prostates from intact rats revealed the characteristic pattern of tall columnar glandular epithelium and highly dilated lumen. By 3 days following castration, the columnar glandular epithelium is no longer maintained, the glandular cells are now cuboidal, and numerous apoptotic bodies are evident, either singly or in small groups. In contrast, prostates of 3 day castrated rats treated with nifedipine pellets demonstrated involuted cuboidal glandular epithelial cells, but the incidence of apoptotic bodies was distinctively reduced compared to the castrated placebo group (38, 50). In ventral prostates from rats castrated and given nifedipine, the degree of DNA fragmentation is also significantly decreased as compared to castrated-placebo group and this inhibition correlates well with the degree of inhibition obtained in prostatic weight and DNA loss by nifedipine (38).

Additional studies have demonstrated that it is possible to use prostatic organ culture to study the effects of increased Ca^{+2} levels on prostatic cell death *in vitro* under conditions in which neither drug toxicity to the host nor lymphocytic infiltration into the gland is a problem. To do this, adult male Copenhagen rats are castrated and the animals left untreated for two weeks to induce the involution of the ventral prostate.

After two weeks of involution, the castrated rats are implanted subcutaneously with a testosterone containing silastic implant which restores and maintains the serum testosterone level within the 1-2 ng/ml physiological range. After three days of androgen stimulation, a time when the rate of androgen-induced cell proliferation is maximal, the animals are killed and the ventral prostates removed. The glands are cut with a razor blade into small pieces of approximately 1 mm³ and ≈ 15 pieces per dish are placed in 30 mm organ culture dishes. The culture medium is Medium 199 with Earle's salts containing L-glutamine 120 mg/l, Penicillin 100,000 IU/l and Streptomycin, 25 mg/l which is equilibrated with 40% O₂, 5% CO₂, and 55% N₂ as the gas phase. The DNA of the proliferating cells in the explants are labelled with 0.5 μ l/ml ¹²⁵I-iododeoxyuridine for 1 day, and the labelling medium contains insulin (2 μ g/ml) and testosterone (10⁻⁷M), a combination which yields the maximal rate of *in vitro* DNA synthesis. After ¹²⁵I-DNA labelling, the explants were washed with cold medium and cultured further for 24 h in the presence of testosterone, insulin and corticosterone (10⁻⁷M), to wash out any unincorporated free ¹²⁵I-iododeoxyuridine radioactivity. After this washing, the starting radioactivity in each dish is individually recorded. For this purpose, a special gamma counter is used which is equipped with a well-type detector able to count ¹²⁵I in individual 30 mm culture dishes. Since >85% of this starting ¹²⁵I-radioactivity is incorporated into the DNA of the glandular epithelial cells within the organ culture, the starting radioactivity can be used as an index of the number of glandular epithelial cells alive at the start of the test period. After this initial counting, the dishes are randomized to contain either culture media with or without 10⁻⁷M testosterone. Once the labelled dishes are randomized, the appropriate media is changed every two days with the total radioactivity per dish recorded after each media change. This is continued for a period of 2 weeks. The dpm per each dish at each time period in culture is divided by the starting dpm per dish at time zero to determine the percentage of starting cell still alive at the particular time points (i.e. equivalent to the percentage of starting radioactivity still present at the particular time). The log of percentage of starting radioactivity present *versus* the time of incubation is then plotted and a linear regression of data performed to determine the slope of this plot. This slope is then multiplied by -2.3, to calculate the average percentage of cell dying per day in the particular dish. Using this method, it has been demonstrated that ventral prostatic glandular epithelial cells can be maintained in G₀ organ culture for a period of up to 14 days with a low rate of cell death (i.e. $\approx 5\%$ per day) if androgen is included in the media. If androgen is not included in the media, the rate of glandular cell death increases to $\approx 12\%$ per day.

Using this organ culture system, it has been demonstrated that rate of programmed death of the glandular epithelial cells can be shifted from 5% to $\approx 12\%$ of the cells dying per day when testosterone plus 10 μ M of the Ca ionophore, A23187 are both in the media. Thus, in the presence of the ionophore, the rate of cell death in the presence of testosterone is identical to that induced when testosterone is not present. Additional studies have demonstrated that if the organ cultures are maintained in media lacking testosterone, but containing 10 μ M of the Ca⁺² channel blocks, nifedipine, the rise in the rate of cell death from 5% to 12% of the cells dying per day usually induced can be totally prevented (i.e. in the presence of nifedipine the rate is also 5%). These results suggest that increases in intracellular free Ca⁺² probably derived from extracellular Ca⁺² pools, are a critical early event involved in triggering the subsequent process of programmed cell death (i.e., specifically DNA fragmentation) in the rat ventral prostate following androgen ablation.

Regardless of the specific mechanism, once a prostate cell fragments its genomic DNA into such small pieces, the DNA is no longer functional for cell replication or gene expression, as evidenced by the fact that the m-RNA levels for the major secretory proteins decrease abruptly (53) and thus the cell is terminally committed to death. The terminal process of prostatic cell death following castration is not known with certainty. Based upon data in other systems in which programmed cell death has been studied (54-57) particular glucocorticoid induced programmed cell death of thymocytes (57), this may involve activation of the poly (ADP-ribose) synthetase pathway within the nucleus of the prostatic cells. In these other cell death systems, this nuclear enzyme is specifically activated by the fragmentation of the DNA (54-57) and its chronic activation results in the enzymatic depletion of the nucleotide co-factor NAD⁺. Due to the unique role of NAD⁺ in both glycolysis and oxidative phosphorylation, depletion of NAD⁺ results in the eventual depletion of ATP. It is this rapid depletion of NAD⁺ and ATP induced by increased poly ADP-ribosylation which is believed to be the actual cause of the rapid demise of the cell (54-57).

Following androgen ablation, not only is there an activation of programmed cell death, but also a decrease in cell proliferation occurs (27). Since it has been demonstrated that transforming growth factor- β (TGF- β) is a potent inhibitor of cell proliferation of both normal and malignant prostatic epithelial cells, (58), the expression of transforming growth factor β in the rat ventral prostate was studied following castration (49). Steady state levels of TGF- β mRNA were determined by Northern blot analysis and compared with mRNA levels for prostatein C₃ (53) the major androgen-dependent secretory protein of ventral pros-

tate and also with mRNA levels for TRPM-2, a gene that is specifically expressed during castration induced prostatic cell death (47). The transcript levels encoding TRPM-2 initially increased 10-fold within the first day after castration and by day 4 post castration there is a dramatic increase (approx. 50-fold) which correlated well with the maximal rate of cell death of the androgen-dependent prostatic glandular epithelial cells. Within the first day following castration there is a dramatic increase in the levels of TGF- β mRNA in the ventral prostate (approx. 10-fold) and by 4 days post castration (49), TGF- β mRNA is maximally expressed (approx. 40-fold increase), by which time the androgen-dependent C₃ secretory protein mRNA transcripts diminish to undetectable levels. Androgen administration to 4-day castrated rats leads to a marked decrease in TGF- β mRNA to a level comparable to its constitutive expression obtained in the intact control animals, indicating that expression of TGF- β in the rat ventral prostate is under negative androgenic regulation (49). Scatchard analyses of the binding of TGF- β to membranes from rat ventral prostate reveals the presence of high affinity (K_d = 140 pM) saturable binding sites for [¹²⁵I] TGF- β to prostatic membranes, which are displaced in the presence of excess unlabeled TGF- β , but are, unaffected by epidermal growth factor, nerve growth factor, fibroblast growth factor, or insulin, indicating the specificity of binding (59). Castration results in a significant increase in the total [¹²⁵I] TGF- β binding per total prostate, with no apparent change in the affinity of membrane receptors for TGF- β . These studies demonstrate that additional TGF- β receptor are synthesized during the first 4 days following castration since the total number of receptors per gland increases 2-3 fold even though the total number of cells per gland decreases 1/3 during this time period (59). Additional studies demonstrates that direct exposure of the ventral prostate to exogenous TGF- β delivered via osmotic minipumps in intact male rats produces $\approx$ 20% reduction in prostatic cell number (47). These results suggest that TGF- β is not a major factor involved in the induction of cell death following castration. The elevated TGF- β expression, however, may well be a major mechanism for the inhibition of cell proliferation observed following castration.

Programmed Cell Death of Human Prostatic Cancer Cell Following Androgen Ablation

Recent completed studies have demonstrated that not only normal rat prostatic cells but also human androgen-responsive prostatic cancer cells activate the pathway of programmed cell death following androgen ablation. The PC-82 human prostatic cancer is highly androgen-responsive when grown as a xenograft in nude mice (60). If intact male nude mice are inoculated with human PC-82 prostatic cancer, continuously growing tumors are produced. If the host mice is castrated when the PC-82 tumor is $\approx$ 0.5cc in size the rate of cell proliferation decrease $\approx$ 7-fold from 3.5% of the cells proliferating per day to 0.5% of the cells proliferating per day and the rate of cell death increases $\approx$ 11-fold from 0.5% of the cell dying per day to 4.7% of the cells dying per day. Due to these changes the tumor involutes rapidly following castration reaching $\approx$ 1/2 of its starting size within three weeks of castration. Biochemical analysis during this involution period has demonstrated that TRPM-2 and TGF- β m-RNA levels and DNA fragmentation into nucleosomal size pieces are detectably increased within the first day following castration. The levels of all of these parameter increase to a maximum on day three following castration. In addition, if exogenous androgen is given back to the castrated host, DNA fragmentation ceases, TRPM-2 and TGF β levels drop, involution stops and growth of the tumor resumes.

Programmed Cell Death in Androgen-Independent Prostatic Cancer Cells

While androgen-independent prostatic cancer cells do not activate the program of cell death following androgen ablation, these cells still retain the major portion of the program cell death pathway. This has been demonstrated using a series of Dunning R-3327 androgen-independent prostatic cancers established as continuously growing *in vitro* cell lines. For example, the Dunning AT-3 androgen-independent, highly metastatic, anaplastic prostatic cancer cells have been treated *in vitro* with a variety of non-androgen ablative agents which induce "thymine-less death" of the cells [e.g. cells treated with 5-fluoro-deoxyuridine (5-Fdur) or trifluorothymidine (TFT)]. Analysis has revealed that "thymine-less death" results in an increase in the expression of the TRPM-2 gene and an increase in the nuclear Ca²⁺Mg²⁺ dependent endonuclease with the resultant fragmentation of the genomic DNA of the AT-3 cells into a similar nucleosomal ladder, as seen in the death of androgen-dependent prostate cells following castration (61). This cascade of events requires 6-12 hrs before fragmentation of the DNA is complete. The AT-3 cells are not "dead", as defined by their ability to metabolize a mitochondrial vital dye (MTT), however until 24 hrs of treatment. This demonstrates that the fragmentation of the genomic DNA is an early, irreversible, commitment step in the of programmed cell death of even androgen-independent prostatic cancer cells (61). If the AT-3 cells are treated with osmotic shock induced by exposure to distilled water or agents which inhibit the plasma membrane ATPase activity (i.e. ouabain or iodoacetate), the cells rapidly lyse in less than 3 hours after treatment and do not metabolize the MTT vital dye (i.e. they are dead) even though they do not fragment their DNA into nucleosomal size pieces nor does they elevate TRPM-2 mRNA levels (61). This

data demonstrates that agents which induce necrotic death of the AT-3 cells (i.e. osmotic effects) do not lead to the activation of the programmed cell death of these cells. Programmed cell death can be activated, however, even in androgen-independent prostatic cancers by specific agents (e.g. those able to induce a "thymine-less" state).

The problem with agents of this latter type, however, is that cell proliferation is required for the "thymine-less" state to activate the program of cell death in these AT-3 cells. Therefore some type of agent which can likewise activate this death program in androgen-independent prostatic cancer cells not in the cell cycle and not requiring the cell to attempt to proliferate, still must be identified. The effect of TGF β on AT-3 cells *in vitro* have also been studied (58). At a concentration of 0.1 ng/ml, TGF- β has a profound inhibitory effect upon the growth of AT-3 cells in culture (i.e. >80% reduction in growth rate). TGF- β , even at a concentration of 20 ng/ml, however, does not induce death of the AT-3 (i.e. TGF β is cytostatic not cytotoxic to these cells).

If AT-3 cells are treated *in vitro* with 10 μ M of either the calcium ionophores, A23187 or ionomycin, cell death can be induced. Using microfluorescence image analysis (62) on AT-3 cells loaded with the fluorescent dye "fura-2" to measure intracellular free Ca⁺² level such ionomycin treatment has been demonstrated to elevate the intracellular free Ca⁺² levels from < 80 nM to > 200nM within the first minute of treatment. After the first few minutes, the intracellular free Ca⁺² returned to $\approx$ 50-100nM. Such sustained elevations in intracellular free Ca⁺² results in cell proliferation stopping within hours of treatment and then the cells begin to die after $\approx$ 48-72 hours. Biochemical analysis during this time course demonstrated that DNA fragmentation into nucleosomal oligomers begins as early as 6 hours after Ca⁺² ionophores treatment. Interesting, there is no elevation of the TRPM-2 mRNA levels during the chronic elevation of intracellular free Ca⁺² induced by ionophore. This may be significant since such chronic elevation in free Ca⁺² levels does activate the DNA fragmentation suggesting that TRPM-2 elevation is not required for this step in the process of cell death. These results suggest that TRPM-2's site of action in the cell death may be involved in inducing an increase in intracellular free Ca⁺², which the ionophores is fully capable of doing without any TRPM-2 involvement. This possibility is further strengthened by the recent clarification that TRPM-2 is highly related if not identical to the previously identified sulfated glycoprotein 2 normally secreted by rat sertoli cells (63). This SGP2 protein has been demonstrated to be secreted by sertoli cells and to bind to the acrosomal membrane of sperm (64). This may be significant since sperm in order to undergo the capacitation reaction must proceed through an acrosomal reaction step which involves the breakdown of the acrosomal membrane, a process which is known to involve Ca⁺².

CONCLUSIONS

To increase survival for men with metastatic prostatic cancer what is desperately needed is a modality which can effectively eliminate the clones of androgen-independent cancer cells already present even before therapy is begun within individual heterogeneous prostate cancers. By combining such an effective modality with any of the various types of androgen ablation presently available, all of the populations of tumor cells within individual heterogeneous prostatic cancer can be affected thus optimize the possibility for cure. Unfortunately, such an effective form of therapy for the androgen-independent prostatic cancer cell is not presently available. Effective chemotherapy for the androgen-independent prostatic cancer cell will probably require two types of agents; one having anti-proliferative activity affecting the small number of dividing androgen-independent cells, and the other able to increase the low rate of cell death among the majority of non-proliferating androgen-independent prostatic cancer cells present. Androgen-dependent prostatic epithelial cells can be made to undergo programmed death, even if the cells are not in the cell cycle (i.e. G₀ cells), simply by means of androgen ablation. Androgen-independent prostatic cancer cells retain the major portion of this programmed cell death pathway, only there is a defect in the pathway such that it is no longer activated by androgen ablation. The long term goal, therefore, is to develop some type of non-androgen ablative method to activate this programmed cell death cascade in androgen-independent prostatic cancer cells distal to the point of the defect.

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DISCUSSION

Question from the floor:

When you see this calcium magnesium dependent nucleus working, and when there are changes in calcium levels, why don't you see the nucleus being activated at different times?

Dr. John Isaacs:

The reason you don't is that it requires a sustained elevation in calcium. In other words, you have to have a sustained (i.e. multiple hours) increase in nuclear free calcium. Dr. Arenius and others have suggested that there is a calcium pump on the nuclear membrane which is ATP dependent, and that elevations that occur and are terminated in one or two minutes do not lead to chronic elevation in the calcium concentration in the nucleus. So, it seems that you have to have chronic elevation in the nucleus. I think that's what prevents activation normally.

Question from the floor:

So, it's more than just a transient increase that they usually see in cell signalling. It's more of a sustained higher level of calcium.

Dr. John Isaacs:

Yes. Well, it's not so high. That's the interesting point. It turns out that in the cell, the intracellular free calcium is between 20-40 nanomolar. All it takes is an elevation of about 120-140 nanomolar in the cytosol, because this can lead to an increase in the nucleus of between 400-600 nanomolar. You have to get above several hundred nanomolar in the nucleus to activate this enzyme.

Dr. Lamb:

Is the TRPM 2 protein the one that is highly expressed in Sertoli cells? Is that the same?

Dr. John Isaacs:

The TRPM gene from the prostate has been sequenced, and it has high similarity to the sulphate glycoprotein 2 which is produced by Sertoli cells. My reading of the literature is that SGP-2 and TRPM-2 are probably related proteins. They may not be the identical protein.

Dr. Bilhartz:

In these animal models, have you noticed any difference in the actual clinical response rate or survival of the animals after endocrine therapy for the tumor, similar to prostate cancer after the patients after they have been on Nifedapine or other calcium channel blockers?

Dr. John Isaacs:

What we tried to do in our studies was to give the Nifedipine in an indwelling pellet which we actually placed right in the prostate to achieve high concentrations. We have had very little luck trying to give Nifedipine systemically without producing major toxicity. You're talking about trying to give a calcium ionophore, but the ionophores are chemotoxic, and they produce major problems. Nifedipine should block cell death.

Dr. Bilhartz:

That's why I'm asking, because so many of our patients that we treat with endocrine therapy are on Nifedapine or other calcium channel blockers, and I'm wondering if maybe we should change them to a different medication if there were any clinical indications that this was going to slow cell death.

Dr. Buttyan:

I would like to comment on this question. Regulation of the intracellular calcium ion levels is a critically important mechanism to control many cellular responses. Intracellular calcium ion levels are increased during cell proliferation, and, as discussed by Dr. Isaacs, during cell death, and also during other forms of intense stimulus-response. Similar to Dr. Isaacs, we have also reported that calcium channel blockers can delay prostate cell death, and in experiments currently in progress, we have some evidence that these same drugs can slow proliferation of androgen-dependent tumor cells as well as slow their death. Since calcium channel antagonists might be able to slow down both of these processes, growth and death, this would suggest that the effects of these drugs on the normal prostate gland may balance out and not show any clinically relevant change.

Dr. Thompson:

Is it possible to do cyclohexamide experiments in any of your systems? In other words, is protein synthesis required for apoptosis?

Dr. John Isaacs:

It is possible. Using the thymocyte system, you can give dexamethazone and induce apoptosis and DNA fragmentation within 6-8 hours. In the system we're looking at, even with the ionophore, the cells take 18-24 hours to undergo profound cell death. They begin to fragment their DNA, but they do not die for a period of about 24 hours or more. So, you begin to have problems, such as too general a protein synthesis inhibition.

Dr. Miller:

In the first part of your talk, it seemed as though you were saying that G0 cells were the cells you expect to go into the apoptotic compartment. Later, however, it seemed as though you were saying that perhaps cells go directly out of the cycling cell cycle into apoptosis. The reason I'm asking is that if that's what you were really saying, I wouldn't expect that all of the cells that sluff off after castration are actually in G0. If you were to stain prostate epithelium with PCNA, I think you would find a lot of those cells are still in the cell cycle. They are not necessarily G0 cells.

Dr. John Isaacs:

In which cells?

Dr. Miller:

The mature epithelial cells in the rat.

Dr. John Isaacs:

We spent considerable time quantitating the number of cells in the cell cycle. Only about two percent per day are dying and 2% are proliferating.

Dr. Miller:

By what method?

Dr. John Isaacs:

By using labelling techniques.

Dr. Miller:

But labelling is only going to show you the S phase cells.

Dr. John Isaacs:

The percent takes into consideration that the S phase is some fraction of the total cell cycle. We're adjusting for the total cell cycle time. We've done other things to look how long S is versus G2 and G1. Those calculations aren't based on the percent just in S. Following castration about fifty percent of prostate cells die within a week. If you multiply two percent cell proliferation over that period, only 10 percent of cells are dying in cycle.

Dr. Miller:

Right. That was really the question. You answered it the opposite direction. The question is: Do cells, other than G0 cells, enter apoptosis?

Dr. John Isaacs:

Yes. We've done it in renal cancer cells, L cells and in other systems which are actively proliferating. The one thing that is different though, is that in those proliferative systems, calcium may not be increased. In other words, because the cells are in S, they seem to be able to fragment their DNA without having to have a measurable increase in free calcium.

Question from the floor:

It seems possible that following castration in the rat prostate, that there could be alterations in membrane calcium pumps. Is there any indication that other alterations might be occurring?

Dr. John Isaacs:

We are attempting right now, working with Bob Tucker, to take cells that are isolated directly from intact animals versus several hours following castration. The first 24 hours is the most interesting window to look at following castration. So, we are trying to isolate those cells and then do the determination of the intracellular free calcium. The problem is that intracellular free calcium determinations require the cells (it works beautifully on tissue culture cells). So, we need a cell culture system for these studies.

CELL MOTILITY AND STRUCTURAL HARMONICS IN PROSTATE CANCER

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INTRODUCTION

Cancer cells exhibit a wide variation in function (pleiotropism) that is accompanied by large variations in structure (pleiomorphism). This variation of structure may be a driving force in the development of genetic instability (1) as well as in the alteration of timing mechanisms within tumor cells. A wide variety of harmonic and periodic events occur within cells. The vibrational or oscillatory properties of these events or elements within the cell may have potential regulatory importance and may be related to biologic clocks or timing mechanisms. Cell events such as signal transduction and motility occur within spatial and temporal constraints and have complex harmonics that can be identified and analyzed (2-4). A cell matrix system has recently been identified which extends throughout the cell from the cytoskeleton, to the nuclear matrix and finally to DNA. The cell matrix has dynamic and structural properties and could provide a direct mechanical mechanism for linking cell signals. Therefore, the dynamic cell matrix system is an important part of an integrated cellular information transfer system which couples oscillating elements within the cell both spatially and temporally. The cell matrix system allows a cell to sense, process, and act on information and signals from the peripheral environment. These coupled interactions are accomplished through harmonics transmitted directly through a chemomechanical model of tensegrity that will be described in more detail in this article.

The cancer cell is identified by alteration in cell structure. Cell harmonics are altered when structure changes within a cancer cell and this change in coupled information transfer may be central to many aspects of tumor biology.

Cell Motility

In 1940, George Gey was the first to use time-lapse cinematography to show living cells in motion (5). In 1966, Wood *et al.*, using a transparent chamber in the rabbit ear, demonstrated that leukocytes, macrophages, and J-2 carcinoma cells were all highly motile (6). Since that time, several studies have demonstrated the motility of cells *in vivo* as well as *in vitro* (7-12). Fulton recognized three types of cellular motion; swimming due to flagella and cilia, ameboid motion by pseudopodal extension, and bleb formation and ruffling (13). These different motions allow the cell to perform different functions and indicate the dynamic processes which underlie structure. Partin *et al.* (2) have developed a system for quantitating all aspects of cell motility by Fourier analysis that defines the harmonics of cell motion. The subtypes of cell motility can be captured with time-lapse video recordings. Motility mimics wave-form motions that may be analyzed by mathematical techniques (Fig. 1). The complex shape of serial cell contours can then be decomposed into spatial harmonics and analyzed via Fourier analysis. Fourier analysis takes advantage of the fact that shapes can be described as wave-forms which can in turn be described as sinusoidal frequencies. Partin *et al.* (2) demonstrated that changes in cell harmonics and motility correlate with metastatic ability (Fig. 2). The quantitative analysis of Partin *et al.* demonstrated the dynamic motion of the cells in space as well as time and prompted us to look for other types of harmonic motion within the cell.

Biological Oscillations within the Cell

The dynamic nature of the intracellular milieu has been demonstrated on several levels. Molecules, and the atoms which compose them, oscillate according to the total energy of the system in which they reside. DNA sequences oscillate according to their base pair composition (14, 15) (Table 1). Structures containing G-C pairs vibrate at a higher frequency than those containing A-T pairs. Sequence-defined vibrations of DNA may determine the properties of DNA such as protein binding and template function. In this regard, Rudolph and Case (15) have demonstrated that drug binding to the DNA strand is partially dependent on the vibrational frequency of the DNA.

Proteins vibrate in the range of 10^{12} - 10^{15} per second (16-18). These oscillations produce an average structure which can be observed by x-ray crystallography. Karplus and McCammon (18) have demonstrated the physiologic significance of these oscillations by revealing that the vibrations of the myoglobin molecule allows for transport of oxygen molecules in and out of the protein. No single rigid protein structure allows for the movement of oxygen on the observed time scale. It is also believed that protein structural vibrations are important components of enzyme active sites (17, 18).

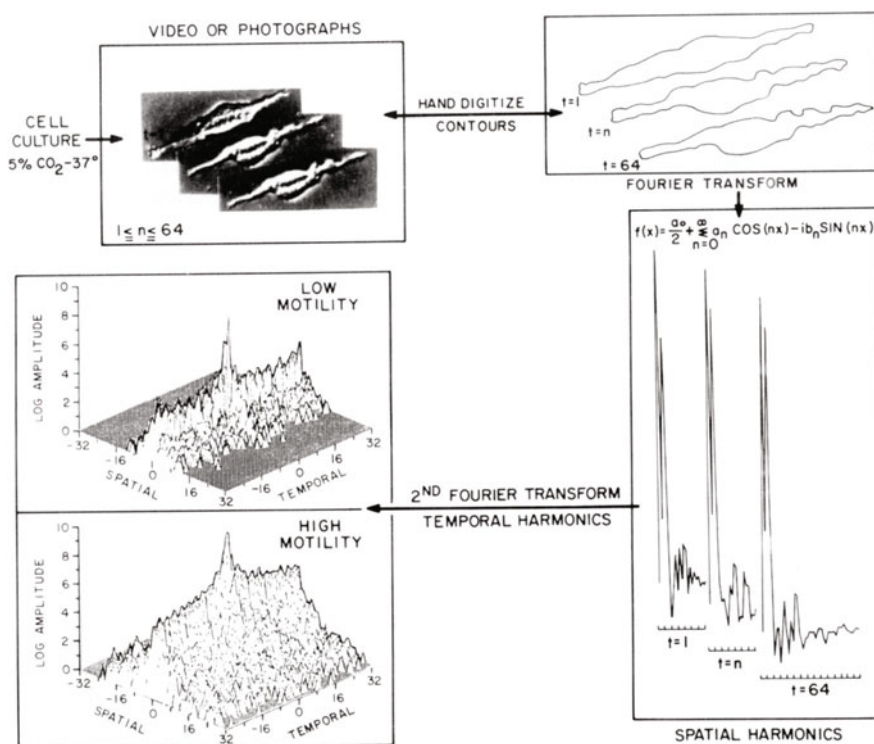


Figure 1: Fourier measurement of the motility of single cells. Cells were digitized at 60-sec intervals for 64 min. Cell contours were manually traced from the digital images with a digitizer tablet. The coordinates were then transformed with a complex fast Fourier transform to determine the spatial Fourier coefficients describing cell shape. The spatial Fourier coefficients were combined into a matrix and a second temporal fast Fourier transform measured the temporal fluctuations in the amplitude of the spatial harmonics. This is graphically depicted in a two-dimensional plot of the spatial-temporal Fourier analysis of the motility of a single cell. For graphical representation, the log of the amplitudes of the Fourier motility coefficients are shown. The results of analysis of a highly motile cell are compared to that of a low-motility cell. [From Partin *et al.*, (2)].

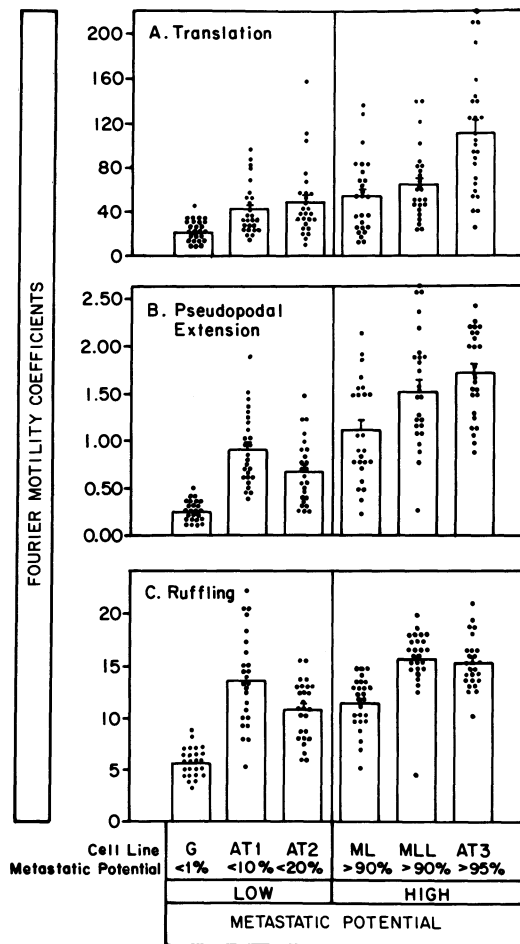


Figure 2: Comparison of Fourier motility coefficients for six Dunning tumor cell lines with various metastatic potential. Twenty-six cells from each of 6 Dunning cell lines were analyzed with the spatial-temporal Fourier method described in Fig. 1. Fourier motility coefficients were determined for translation (A), pseudopodal extension (B), and ruffling (C). Metastatic potential is arbitrarily defined as low metastatic when <20% of rats develop distant metastases and high metastatic when >90% develop distant metastases. Bars represent the mean $\pm$ SEM for 26 cells from each cell line. [From Partin *et al.* (2)].

Ions oscillating between intracellular or intercellular compartments has been demonstrated in several systems. The oscillation of calcium across the cell membrane in Purkinje's cells causes the heart to beat (19). Calcium oscillates and forms wave motions of concentration between intracellular compartments and the cytosol at a rate of 0.5-3 cycles per minute (20-23). Potassium has also been shown to oscillate across intracellular compartments.

In cells we have measured the dynamics and periodicity of structural wave motions including membrane blebbing, membrane ruffling, and pseudopodal extension using the rat prostate Dunning adenocarcinoma line. Membrane blebbing occurs at a rate of 4-6 cycles per minute; ruffling occurs approximately every 6 minutes; pseudopodal extension appears to occur in oscillations of 30, 60, and 120 minutes.

Table 1: Biological Oscillations

Element	Period	Reference
DNA		
-G-C b.p.	3.3×10^{-12} sec	(14, 15)
-A-T b.p.	2.8×10^{-12} sec	(14, 15)
Strand	5×10^{-11} sec	(14, 15)
PROTEIN	1×10^{-12} sec	(16, 18)
IONS	.4-100 sec	(19-23)
MEMBRANES		
Blebs	6-10 sec	
Ruffles	6-15 min	
Pseudopods	30-120 min	
CELL CYCLES	18-24 hours	(24-26)

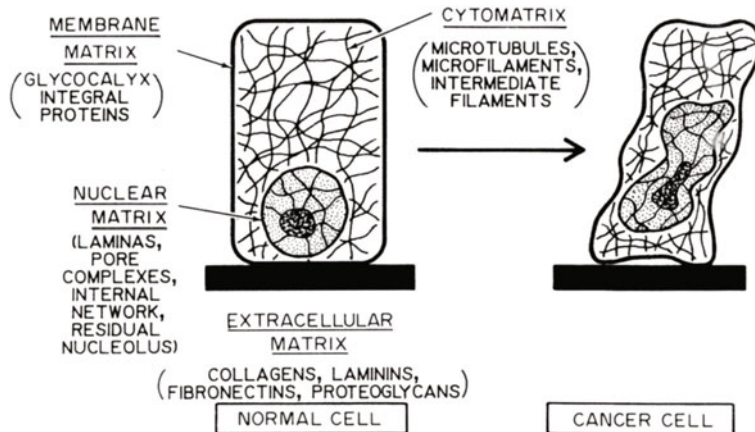


Figure 3: The tissue matrix system. The shape of the cell is dependent on dynamic interactions of its structural components. The nuclear matrix is connected to the cytomatrix. The cytomatrix in turn is attached to the extracellular matrix via the membrane matrix. Virtually every subcomponent of this matrix system has been shown to be altered in the cancer cell. [From Pienta *et al.* (1)].

The mechanisms involved in the control of the periodicity of the mitotic cycle have been partially elucidated. Oscillations in maturation promoting factor and its subcomponents of cyclin and CDC-2 kinase play a critical role in the timing of events within the mitotic cycle (24-26). Elements within the nucleus and cytoplasm appear to be coupled in a coordinated fashion between a nuclear cycle and cytoplasmic cycle. How this coupling occurs is still largely unknown. By tying these events together directly through structure, the cell matrix system is poised to fill this role as the coupling mechanism.

The Cell Matrix System

Skeletal components exist at every level of the cell. The cell matrix system (Fig. 2) consists of the nuclear matrix, the cytoskeleton, and the extracellular matrix (ECM). Fey *et al.* (27) have provided 3-dimensional electron micrographs of the cell matrix system that show the ECM is contiguous with the intermediate filaments of the cytoskeleton which in turn are contiguous with the nuclear matrix. These three components interact to form a cell matrix system. The cell matrix system can be defined as the dynamic structural subcomponents of the cell which interact to organize and process spatial and temporal information to coordinate cell functions.

The nuclear matrix is defined as the dynamic structural subcomponent of the nucleus that directs the 3-dimensional organization of DNA into loop domains and provides organizing sites for the specific control of nucleic acid function and intranuclear and particulate transport (28, 29). It consists of peripheral lamins and pore complexes, an internal ribonucleic protein network, and residual nucleoli. The nuclear matrix plays a pivotal role in many nuclear events including morphology, DNA organization, DNA replication, RNA synthesis and processing, and gene regulation. As such, it is ultimately involved in almost every aspect of cell structure and function. It is probable that the nuclear matrix controls not only production of genetic messages, but also directs them towards their ultimate destination within the cell.

The cytoskeleton is a cytoplasmic matrix system composed of interacting networks of actin microfilaments, intermediate filaments, and microtubules (30, 31). The dynamic nature of microfilaments and microtubules has been well documented (13, 32). The intermediate filaments are a heterogeneous group of proteins which appear to be tissue specific (33, 34) as well as taking part in bridging functions between cytoskeletal compartments, as well as between the cytoskeleton and ECM and cytoskeleton and nuclear matrix.

The ECM is composed of a diverse group of proteins which form an organized network between cells (35, 36). The proteins of the ECM bind to the cytoskeleton through specific receptors called integrins and cell adhesion molecules (CAMs) (37). The ECM has been shown to function in controlling cell shape (38, 39), embryonic differentiation (40, 41), and gene expression (42).

There has been much discussion within cell biology about the various role each skeletal or matrix component plays in cell function (28, 33, 35, 43). It is now clear that these structures must be viewed as a coupled integrated cell matrix system that may be altered in cancer cells. The cell matrix system directs cell structure and function in a dynamic fashion through the concept of tensegrity.

Tensegrity

The term tensegrity was coined by R. Buckminster Fuller in 1948 from the words *tensional integrity*. Fuller defined tensegrity as a structural system composed of discontinuous compression elements connected by continuous tension cables, which continually interact in a dynamic fashion. The human body can be viewed as such a system with the bones acting as compression elements and muscles, tendons, and ligaments acting as tension cables. Ingber and colleagues (44, 45) have first applied these principals to cell architecture by demonstrating that cell shape, and therefore cell function and regulation, are under the control of an interacting tension and compression system. Some cytoskeleton components such as actin are tension cables while others like microtubules may serve as compression elements; together they form a tensegrity structure. Indeed, Dennerll *et al.* (46) have measured a tensegrity system operating in neurites with microtubules as compression elements and actin microfilaments as the tension cables. Fuller points out the power of a tensegrity structure to interact with its environment. "If you just tauten one point in a tensegrity system, all the other parts of it tighten evenly. If you twang any tension member anywhere in the structure, it will give the same resonant note as the others. If you tauten any one part, the tuning goes to a higher note everywhere in the structure. Until its tension is altered, each tensegrity structure, as with every chemical element, has its own unique frequency" (47). Tensegrity

allows for a dynamic skeleton which can not only sense its environment and individual subcomponents, but act on the information it gathers. To explain this gap between input of data and output of action, one must hypothesize that a tensegrity system can transfer information through the vibration of the structure.

Harmonic Information Transfer

The tensegrity cell matrix system allows for the direct transfer of vibrational energy through harmonic wave motions. Wave propagation along a tensor can pass information through the amplitude and frequency of the wave propagating along it. This energy can be tuned by placing compressive elements along the tension cable, much as a guitar or violin string is tuned to different notes. Mathematical formulae have been developed to explain how complex harmonic waves can be deciphered into their primary frequencies and periods and thus interpreted. Fourier transform equations (48) break complex wave functions into simple wave functions which hold the information of individual waves via their amplitude and phase. Hartley developed a separate theory of information transfer which demonstrates that the amount of information that a tensor can pass is equal to the width of the frequency band of the waves of information and the total time they are available for interpretation (49). By providing a branching network throughout the cell the cell matrix is set up both in series and parallel to allow the quick and directed flow of information utilizing these wave form systems.

Biological Clocks

A review by E. Bunning on the master biological clock stresses the need for special properties to account for the vast and varied experimental data accumulated on clock systems. Bunning summarized the mechanistic requirements in his statement: "...the clock operates by an alteration of tension and relaxation, that is by relaxation oscillations" (50). It is still unknown whether the periodicity and harmonics of these tensegrity cell matrix systems are involved in temporal events such as cell cycles and biological clocks. However, by utilizing the principles of tensegrity, the cell matrix is poised to fulfill the function of the master biological clock by providing a coupled harmonic oscillator that regulates periodicity within the system.

CONCLUSIONS

Life processes, in their simplest form, may be viewed as a series of coupled chemomechanical reactions. The ECM, cytoskeleton, and nuclear matrix provide a structural system to coordinate and mechanize the chemical reactions of the cell. The tensegrity cell matrix system allows for specific coupling of the nucleus and cytoplasm with the environment through harmonics. The dynamic nature of the cell matrix allows it to sense, transfer, and act on intracellular and extracellular events. Disruptions of the cell matrix system in cancer cells may help to explain genetic instability and tumor cell heterogeneity, two of the hallmarks of tumor biology. All carcinogenic processes, chemical, viral, irradiation, and spontaneous, change cell shape and pathologists view this as morphological transformation. These carcinogenesis processes uncouple or detune the interlocking matrix systems, changing the structural harmonics and motility of the normal stable cells into motile, unstable cancer cells.

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PANEL DISCUSSION

Intracellular Communications

Panelists: Robert Getzenberg, Tomohiko Ichikawa, Dolores Lamb, Edward Sherwood, and Donald Tindall

Dr. Barrack:

Dr. Smith, did you say that the DDT1 cells are fibroblast-like and the R3327 cells are epithelial-like. Is that correct?

Dr. Smith:

That's correct.

Dr. Barrack:

Were all of the effects that you described for the DDT1 cells? Would the story be any different if you were talking about control of epithelial cells compared to the control of fibroblast cells?

Dr. Smith:

Possibly so. What I can tell you about the G8A1 cells, that I mentioned very briefly, is that growth control is mediated through a different pathway. You can overcome glucocorticoid inhibition with a phorbol ester such as TPA. However, cyclical AMP will further inhibit the growth of this epithelial-like tumor cell. In contrast, cAMP will stimulate the growth of DDT1 cells and TPA is inhibitory. So, you've got inverse growth control mechanisms occurring. The interesting thing is that glucocorticoids inhibit both cell types. So, by looking at the glucocorticoid regulation of one cell type versus the other, you may be able to find a common downstream link for the control of growth of both types of cells.

Dr. Barrack:

Do you think that the difference is because one is stroma-like and one is epithelial-like?

Dr. Smith:

I would like to think so, but I don't know.

Dr. Barrack:

Are there any other studies where one can demonstrate a difference in the mechanisms that control epithelial cells versus stromal cells?

Dr. McKeenan:

There's now emerging a lot of data that shows the profile of responsiveness of both isolated epithelial and isolated mesenchymal cells is quite different. I'll talk about a few of them in my talk later- The Origin of Growth Factor Genes that are Activated During Proliferation. The epithelial cells don't even seem to have a receptor for the platelet derived growth factor family. So, one can start to categorize these as being quite different, but in response directly to the androgen story, I think that's just as complicated as ever. Questions about which is the major target of androgen, and whether or not their signal is transmitted back and forth between the epithelial mesenchymal cell, are still being sorted out.

Dr. Smith:

The epithelial cells are much more responsive to EPF. Under serum containing conditions, these cells are not. We weren't able to see a response to EPF at all, where the GA1 cells, which were more epithelial-like, were extremely sensitive to EPF.

Dr. Young:

You have talked about genetic instability in the prostate cancer cell. Do you have any idea where the hotspots are in chromosomes in terms of the genetic change?

Dr. Coffey:

No, I do not. One form of genetic instability which occurs with aging has been looked at in fibroblasts by the NCI group. As you age, you increase all forms of genetic instability. In cancer, people are looking at genetic instability in the order of sequence, for instance polyps in colon cancer. And those sort of experiments are being started in prostate and others, and as yet, no one has mapped out, to my knowledge, the series of events that occur in the prostate in genetic instability, in either human models or animal models.

STUDIES OF THE ENDOCRINE AND PARACRINE EFFECT OF TUMOR PRODUCED FACTORS IN HUMAN GENITOURINARY CANCERS

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Both prostate as well as kidney cancer are common malignancies in the United States. It is projected that in 1990, prostate carcinoma will be the most common cancer in men and will result in 20,000 deaths. Although hormonal manipulation is the mainstay of therapy of patients with advanced prostate carcinoma, there is no currently available effective therapy for patients with this malignancy. Over 20,000 patients develop kidney cancer per year in the United States, and there are over 9,500 deaths from this cancer. Recent studies have reported approximately a 95%, 5 year survival for patients with Stage T1, No renal cell carcinoma and a 92% survival for patients with Stage T2, No disease (1,2). While the five year survival for patients with N+ (local nodal involvement) renal cell carcinoma is 20% (3,4), patients with distant renal cell carcinoma metastases have only a 20% five year survival (5).

As part of an effort to increase our understanding of the paracrine, endocrine and autocrine effects of genitourinary tumor-produced growth factors as well as to develop new therapeutic strategies to treat patients with these malignancies, we and others have studied *in vivo* as well as *in vitro* effects of prostate as well as kidney carcinoma. There are a number of metabolic abnormalities of mineral metabolism which have been identified in patients with advanced prostate carcinoma which may be evidence of endocrine or paracrine activity of this tumor *in vivo*. Hypophosphatemia has been observed in a number of patients with metastatic prostate carcinoma (6-8). Although a few patients with advanced prostate carcinoma have been reported to have elevations in serum calcium (7,9,10), most patients with advanced prostate carcinoma have either normal or decreased serum calcium (7,8,11-17). Up to 50% of patients with metastatic prostate carcinoma have been found to have osteomalacia (18). Osteomalacia has been found in association with a low serum phosphorus and a decreased 1,25(OH)₂cholecalciferol level. Studies by various investigators have suggested that cancer associated with osteomalacia may be secondary to the production of a factor which 1) inhibits 1 α hydroxylase, the enzyme which regulates the conversion from 25(OH)cholecalciferol to 1,25(OH)₂cholecalciferol and 2) inhibits the proximal renal tubular reabsorption of phosphorus, thus resulting in hypophosphatemia. Lyles, et al performed a study of patients with metastatic prostate carcinoma. In some patients these investigators observed a decrease 1,25(OH)₂cholecalciferol, decreased serum phosphorus and osteomalacia. The findings in this study raised the possibility that prostate carcinoma could produce a factor which induces hypophosphatemia and a decreased serum 1,25(OH)₂cholecalciferol. Other investigators have reported elevated 1,25(OH)₂cholecalciferol levels in patients with advanced prostate carcinoma which returned to control levels after the patients underwent orchiectomy (19). The role of phosphorus metabolism and/or abnormalities in 1,25(OH)₂cholecalciferol in patients with advanced carcinoma is incompletely understood.

A tumor model was developed to study the paracrine and endocrine effects of prostate carcinoma-produced growth factors. 9479 is a human prostate carcinoma xenograft which grows in nude mice which was developed from a patient with metastatic prostate carcinoma and hypophosphatemia (20). Lyles and coworkers reported that athymic nude mice bearing this human prostate carcinoma developed hypo-

was developed from a patient with metastatic prostate carcinoma and hypophosphatemia (20). Lyles and coworkers reported that athymic nude mice bearing this human prostate carcinoma developed hypophosphatemia and had an increased urinary phosphorus excretion index. (21). In order to further study the metabolic effects of 9479 in tumor bearing animals, 9479 was implanted in male athymic nude mice and serum phosphorus, calcium and $1,25(\text{OH})_2\text{cholecalciferol}$ were measured. Urinary phosphorus, calcium and creatinine were measured in tumor bearing as well as age-matched, control animals. (17). In this study, there was a decrease in the serum phosphorus levels observed in animals bearing 9479 versus control animals [$3.4 \pm .3 \text{ mg\%}$ (tumor bearing) versus $7.3 \pm 0.2 \text{ mg\%}$ (control), $p < 0.0001$]. In spite of the fact that the tumor bearing animals had a low serum phosphorus, there was an increased urinary fractional excretion in tumor bearing versus control animals. When 9479 was removed from tumor bearing animals, serum phosphorus returned to normal levels. Six animals bearing another tumor (102) had no change in their serum phosphorus (17).

Next, calcium levels were evaluated in tumor bearing animals. In the animals bearing large 9479 tumors there was a significant increase in serum calcium versus control animals ($18.9 \pm 0.5 \text{ mg\%}$ versus $9.0 \pm 2 \text{ mg\%}$). after the tumors were surgically resected in 8 animals, serum calcium returned to normal levels (17). In order to assess whether or not the increase in serum calcium was due to immunoreactive parathyroid hormone, serum PTH levels were measured by an antibody with mid-molecule specificity. In spite of the elevated serum calcium, there was a decrease in the immunoreactive PTH levels in the 9479-bearing animals versus age matched control animals. This data suggested that the increase in serum calcium in the tumor bearing mice was not due to an increase in immunoreactive PTH and that it might be due to another systemically acting factor. In the 9479-bearing animals there was also an increase in the $1,25(\text{OH})_2\text{cholecalciferol}$ levels versus control animals. Whether this increase was secondary to the hypophosphatemia in tumor bearing animals, due to stimulation of $1,25(\text{OH})_2\text{cholecalciferol}$ production by a tumor produced factor, or due to other effects in the tumor bearing animals is not known (17):

There are a number of potential factors which could be responsible for the increased calcium and decreased phosphorus observed in the tumor bearing animals. It has been suggested that agents such as a PTH-like factor, transforming growth factor alpha ($\text{TGF}\alpha$), transforming growth factor beta ($\text{TGF}\beta$), PDGF or other tumor-produced factors could have endocrine and/or paracrine effects and induce bone resorption. In order to assess for the production by 9479 of a factor with the PTH-like bioactivity, ROS 17/2.8, 1 PTH-sensitive clonal rat osteosarcoma cell line, was used (22). When PTH is added to the culture media, measurement of intracellular cAMP is used to quantitate PTH or PTH-like bioactivity. Tumor extracts prepared from 9479 tumors were found to stimulate intracellular cAMP production in the ROS 17/2.8 osteoblastic-like cells (17). The PTH analog [^8Nle , ^{18}Nle , ^{34}Tyr]bPTH-(3-34)amide, which has been shown to inhibit the PTH-mediated increase in intracellular cAMP in ROS 17/2.8 cells, inhibited the increase in cellular cAMP induced by both PTH as well as 9479 tumor extract (17). This study demonstrating that in the athymic nude mouse the 9479 human prostate carcinoma could induce hypercalcemia, hypophosphatemia, an increase in $1,25(\text{OH})_2\text{cholecalciferol}$ and increased urinary cAMP, suggests the possibility that this tumor produces a systemically acting factor which has PTH-like bioactivity.

In order to test for bone resorbing activity by prostate carcinoma, conditioned media derived from PC-3, a human prostate carcinoma cell line, was evaluated in a neonatal mouse calvarial assay and was found to have bone resorbing activity which had some transforming growth factor-like characteristics (Robertson, in preparation). Tashjian and co-workers have demonstrated that systemic administration of $\text{TGF}\alpha$ can induce hypercalcemia in rodents, raising the possibility that in rodents, and potentially in man, a tumor-produced transforming growth factor could have systemic effects (23). Derynck et al have demonstrated that human prostate carcinoma expresses both $\text{TGF}\alpha$ as well as $\text{TGF}\beta$ (24). Whether the metabolic changes observed in patients with advanced prostate carcinoma are due to the endocrine or paracrine activity of a PTH-like factor or a factor such as $\text{TGF}\alpha$, $\text{TGF}\beta$ or PDGF is not known.

Most patients with advanced prostate carcinoma have either normo-orhypocalcemia. A question that has been raised is why nude mice bearing the 9479 prostate carcinoma xenograft might develop hypercalcemia while patients with advanced prostate carcinoma rarely do. One possible explanation is that rodents might have a different sensitivity to tumor produced factors than does man. Another potential explanation has to do with the osteoblastic effect of prostate carcinoma in man. Most patients with advanced prostate carcinoma develop osseous metastases, and most often there is an osteoblastic component to the osseous metastasis. It is possible that in man, when prostate carcinoma spreads to bone and induces an osteoblastic effect, that the increased osteogenic demand of the developing osteoblastic metastasis could absorb calcium and inhibit the development of clinically detectable hypercalcemia. Whether prostate

carcinoma produces two factors, an osteolytic as well as an osteoblastic factor, or whether a single factor induces osteolytic as well as osteoblastic activity is currently not known.

Studies of the paracrine and endocrine effects of renal cell carcinoma, another genitourinary tumor with many properties similar to prostate carcinoma, may help increase our understanding of these aspects of prostate carcinoma. Like prostate carcinoma, renal cell carcinoma has been reported to be associated with systemic effects of what appear to be tumor produced factors. Hypercalcemia, polycythemia, neuromyopathy, pyrexia, cachexia, and abnormal liver function have all been reported in patients with advanced renal cell carcinoma (25). Strewler, et al have demonstrated that human renal cell carcinoma cells induce hypercalcemia in the nude mouse xenograft model and produce a protein with PTH-like bioactivity (26). Gomella, et al have demonstrated that there is increased expression of TGF α and TGF β in human renal cell carcinoma (27) and have shown that there is also increased expression of the EGF receptor (28). Gomella, et al have also shown that TGF β inhibits the growth of human renal cell carcinoma (29) and that TGF β is produced by renal cell carcinoma in a latent form (Sargent, in preparation). It has been hypothesized that in prostate and kidney cancer TGF β production in a latent versus an active form may be incapable of autocrine inhibition of tumor growth (30). Conversely, TGF β produced by prostate or kidney cancer could enhance tumor growth by exerting a negative immunoregulatory effect on the host system. TGF β has been shown to inhibit interleukin-2 induced growth as well as cytolytic activity of lymphokine activated killer cells (31,32) as well as mitogen induced growth of peripheral blood T lymphocytes (33). Advanced prostate and kidney cancer often spread to bone, which is thought to be one of the sources of TGF β in the body. It is possible that TGF β , either produced by the tumor or released from bone by either a PTH-like factor or a factor such as TGF α , could inhibit the host immune system resulting in increase proliferation or spread of the tumor (30).

A strategy has been developed to inhibit the growth of human malignancy using agents with anti-growth factor activity. One agent currently being evaluated is suramin, a polysulfonated naphthylurea that has been used in the treatment of trypanosomiasis (34). This agent, which has been shown to inhibit reverse transcriptase activity *in vitro* (35), has also been shown to block the binding of a number of growth factors including EGF, PDGF, and TGF β (36-38) and to inhibit the effect of these factors on tumor cells in culture (Wade, submitted for publication). A recent study by Stein, et al demonstrated that systemic administration of this agent in patients with advanced malignancy, including a patient with renal cell carcinoma, can be associated with regression of disease. Currently, clinical trials are ongoing to evaluate the effect of suramin in patients with advanced carcinoma. Further studies to evaluate the effect of suramin as well as other anti-growth factor agents in prostate as well as renal cell carcinoma and to attempt to determine the mechanism of action of this form of therapy are currently underway and will hopefully lead to the development of effective forms of therapies for patients with these malignancies.

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FIBROBLAST GROWTH FACTOR: IMPLICATIONS IN THE ETIOLOGY OF BENIGN PROSTATIC HYPERPLASIA

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INTRODUCTION

The development of benign prostatic hyperplasia (BPH) is considered an inescapable phenomena for the aging male. Ninety percent of men will develop histologic evidence of BPH by the age of 80 (1). Bladder outlet obstruction is the major consequence associated with BPH. It has been estimated that 20-25% of all men over the age of 50 will require a prostatectomy for relief of bladder outlet obstruction (2). Approximately 350,000 surgical procedures are performed each year in the United States for BPH, resulting in an overall medical cost that exceeds 1 billion dollars annually (3). These statistics emphasize the need for new approaches to the study of BPH, for despite many years of study, the etiology of the disease has not been resolved.

PURIFICATION OF BASIC FIBROBLAST GROWTH FACTOR FROM BPH TISSUE

In 1979, Jacobs and associates (4) reported that extracts of human prostate were mitogenic for cultured cells. Cells responding to prostatic extracts included osteoblasts derived from fetal rat calvaria, fetal rat skin fibroblasts, and human foreskin fibroblast. Extracts from normal post-pubertal prostate, BPH prostate, and well-differentiated prostate cancer were found to stimulate 3H-thymidine incorporation by the cultured cells; whereas, extracts of human muscle and kidney contained little if any mitogenic activity. The growth stimulating factor, termed Prostate Growth Factor, had properties of a protein. At that time, it was postulated that the prostate contained a protein growth factor that may function in the development of BPH and in the osteoblastic response to prostate cancer. The next several years were spent in isolating and characterizing the growth factor from BPH tissue (5-8). The major breakthrough in purifying the growth factor occurred when it was realized that the physical and chemical properties of prostate growth factor were similar to the heparin binding growth factors (9,10).

Tissue was obtained at the time of transurethral resection of the prostate to relieve urinary tract obstruction due to BPH. The tissue was homogenized in high ionic strength buffer (pH 7.6), containing protease inhibitors, precipitated with ammonium sulfate, and fractionated by 2 cycles of heparin-Sepharose affinity chromatography (11). The predominant growth factor activity determined by bioassay eluted from the second heparin column between 1.4 and 2.0 M NaCl. This elution profile was consistent with the properties of basic fibroblast growth factor (bFGF). The growth factor activity was enhanced 8,488 fold and recovery was 34%. Previous attempts to purify the growth factor from BPH tissue that were not based on the properties of binding to heparin increased activity by only 1400 fold and recovery was only 1%. The preparation when electrophoresed on SDS-PAGE gels contained a predominant protein of Mr 17,600 but also contained less prominent proteins. The 17,600 Mr component was further purified by cation-exchange chromatography. In collaboration with Dr. Joachim Sasse, who provided antisera to synthetic bFGF synthetic peptides, the immunologic identity of Prostate Growth Factor and bFGF was confirmed (11).

Bovine pituitary bFGF had been sequenced by Esch and collaborators (12) and was reported to be a 146 amino acid peptide. However, the 17,600 Mr growth factor that we had isolated appeared larger than the 16,415 Mr peptide determined by sequence analysis. Therefore, it was likely that the growth factor that we isolated from prostate had an additional sequence not present in (1-146)bFGF. In collaboration with Dr. Fred Esch, the growth factor was found to contain an amino-terminal, 8 amino-acid extension not found in (1-146)bFGF (13). The extended form of the molecule had the sequence, Ala-Ala-Gly-Ser-Ile-Thr-Thr-Leu. This sequence agreed with the sequence of 8 of the 9 extra residues at the amino-terminus of bFGF predicted from the nucleotide sequence of a bovine brain clone encoding bFGF that was reported by Abraham, et al. (14). The amino-terminal methionine residue was not present. The evidence indicated that the form of bFGF that exists in BPH tissue is (-8-146) and that variations in the form of the molecule isolated are related to the homogenization procedure. Homogenization of BPH tissue in high ionic strength alkaline buffer containing protease inhibitors lead to the isolation of (-8-146)bFGF. When the growth factor was isolated from BPH tissue by a similar protocol used to isolate bovine pituitary bFGF, the yield of growth factor was greatly reduced and a smaller form that lacks the amino-terminal extension, likely (1-146), was recovered (13). The (1-146) form and a (16-146) form of bFGF that was isolated from various tissues at acid pH in the absence of protease inhibitors (15-17) seems to be a result of tissue specific enzymes, activated during extraction under acidic conditions, which degrade the growth factor.

A WORKING MODEL FOR THE ROLE OF bFGF IN THE ETIOLOGY OF BPH

Our working model for the role of bFGF in the etiology of BPH is depicted in the Figure. We propose that injury to the prostatic ducts in the periurethral zone in the proximal region of the prostate located between the bladder neck and the verumontanum releases bFGF. Once released from damaged prostatic epithelial or stromal cells, or from extracellular reservoirs, the mitogen is free to react with stroma cell surface receptors and initiate their proliferation. The primary stromal nodule induces the ingrowth of epithelium, that is maintained by androgen, producing the mixed nodules characteristic of BPH.

PATHOPHYSIOLOGY OF BPH

There is general agreement that BPH begins in the inner region of the prostate adjacent to the urethra between the verumontanum and the bladder neck. The close proximity of the hyperplastic growth to the urethra accounts for the high incidence of urinary obstruction in BPH. In 1925, Reischauer (18) described the first lesion of BPH as a fibromyomatous nodule. He stated that the stromal nodules induced a very early and rapid penetration of glandular elements. Subsequent morphologic studies by Deming and Neumann (19), LeDuc (20) and Moore (21) confirmed this thesis. McNeal (22) reported that BPH originates in distinct foci in the periurethral portion of the prostate. The first lesions appear in the third and fourth decades of life and thus precede the clinical symptoms of BPH by 10 to 30 years (23). McNeal (23) described the cells of the periurethral predominantly stromal nodule as resembling embryonic mesenchyme, with an abundance of pale ground substance and only a few fine collagen fibers. Most transitional zone nodules, on the other hand, appeared glandular with little stroma. With advancing age, glandular and stromal nodules increased in number but large nodules were uncommon until well into the 7th decade. In advanced BPH, there is a marked coordinated enlargement of most of the nodules within the prostate. The true prostatic tissue is displaced caudad and posteriorly as the lobes of benign hyperplasia grow and distort the original anatomy of the gland. The lateral and medial lobes seen in patients with BPH are masses of hyperplastic tissue that have displaced the true prostatic tissue to the periphery (24). McNeal (23) suggested the genesis of the BPH nodule results from a disturbance arising at the tissue level within the susceptible region of the prostate that results in the reversion of a clone of stromal cells to the embryonic state. The foci of embryonic stroma then induces the growth of adjacent epithelium giving rise to the glandular nodule. The duct formation in epithelial hyperplasia appeared greater on the wall facing the embryonic stroma suggesting that the inductive influence of the stroma may be mediated by diffusible inducers.

McNeal's hypothesis was based entirely on his extensive morphologic observations of the prostate. Bartsch, et al. (25) have published the only cytomorphometric analysis of the human prostate. They reported that the ratio of the stroma:epithelium in prostates from young men compared to enucleated prostates from men with BPH was 2:3 and 5:1, respectively. The conclusion that BPH is primarily a stromal process was concluded from this analysis of seven enucleated prostatectomy specimens and nine organ donor prostates. The heterogeneity of the prostate seen in enucleated BPH specimens clearly warrant further cytomorphometric analysis.

STROMAL-EPITHELIAL INTERACTION IN THE PROSTATE AND THE ROLE OF ANDROGENS

Development of the prostate from the embryonic urogenital sinus and maintenance of the morphology and secretory function of the adult prostate is depended upon androgens. Castration results in reduction of prostatic weight, cessation of secretory activity, and atrophy and death of the prostatic epithelium (26, 27). These events can be restored to the pre-castrated condition by androgen replacement. Insight into possible mechanisms involved in androgen induced development and maintenance of prostatic epithelium is found in the work of Cunha and co-workers (28). Their approach involved recombination experiments with urogenital sinus epithelium and mesenchyme from normal, wild-type, mouse embryos and from mouse embryos with testicular feminization syndrome (Tfm). The prostates of mice with Tfm do not develop because of a mutation affecting the androgen receptor. Their studies clearly showed that prostatic development and secretory function only occurred in tissue recombinants prepared with wild-type mesenchyme (androgen-receptor positive), irrespective of the source of the epithelium, wild-type or Tfm (androgen-receptor negative). They also demonstrated that wild-type urogenital sinus mesenchyme would induce adult urinary bladder from either wild-type or Tfm mice to differentiate into prostate and that the induction was androgen dependent. These studies indicated that mesenchyme and not the epithelium is the target and mediator of androgen-induced prostatic glandular development. These observations are best explained by a hypothesis in which androgen-responsive stromal cells produce a growth factor that elicits epithelial proliferation. It is tempting to speculate that bFGF might play such a role.

Aside from its potent mitogenic properties, bFGF has been implicated as a mesenchymal inducer during embryonic development. Basic FGF mRNA and protein have been identified in *Xenopus* oocytes and early embryos (29). Slack, et al. (30) reported that the application of bFGF to ectodermal explants of early *Xenopus* embryos induced mesenchymal differentiation. It is not known if adult tissue can respond to the inductive potential of bFGF and if it might be the inducer of mesenchymal "reawakening", suggested by McNeal (23) to be the initiator of the prostatic hyperplastic process.

There is general agreement that androgens are required for the development of BPH. The observation that BPH does not develop in men castrated prior to puberty supports this conclusion (31). Agreement on whether castration inhibits hyperplasia is by no means universal. Walsh (1) has reviewed this topic and concluded that castration may be most influential in types of BPH characterized by epithelial outgrowth and least influential in those cases where the pathology is primarily stromal in nature. A recent study by Peters and Walsh (3) supports this conclusion. They showed that Nafarelin acetate, a potent luteinizing hormone releasing agonist, decreased prostatic volume and improved symptoms of obstruction in BPH. The major morphologic feature resulting from androgen deprivation was regression of the glandular epithelium.

Attempts have been made to determine which cells of human BPH contain androgen receptors. These studies have given conflicting data. Kreig, et al. (32) found similar levels of androgen receptor in both epithelium and stroma. Lahtonen, et al. (33) reported a higher concentration of androgen receptors in the stroma than in the epithelium. Autoradiographic analysis of human glandular BPH showed androgen receptor localization almost exclusively within the epithelial nuclei; whereas in fibromuscular BPH, higher levels of nuclear androgen receptors were found in the stromal cells than in epithelial cells (34). Similar conclusions were reached when the analysis of androgen receptors in prostate was performed by immunohistochemical staining with recently available antiserum to the androgen receptor (35).

The study by Peters and Walsh (3) with Nafarelin acetate could be explained by a direct effect of androgen on the prostatic epithelium. An alternative explanation is that the stromal cells are stimulated by androgens to produce factors which stimulate epithelial cell growth. In the case of stromal hyperplasia, the stromal cells might produce factors which stimulate their own growth (autocrine regulation) or the epithelium might produce factors which stimulate the stroma (paracrine regulation). The recognition of the prostatic cell population that is responsible for growth factor synthesis and the identification of cells with the capacity to respond to these signals will provide valuable information for choosing between these possibilities.

PROPERTIES OF bFGF THAT INVOKE AN INJURY HYPOTHESIS IN BPH DEVELOPMENT

Basic FGF is present in many mesoderm-and neuroectoderm-derived tissues including prostate (36). The basic form of the molecule shares a high degree of structural homology (55%) with an acidic form (aFGF) suggesting that both growth factors were derived from a common ancestral gene (12). The tissue

distribution of aFGF is more restrictive than the basic form (36). Both mitogens possess binding sites for heparin (37) that explain their association with heparin sulfate-containing proteoglycans (GAG) that are components of the extracellular matrix (ECM) and basement membranes of all tissues.

The structure of bFGF has been highly conserved in species ranging from amphibians to mammals. This suggests that the growth factor has an important function in regulating growth and development. The peptide is a potent mitogen for fibroblasts and many other types of cells *in vitro*. Studies with cultured endothelial cells have provided important insight into how bFGF may regulate normal growth processes and have suggested mechanisms by which these physiologic processes might be altered in disease states.

Fibroblast growth factors are mitogenic for cultured endothelial cells. The growth factors are known to be angiogenic factors; a property of the molecule demonstrated both *in vitro* and *in vivo* (38). Fibroblast growth factors are also produced by cultured endothelial cells. The growth factors have been identified in low abundance in the conditioned medium, but the majority of the growth factors are cell-associated or are found in the ECM. Heparin sulfate-containing GAGs are a major component of the ECM. These structural elements bind, and protect FGFs from enzymatic degradation (16,39). The evidence for association of bFGF with heparin-sulfated GAGs was provided by Baird and Ling (40) and Vlodavsky, et al. (41). Their studies with cultured cells showed that bFGF could be released from ECM reservoirs by treatment with heparinase or heparitinase, or by brief exposure to high salt or heparin that did not disrupt cell membrane integrity. The cDNA for bFGF sequenced from bovine pituitary (14) and from human fibroblasts (42), indicates that the growth factor lacks a consensus signal peptide. The mechanism whereby bFGF exits the cell to be deposited in the ECM is unclear. The growth factor may complex with GAGs inside the cell and be transported with GAGs. Alternatively, there may be as yet unidentified secretory mechanisms that allow the intracellular bFGF to exit without activating cell surface receptors.

At this time it is not clear why bFGF synthesized by cells is not mitogenic for the same cells unless added exogenously. The storage of the growth factor in the ECM appears to make it inaccessible to its cell surface receptor thus providing a mechanism to regulate its mitogenic potential. Structure-function studies with synthetic bFGF peptides have identified two sequences that cross-react with the FGF receptor (37). Both functional domains share the capacity to bind heparin. This emphasizes the importance of GAGs in modulating the mitogen activity of the growth factor *in vivo*.

Heparin-sulfate degradation is a major enzymatic step in the breakdown of the ECM. The degradation of these GAGs, produced experimentally by heparinase and heparitinase treatment (40), results in decreased absorption of newly synthesized bFGF to the ECM and releases the growth factor from the ECM. Thus, one of the mechanisms by which tumors and perhaps some normal cells induce neovascularization and cell proliferation, might be by releasing heparinase-like enzymes. The observation that several tissues, including tumors, are a rich source of heparinase-like enzymes (43,44) support this hypothesis.

In the proposed model for the role of bFGF in BPH, injury perhaps resulting from infarction, infection, reflux of urine, or ejaculation, could release bFGF. The growth factor can be released upon cell lysis or by enhanced enzymatic turnover of the ECM. Heparin-like molecules are products of vascular endothelial and smooth muscle cells (45). Damage to these cells could release these molecules that are known to compete with bFGF for binding to the ECM. Infiltrating lymphocytes, mast cells, and macrophages may contribute to the mobilization of bFGF. These cells are also known to release heparin and are a source of enzymes which degrade heparin-sulfated GAGs. Once released, bFGF is free to bind cell surface receptors known to be localized on prostatic fibroblasts and perhaps epithelial cells. The proliferative response of these cells to bFGF could be responsible for the initiation of the fibrostromal nodule believed to be the earliest pathologic feature of BPH.

DISTRIBUTION OF bFGF IN NORMAL PROSTATE AND BPH

Our previous studies demonstrating bFGF in prostate utilized whole tissue homogenates. BPH originates in distinct foci in the proximal periurethral regions. If bFGF plays a role in the hyperplastic process, it may be that growth factor levels will be elevated in the periurethral zone of prostates obtained from young men and in the adenomas of progressive BPH. This hypothesis is being tested by quantitating bFGF in various regions of the normal and hyperplastic prostate. Dr. Frank Begun in our laboratory, has devised a sampling technique whereby a 4-5mm cross-section is made through the whole prostate of organ donors and through the enucleated gland of BPH. The section is taken between the bladder neck and the verumontanum. The cross-section is bisected and the two halves placed on top of each other. The top specimen is cut into 2-3mm pieces, scoring the bottom section in the process. A "map" is made depicting

the location of each small piece of tissue. Specimens are homogenized and assayed for bFGF by RIA. The underlying scored cross-sectioned specimen is fixed and processed as a whole-mount for histologic study. This technique allows growth factor levels to be localized within the prostate and permits histologic assessment of the adjacent tissue. The number of glands examined to date (Table) is small and bFGF levels have not been compared with tissue histology. The preliminary observations indicate that mean immunologic bFGF levels were higher in the periurethral zone and adenomas of BPH than in the periurethral zone of adult normal prostates. In addition, higher levels of the growth factor were found in the periurethral region of the adolescent prostate than in the peripheral or capsule regions; suggesting that bFGF levels may be related to the proliferative state of the tissue. Alternatively, it may be that bFGF content varies with the amount of stroma and epithelium in the prostate specimen.

PROSTATE-DERIVED FIBROBLASTS PRODUCE bFGF

The cell population(s) responsible for bFGF production in the prostate is not known. Studies to identify the prostatic cells responsible for bFGF production are in progress in our laboratory. Our approach includes: a) isolation of stromal and epithelial enriched cell population, growth of the cells in culture, and determination of growth factor production; b) immunohistochemical staining for bFGF in cultured cells and in normal prostate and BPH specimens; and c) Northern analysis and in situ hybridization for bFGF mRNA.

Thus far our studies have focused on cultured fibroblasts derived from the prostate (46). These studies showed that fibroblast cell lysates were mitogenic for prostate-derived fibroblasts in vitro. Thus, prostatic fibroblasts contain a mitogen that is capable of stimulating their own growth. The mitogen in fibroblast cell lysates competed with radiolabeled bFGF for binding to antiserum to bFGF. The mitogen had an identical elution profile as authentic bFGF from a column of heparin-Sepharose and the eluted protein reacted with antiserum to bFGF on Western analysis. Furthermore, prostatic fibroblasts incorporated ³⁵S-methionine into protein that was precipitated with bFGF antiserum and had the same molecular weight as bFGF. The conclusion is that cultured prostate-derived fibroblasts synthesize a growth factor that stimulates their growth with properties that are indistinguishable from bFGF. It remains to be determined if bFGF is also synthesized by prostatic fibroblasts in vivo. In addition, it will be important to determine if bFGF synthesis and regulation is the same or different in fibroblasts derived from normal prostate and BPH.

It remains to be determined if prostatic epithelial cells also produce the growth factor. Advances have been made in the propagation of prostatic epithelial cells from both rat (47) and human prostates (48, 49). The requirements for in vitro propagation of prostatic epithelial cells differ considerably from the

Table 1: Prostate bFGF Levels by Geographic Location

Prostate and Geographic Location	bFGF(ng/g)	n
Normal prostates		
Periurethral		
adolescents	125 ± 20	3
adult	84 ± 6	2
Central		
adolescents	129 ± 49	3
adult	74 ± 12	2
Anterior	78 ± 13	5
Peripheral	78 ± 18	6
Capsule	72 ± 20	3
BPH		
Periurethral	208 ± 54	6
Adenoma	170 ± 29	6
Anterior	118 ± 57	4
Capsule	72 ± 20	3

requirements for fibroblast growth. The stromal cells require serum. The epithelial cells can be grown in serum-free medium supplemented with insulin, transferrin, selenium, glucocorticoid, cholera toxin, EGF, and bovine pituitary extract. Bovine pituitary extract (Collaborative Research) contains immunoreactive bFGF and perhaps other growth factors (unpublished observations). Controversy exists as to the requirement for FGF for growth of prostatic epithelial cells in culture. McKeehan, et al. (50) reported that the requirement for bovine pituitary extract could be replaced by FGF. Kabalin, et al. (51) were unable to substitute purified FGF for bovine pituitary extract but did find that co-cultivation of epithelium with stroma cells or stroma cell-conditioned medium stimulated epithelial cell proliferation. These observations support the notion that soluble factors produced by prostatic fibroblasts regulate growth of the epithelium.

Despite the requirement for androgen *in vivo* the propagation of prostatic fibroblasts and epithelial cells *in vitro* is not dependent on androgen (47, 48). It may be that the cells able to adapt to growth *in vitro* represent a small subset of the cell population in prostate. Alternatively, it is possible that prostatic epithelial cells do not require androgen for growth in culture because their requirement for androgen is met by bFGF or other factors produced by stroma cells. Despite attempts to demonstrate that bFGF production by prostatic fibroblasts is influenced by steroid hormones, we have not observed altered expression of the growth factor by androgen, estrogen, or combinations of these hormones. Our studies, however, do not rule out that steroid hormones may indirectly regulate bFGF synthesis. In this regard, we have recently found that prostate-derived fibroblasts treated with transforming growth factor beta 1 (TGF β 1) exhibit elevated levels of bFGF. Transforming growth factor β 1, like the FGFs, is widely distributed in mammalian tissues. Transforming growth factor β 1 has not been reported in human prostate. This regulatory agent has been found to stimulate the production of fibronectin and collagen by cultured fibroblasts and the synthesis of GAGs in arterial smooth muscle cells (52, 53). The growth factor is present in high concentration in platelets and is also expressed by macrophages and lymphocytes (54). Thus, TGF β 1 is also believed to be involved in wound healing and in normal tissue repair. Unlike bFGF, TGF β 1 inhibits the growth of most cultured cells including prostatic fibroblasts. It will be important to determine if this modulator of cell proliferation and bFGF production is present in human prostate, and if its production or mode of action is under androgen control.

SUMMARY

A clear understanding of the possible role of bFGF in prostate is still in its early stage. However, it is important to consider a role for bFGF in the etiology of BPH because the growth factor is intimately associated with the prostatic stroma and thus is of potential importance to the development of the fibrostromal nodule seen in BPH. We believe that our goals to: a) determine the distribution of bFGF in the prostate; b) identify the prostatic cells responsible for bFGF synthesis; c) identify prostatic cells with receptors for the growth factors; and d) identify regulators of growth factor production, will provide new insight into the role of bFGF in normal and abnormal growth processes in the human prostate.

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DISCUSSION

Dr. Lee:

I understood that the basic FGF can stimulate fibroblast proliferation. Did you say the prostate epithelial cell can also be stimulated by basic FGF?

Dr. Story:

The growth of prostate epithelial cells *in vitro* requires supplementation of the culture fluid with bovine pituitary extract (BPE). BPE is rich in FGFs. Dr. McKeehan's group reported that prostatropin, acidic FGF, supports prostatic epithelial cell growth. Dr. Peehl's group have been unable to demonstrate a stimulatory effect of FGFs on human prostatic epithelial cells

Dr. Buttyan:

You showed data suggesting that the concentration of bFGF might be elevated in hypertrophied human prostatic tissues when compared to control normal prostate tissue. Your BPH tissue was obtained from presumed androgenically normal male patients. Since your so called "normal prostates" were obtained from brain-dead tissue donors, and since brain death often involves the shutdown of hypothalamic-pituitary functions, I would like to know if you have analyzed any of these control tissues to ensure that they had appropriate levels of androgenic stimulations at the time of removal? Could androgen depletion lower the levels of bFGF in these tissues?

Dr. Story:

No, we have not. You are right; these are from organ donors. We have not looked at that. You brought this up before. I think it is an important thing we should look at. I would not expect that bFGF in these glands would be reduced very rapidly. Clearly castration in the rat model does not rapidly reduce bFGF levels. I would be surprised if it did, but I agree with you; we should look at other parameters in these tissues.

Dr. Miller:

I want to get back to a couple basic points. At one point in your talk you referred to BPH, I think, as a form of neoplasia. The first question is when you think about growth factors, are you looking at BPH as though it's a form of hyperplasia, or as though it's actually a form of neoplasia? I think the distinction between the two is very important, because in one case you are talking about something that is potentially reversible, and in the other you are talking about something that has already become autonomous. The second question is whether or not the stromal aspect of this disease drives it. If that were true, it would seem to me we would see examples in the prostate of the same thing we see in the uterus; that is, smooth muscle tumors leiomyomas. As a point of fact, we almost never see that in the prostate. Instead we see large nodules which are predominantly epithelial. The question is: is it really the epithelial cell that is driving the stromal cell, or is it the stromal cell that is driving the epithelial cell?

Dr. Story:

Obviously we would like to know the answer to that. In regards to your first question, this is a benign neoplastic growth. It's not by nature metastatic. The growth regulation of this is unknown, at the present time. We know little on how to reverse this process.

Dr. Catalona:

I just missed a point you were developing on the effect of TGF beta on the fibroblast growth factor content. What was that observation, and how did you explain it?

Dr. Story:

In the prostate fibroblast cell model, we have looked at agents that regulate FGF production. We've looked at androgens, estrogens, and combinations thereof, and many agents that Roy Smith talked about. The only thing, so far, that we have found that regulates bFGF production is transforming growth factor-beta 1. This agent is inhibitory for cell proliferation, but it stimulates bFGF production, both on a per cell bases. I think it's an important regulator of bFGF production. If it functions *in vivo* in the prostate, or if it's important in the etiology of BPH, we don't know at this time.

Dr. Catalona:

But how can you explain that observation since you have more of it there and yet it decreases the cell proliferation? Can you hypothesize?

Dr. Story:

It would appear that bFGF is simply not available to the receptor. It may be in the extracellular matrix. We need to look at things that can release bFGF from an extracellular source, and ask the question: is this then proliferative? Do the cells respond to it once it's released?

Dr. Isaacs:

My question is similar to what we've just spoken about here. What is known about the ability of FGF, when it's immobilized on an extracellular matrix, to act as a mitogen? Is that totally inhibitory, and have you looked at, for example, adding bFGF to reconstitute extracellular matrices to see whether or not that has any activity?

Dr. Story:

This has been an interesting question. We know that if one just takes bFGF, for example ion-danated bFGF, and adds it to prostate fibroblast cultures, over 90 percent of the bound label can be competed for by heparin, suggesting that it is extracellular matrix associated. If you add bFGF exogenously, apparently some of this comes in contact with the receptor, and initiates a mitogenic response. The growth factor can be eluted from cell lysates and the extracellular matrix. It is mitogenic when added to cells with FGF receptors. As far as I know, no one has demonstrated that heparin or enzyme that act on the extracellular matrix, and presumably release the growth factor, initiate a mitogenic response.

FIBROBLAST-MEDIATED HUMAN EPITHELIAL TUMOR GROWTH AND HORMONAL RESPONSIVENESS IN VIVO

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INTRODUCTION

Mesenchymal-epithelial interaction is an integral part of embryonic development (1,2). This interaction continues throughout adult life and is responsible for maintaining the differentiative functions of a variety of mature organs (3). Classical approaches demonstrating the inductive activity of mesenchymes on epithelial growth and phenotypic expression have used surgically and enzymatically dissected cellular components (e.g., mesenchyme or epithelium) and recombined them either homo- or heterotypically (4-6). The advantages of this approach are that mesenchymal-epithelial interactions can be studied in purely separated cellular compartments and that the resultant recombinants are capable of developing fully into functional mature organs or body parts.

In adulthood, mesenchymal cells also mediate sex steroid-induced epithelial growth (7,8) and degeneration (9) *in vivo* and growth and gene expression (10-12) *in vitro*. Since both mesenchymal and epithelial compartments are known to contain numerous cell types, including the fibroblast, smooth-muscle, endothelial, nerve, and immune-reactive cells in the mesenchymal compartments and epithelial, basal, and paracrine cells in the epithelial compartment, we have tried a new approach by breaking down the mesenchymal and epithelial compartments into their individual cell types and recombined the relevant cell types *in vivo* and *in vitro* to determine (1) the extent of cellular interaction and (2) the subsequent effect of cellular interaction on the growth, hormonal sensitivity, and tumorigenic potential of the test cell types. This approach has led to the conclusion that the growth and the tumorigenic phenotype of the epithelium can be regulated profoundly by neighboring fibroblasts. In a recent article (13) we reported our experiments demonstrating that coinoculating tumorigenic rat prostatic mesenchymal cells and *nontumorigenic* rat prostatic epithelial cells produced carcinosarcoma, a mixed tumor composed of both carcinomatoid and sarcomatoid components, in both syngeneic and athymic animals.

In this communication, we summarize our most recent results on the use of fibroblasts as mediators to accelerate human epithelial tumor growth in athymic nude mice (14). In addition, we describe a novel method of conferring androgen responsiveness on an androgen-insensitive cell line through its neighboring fibroblasts. In this case, we have found that a human bladder epithelial cell line, WH, can be

induced to form carcinoma *in vivo* and grow in an androgen-sensitive manner by a nontumorigenic but androgen-responsive embryonic fibroblast-mediated pathway.

FIBROBLAST-MEDIATED HUMAN EPITHELIAL TUMOR GROWTH *IN VIVO*

Because rat prostatic fibroblasts appear to recruit nontumorigenic or nontumor-forming rat prostatic epithelial cells to participate in carcinosarcoma growth (13,14), we decided to test whether fibroblasts isolated from rodent species could exert a similar growth-promoting effect on human epithelial cell growth in culture and tumor growth *in vivo*. Table 1 shows that several low tumorigenic or nontumorigenic human epithelial cell lines derived from prostate PC-3 cells, bladder WH cells, and mammary-gland MDA-436 cells and from early passages of human renal pelvic and prostatic cancer cell strains could indeed be recruited to participate in carcinosarcoma growth when coinoculated with a tumorigenic rat prostatic fibroblast NbF-1 cell line. More strikingly, we found that lethally irradiated fibroblasts (albeit less efficient than nonirradiated fibroblasts) and nontumorigenic fibroblasts (e.g., NIH-3T3 cells) were also capable of recruiting human epithelial cell lines to participate in tumor formation. The typical resulting tumors were histologically characterized as carcinomas (Fig. 1, panel A) and were stained positively by cytokeratin antibody (Fig. 1, panel B). Southern hybridization of DNA extracted from the tumors with human-specific *alu*-repetitive DNA as the probe also supported the conclusion that fibroblasts accelerated human epithelial tumor growth *in vivo* (Fig. 2). These results emphasized the critical role of fibroblasts, even lethally irradiated or nontumorigenic fibroblasts, in human epithelial tumor growth.

To document the cell-type specificity and bidirectionality of mesenchymal-epithelial interactions, we performed experiments to determine the extent of cellular interaction between the rat prostatic NbF-1 fibroblasts and the human prostatic PC-3 epithelial cell line. Figure 3 shows that when live NbF-1 cells were coinoculated in athymic hosts with PC-3 cells, a mixed tumor, i.e., carcinosarcoma, was rapidly induced. Irradiated NbF-1 cells, but not irradiated PC-3 cells, also accelerated human PC-3 tumor growth *in vivo* (Fig. 4). Irradiated NbF-1 cells alone did not affect the growth or latency period for NbF-1 tumor growth *in vivo*, suggesting the cellular specificity of mesenchymal-epithelial interaction. Interaction between NbF-1 and PC-3 appears to be bidirectional, because irradiated PC-3 cells markedly accelerated the growth and shortened the latency period (from 20 to 2 days) for NbF-1 tumor growth (Fig. 5).

Interestingly, NbF-1 and PC-3 cells, when cultured together *in vitro*, failed to alter growth rate, indicating the lack of *in vitro* cellular interaction as that observed *in vivo* (Fig. 6). These results suggest that contributory factors from the host and/or intrinsic differences between cell growth *in vivo* and *in vitro* may be important in the promotion of tumor growth resulting from mesenchymal-epithelial interaction.

We have demonstrated conclusively by cytogenetic analysis of tumor-derived cell strains that NbF-1- and PC-3-induced tumors were not the result of cell fusion. Figure 7 represents chromosomal patterns of cells derived from the mixed tumors. The tumor-derived cell strains, like their parental cell lines, retained their species-specific characteristics without any evidence of gross chromosomal mixing between species.

The series of experiments summarized above showed that a mesenchymal component from a variety of sources can promote human epithelial tumor growth *in vivo*. This lack of species specificity in the mesenchyme-mediated acceleration of *in vivo* human epithelial tumor growth is consistent with cellular behavior in other embryonic inductive systems, where stromal-epithelial interactions have been demonstrated to occur between species and among organs that share a similar developmental repertoire (5,15). The fact that lethally irradiated and nontumorigenic mesenchymal cells also capable of promoting human epithelial tumor growth *in vivo* is significant: it suggests that extracellular matrix, growth factor(s) provided by the mesenchymal cells, or both may mediate recruitment of epithelial cells into tumor growth. The ability of nontumorigenic fibroblasts to induce the growth of human epithelial tumors without an apparent fibroblastic component, e.g., as occurs where 3T3 cells stimulate PC-3 tumor growth *in vivo*, is intriguing. It raises the possibility that mesenchymal cell types make potentially important contributions to human carcinoma development and progression, even if the overall mesenchymal contribution is only a minor component of the tumorigenesis. The mechanisms of mesenchymal-epithelial interaction most likely involve growth-factor and extracellular-matrix pathways. Because of the difficulties in demonstrating mesenchymal-epithelial interaction *in vitro*, we suggest that of host factors, the specific conformation of the

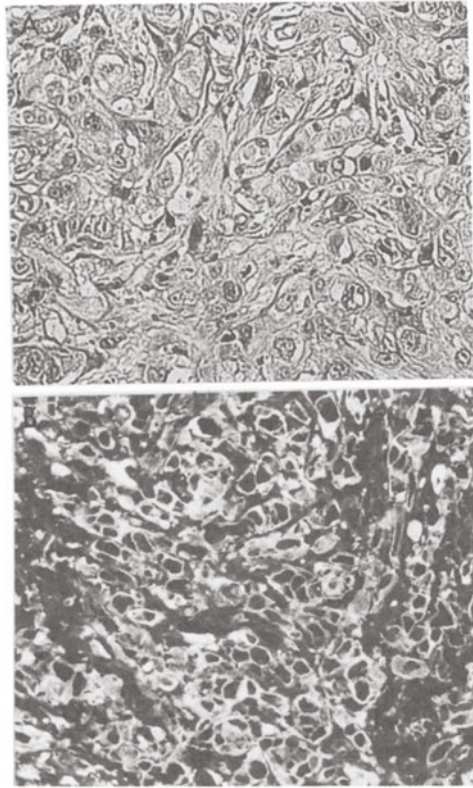


Figure 1: Histology (A) and anticytokeratin antibody staining profile (B) of a representative carcinoma specimen formed by coinoculating nontumorigenic mouse embryonic NIH-3T3 cells and a human prostatic PC-3 cells.

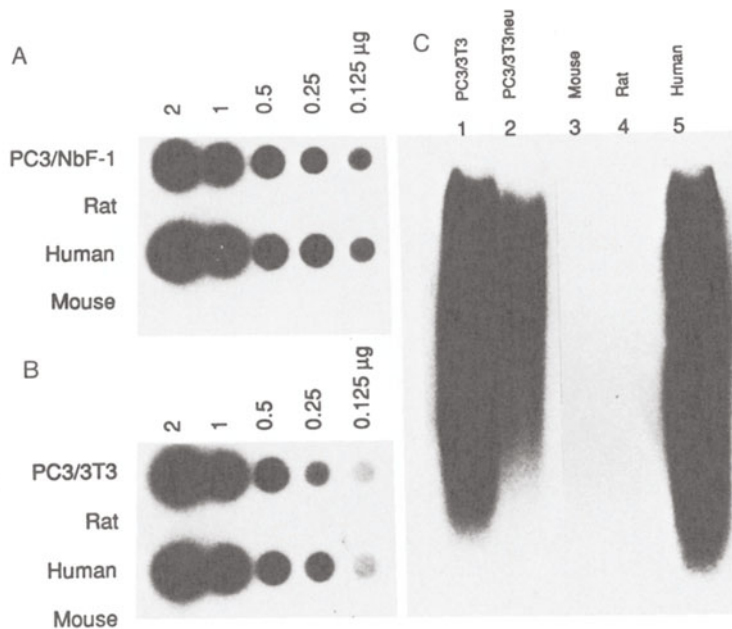


Figure 2: Dot-blot and Southern hybridization of tumor DNA with human-specific repetitive alu cDNA probe.

Table 1: Interaction of rat prostatic fibroblasts with human epithelial cancer cells in tumor formation

Rat prostatic Fibroblasts*	Human Epithelial Cancer Cells	Incidence of Tumor Formation ⁺
Nonirradiated		
None 1 x 10 ⁶ PC-3	0/16	
None	4.5 x 10 ⁶ PC-3	0/6
1 x 10 ⁵ NbF-1	None	0/6
1 x 10 ⁵ NbF-1	1 x 10 ⁶ PC-3	25/28
None 1 x 10 ⁶ MDA-436	0/4 ⁺⁺	
1 x 10 ⁵ NbF-1	1 x 10 ⁶ MDA-436	6/6
None 1 x 10 ⁶ WH	0/12	
1 x 10 ⁵ NbF-1	1 x 10 ⁶ WH	8/8
None 1 x 10 ^{6**}	0/4	
1 x 10 ⁵ NbF-1	(Prostatic ascites) 1 x 10 ^{6**} (Prostatic ascites)	2/4
None 1 x 10 ^{6**}	0/4	
1 x 10 ⁵ NbF-1	(Renal pelvic ascites) 1 x 10 ^{6**} (Renal pelvic ascites)	4/4
Lethally irradiated		
1 x 10 ⁵ NbF-1	None	0/12
1 x 10 ⁵ NbF-1	1 x 10 ⁶ PC-3	13/14
1 x 10 ⁵ NbF-1	1 x 10 ⁶ MDA-436	4/4
1 x 10 ⁵ NbF-1	1 x 10 ⁶ WH	3/3
1 x 10 ⁵ NbF-1	1 x 10 ⁶ (Prostatic ascites)	2/2

* Rat prostatic fibroblast cell line (NbF-1) was derived from Nb strain.

+ Unless otherwise indicated, incidence of tumor formation was recorded 30-36 days postinoculation.

+ + Our results agreed with the original report of Cailleau, et al. (28).

** Human prostatic and renal pelvic ascite cancer cells, one case each in their second through fourth passages were used.

PC-3, MDA-436, and WH cells are human prostate, mammary, and bladder cancer cell lines, respectively.

NbF-1 (1 x 10⁵) or WH (1 x 10⁶) cells, when inoculated alone, were marginally tumorigenic: tumors were detectable after day 18 but before day 30 postinoculation. The coinoculation of NbF-1 and WH or PC-3 cells, however, significantly enhanced the tumorigenicity of the interacting human epithelial cell lines. Here the incidence of tumor formation was recorded at day 18.

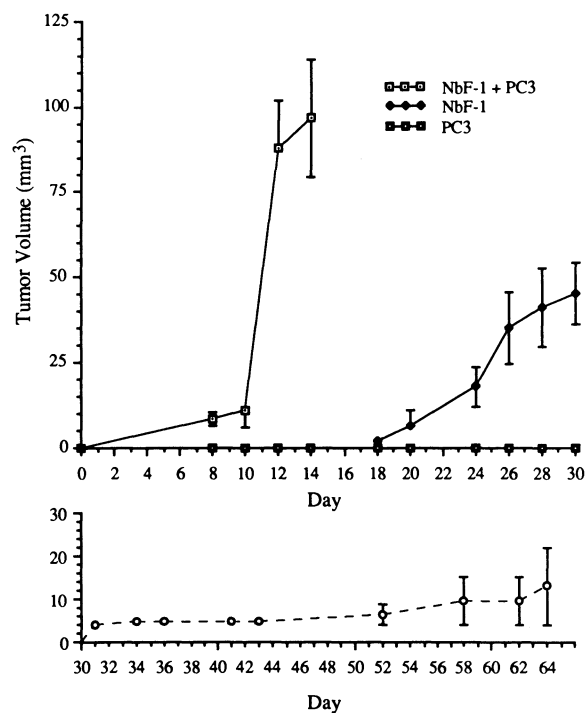


Figure 3: Acceleration of human prostatic PC3 tumor growth *in vivo* by a rat prostatic fibroblast (NbF-1) cell line.

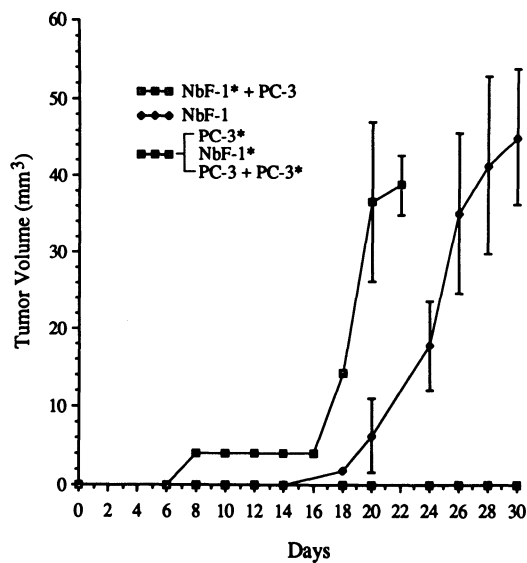


Figure 4: Acceleration of human prostatic PC3 tumor growth *in vivo* by a lethally-irradiated rat prostatic fibroblast (NbF-1) cell line.

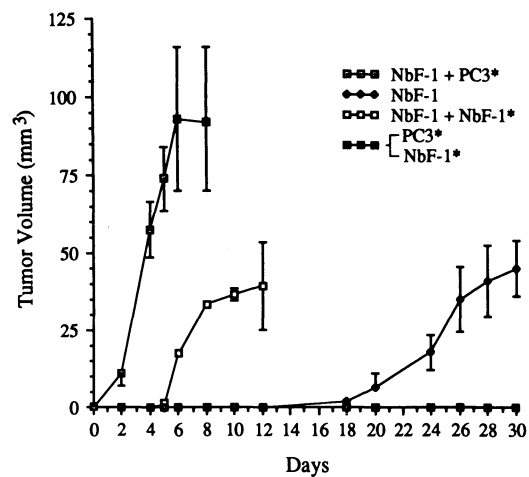


Figure 5: Acceleration of the growth of rat prostatic NbF-1 cells *in vivo* as fibrosarcoma in athymic nude mice by irradiated human PC-3 cells.

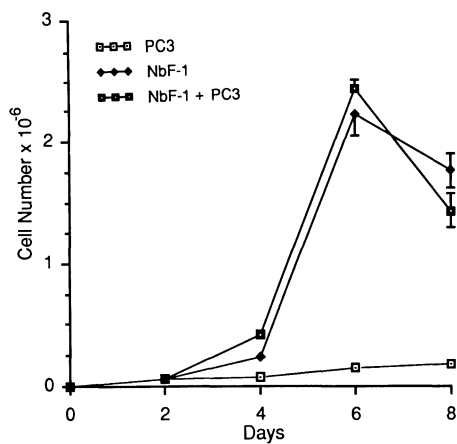


Figure 6: The lack of effect of rat prostatic NbF-1 cells on the growth of human PC-3 cells in culture.



Figure 7: Chromosomal analysis of NbF-1 (B) and PC-3 (A) cells shows the cells retained species-specific characteristics after coinoculation into the athymic hosts.

FIBROBLAST-MEDIATED HORMONAL RESPONSIVENESS OF HUMAN EPITHELIAL TUMORS IN VIVO.

In our previous studies (7,8) using tissue recombination methods, we demonstrated that fetal urogenital mesenchyme (UGM) elicited both growth and differentiative action on adult urinary bladder epithelium. By employing fetal UGM isolated from either wild-type (androgen receptor-positive) or *Tfm/y* (androgen receptor-deficient) mice to induce epithelial growth, we demonstrated that androgen receptors in the mesenchyme are obligatory for androgen-elicited morphogenic and growth responses to occur in the responding epithelium (7). In the present communication, we describe a novel method by which we have successfully conferred androgen sensitivity on an androgen-unresponsive human bladder epithelial cell line (WH) derived from a transitional cell carcinoma. This method involved the coinjection of an androgen-responsive mesenchymal cell line derived from fetal rat UGM (rUGM) with the human bladder epithelial WH cell line in athymic nude mice. Subsequently we analyzed the growth of the chimeric tumors. Figure 8 shows that of several different fibroblast cell lines tested, rUGM markedly enhanced the growth of human bladder WH tumors in the male to a degree about 17-fold higher than that in the female hosts. The sex difference of WH tumor growth *in vivo* was completely abolished when 3T3 cells, a known nontarget of androgenic steroid, were substituted for rUGM in coinoculating with WH cells in the athymic mice *in vivo* (Fig. 9). Because early passages of rUGM was nontumorigenic, the resulting tumors were identified as carcinomas according to both histologic analysis and DNA dot hybridization using a human *alu*-repetitive DNA probe (unpublished results). These results established for the first time that an androgen-unresponsive bladder epithelial cell line (WH) can be grown *in vivo* in an androgen-sensitive manner by coinoculation it with a known androgen-target mesenchymal cell line, rUGM.

We have examined the expression of a prostate-specific antigen (PSA) by the bladder WH epithelial cells in a chimeric tumor formed following coinoculation of WH and rUGM cells. In comparison to that of the human PC-3 cell line, the level of PSA expression by WH tumors was negligible (data not shown). These results established that, despite the rUGM cell-induced acceleration of WH tumor growth *in vivo*, cell type-specific gene expression by WH cells, as evaluated by the PSA expression, was not "reprogrammed" by rUGM toward the typical prostatic phenotype. In this regard, it should be noted that in tissue-recombination studies, fetal UGM tissue was able to mediate an inductive action on the effector cells in the normal adult urinary bladder epithelium to express prostatic-specific properties, including androgen receptors and prostatic-specific proteins (7,16).

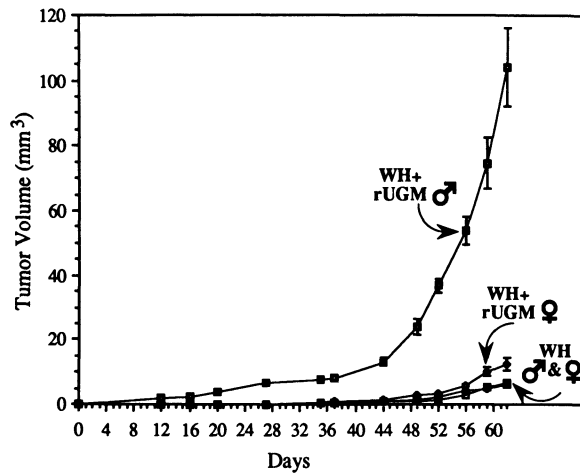


Figure 8: Using male and female hosts, fibroblasts derived from rat fetal urogenital sinus (rUGM) stimulated the growth of a human bladder epithelial cell line, WH, in athymic nude mice in an androgen-dependent manner.

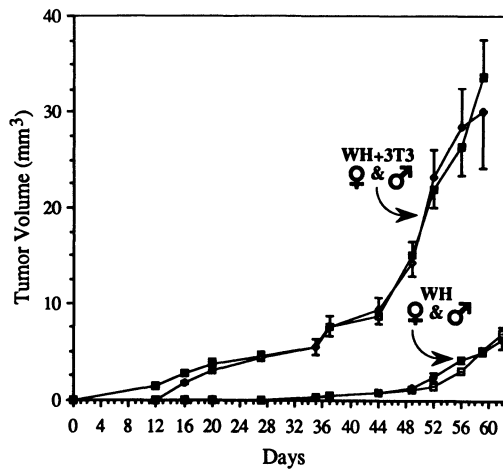


Figure 9: Using male and female hosts, fibroblasts derived from embryonic mouse (NIH-3T3 cells) failed to stimulate the growth of human bladder WH cells *in vivo* in an androgen-dependent manner.

MECHANISMS OF FIBROBLAST-MEDIATED GROWTH OF EPITHELIAL TUMORS *IN VIVO*

By employing the above-described methodology and using well-defined cell types to generate human tumors, we can begin to address questions concerning the cellular and molecular basis of mesenchyme-mediated human tumor growth and hormonal responsiveness *in vivo*. Our approach is focused in two areas:

Role of soluble growth factors in human epithelial cell growth *in vitro*: We have examined the potential mitogenic effect of conditioned medium obtained from cultured rUGM cells on the growth of WH cells and shown that, in comparison to control medium, the rUGM derived conditioned medium stimulated WH cell growth *in vitro*. We have also studied the effect of rUGM-conditioned medium on the growth of a rat prostatic epithelial cell line, NbE-1. These studies have demonstrated that a nondialyzable heparan sulfate-bound fraction, eluted by a high-salt solution (>1.0 M NaCl), appears to have mitogenic action. Similar medium collected from normal rat kidney cells fractionated by heparan sulfate column chromatography failed to exert mitogenic action on the growth of NbE-1 cells. Currently, we are attempting to determine if similar growth factor(s) fractionated and isolated by column chromatography and high-performance liquid chromatography from medium conditioned by rUGM cells can induce WH cell growth and confer androgen sensitivity *in vitro*.

Role of extracellular matrix on human epithelial cell growth *in vitro* and tumor growth *in vivo*: Despite our observations of fibroblast-mediated human epithelial tumor growth and hormonal responsiveness *in vivo*, we have not obtained convincing evidence to suggest that live or lethally irradiated fibroblasts can influence the growth of human epithelial cells *in vitro*. We have also tested the effect of Matrigel, a solubilized tissue basement membrane isolated from EHS (Englebreth-Holm-Swarm) tumor, coated on plastic dishes, on the growth of NbE-1 cells. We have shown that Matrigel failed to stimulate NbE-1 cell growth *in vitro* (unpublished results). In separate experiments however, we have demonstrated that Matrigel, but not laminin alone, induced nontumorigenic NbE-1 cells to form carcinoma *in vivo* as evidenced by the histomorphologic and cytokeratin immunofluorescence staining profile of the resultant tumors. At present, the significance of these findings remains obscure. It is not clear whether extracellular matrix materials, when presented *in vivo*, assume certain physical-chemical properties that then permit the stimulation of epithelial tumor growth *in vivo*.

In a recent study, Williams et al. (17) demonstrated that hemangiomas can be induced in a variety of species by the injecting mouse endothelioma cells expressing the polyoma middle-T oncogene. They found that the hemangioma formation was the result of rapid recruitment of nonproliferating host endothelial cells. Interestingly, these investigators demonstrated that when endothelioma cells were grown on nitrocellulose filters they failed to induce tumor growth in hosts, suggesting that the topology of cells existing in three-dimensional conformation *in vivo* may be crucial to enhanced growth and tumorigenesis. This hypothesis needs to be tested before the relative contributions of extracellular matrix constituents and host factors to *in vivo* tumor growth can be adequately evaluated.

SUMMARY AND SIGNIFICANCE

Malignant growth and progression of human genitourinary cancers are determined by both genetic and epigenetic factors (18-20). The objectives of our studies are to determine specifically the role of mesenchymal-epithelial interaction in human epithelial tumor growth and progression and the mechanisms of hormonal responsiveness of human epithelial tumor growth *in vivo*. Our results indicate that mesenchymal cells, expressing either a tumorigenic or nontumorigenic phenotype or even lethally irradiated, can alter the growth kinetics of human epithelial tumors and shorten the latency period until tumor appearance. These results emphasize the importance of the mesenchymal element in epithelial tumorigenesis. The mechanisms of mesenchymal-epithelial interaction most likely involve growth-factor and extracellular-matrix pathways. Because of the difficulties in demonstrating mesenchymal-epithelial interaction *in vitro* we suggest that of host factors, the specific conformation of the inductive mesenchymal molecules, or both may be involved in *in vivo* mesenchymal-epithelial interaction. We have demonstrated that cell fusion did not occur during the *in vivo* growth of the mesenchymal-epithelial chimeric tumors.

By employing the growth of an androgen-unresponsive human bladder WH cell line *in vivo* as a model, we have successfully conferred androgen responsiveness on this cell line *in vivo*. Our results indicate that a certain growth factor(s) produced *in vivo* by rUGM cells may be critical to androgen-induced growth. The role of extracellular matrix in stimulating *in vivo* WH tumor growth in an androgen-

dependent manner has not been evaluated. This model of conferring androgen responsiveness to an androgen-insensitive cell line supports our early hypothesis that mesenchyme may be the primary target for androgen. Mesenchyme relays growth and differentiative signals to its neighboring epithelial cells.

Our study clearly demonstrates that cell-cell interaction can occur *in vivo* by coinoculating morphologically and biochemically well-defined cell types. This new approach has allowed us to establish a model system that can be employed effectively to define the *in vivo* mechanism of mesenchymal-epithelial interaction and the role of androgen in regulating these processes. We anticipate using this model system to accelerate human tumor growth *in vivo*, and facilitate the testing of potential chemotherapeutic drugs on human tumor growth.

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DISCUSSION

Dr. Smith:

You demonstrated that if you take fibroblasts from a tumor cell, you can accelerate the growth of epithelial tumor cells. Is it possible to take epithelial cells that have been immortalized, say with myc, that are not transformed, and then do the obvious sort of experiment. If you take the fibroblasts from the tumor, can you then completely transform those epithelial cells *in vivo*?

Dr. Chung:

We have not done this experiment, but we have performed experiments to test if the fibroblasts could activate the growth of normal prostatic epithelial cells. Since culture cells could be interpreted as cells that have already been going through the first step of transformation, we inject the fibroblast directly into the prostate trying to see if we can accelerate normal epithelial cell growth. So far, we have not observed epithelial cell growth in the normal prostate. My feeling is that several steps of transformation may have to occur before epithelial cells can respond to growth acceleration by their neighboring fibroblasts.

Dr. Smith:

Yes, it might just work if you were to immortalize them first.

Dr. Buttyan:

To follow up your experiment in which you showed that the simple addition of Matrigel to a cell suspension was sufficient to allow tumor formation by normally nontumorigenic epithelial cells, does the tumor grow continuously or does it reach a particular size and stop growing?

Dr. Chung:

It continues to grow.

Dr. Buttyan:

In this case, would you then think that the Matrigel is important for the initiation of tumorigenesis or is it required for continuous growth even as it is diluted out by the growing tumor?

Dr. Chung:

It probably is important for the initiation of the tumor growth initially, and then the epithelial cells go through perhaps the last few key "steps" of carcinogenesis.

Dr. Matusik:

I was interested when you combined the PC3 cells with irradiated Nb-1 cells, and you showed that you can still form tumors. This suggests either a growth factor still present or some kind of matrix requirement. Have you pursued that in trying to place a membrane between the matrix and the epithelial cells to see if you could block growth or demonstrate that there is a diffusable factor?

Dr. Chung:

We have been pursuing both the extracellular matrix and growth factor experiments. Since the work at this time is inconclusive, I probably should not comment on which growth factor is involved here. In terms of the extracellular matrix, one of the key experiments that we are trying to do is to see if we can irradiate rUGM cells and see if the irradiated cells might still confer androgen sensitivity. We are now looking at about four to five growth factors at this time.

Dr. Matusik:

You combined a cell to cell culture and showed that they did not become additive and they did not stimulate proliferation, which then suggested to you that there is something present in the nude mouse that's contributing. But, now I'm coming into a contradiction, because if the irradiated cell is dead, what could it be contributing?

Dr. Chung:

I believe the *in vivo* topology of cells in 3-dimensional structures are extremely important. I don't know about a lot of things that might be happening *in vivo*. But I know the appropriate model for *in vivo* growth is extremely difficult to demonstrate *in vitro*.

Dr. Matusik:

Can I make a suggestion? Perhaps when you do the matrix combination with the dead cell with the PC3 cell in a nude mouse, that it now makes that PC3 cell capable of responding to something like androgens that previously it couldn't respond to. So the component may be a matrix interaction that now lets the cell respond.

Dr. Chung:

The PC3 cells are supposed to be androgen independent.

Dr. Matusik:

I know.

Dr. Miller:

I would like to ask a question as a diagnostic pathologist. When we refer to carcinosarcomas in the bladder, we define them on the basis of the fact that both of the components are malignant. You can, therefore, find metastases from the sarcoma and metastases from the carcinoma. When you develop your primary tumors, what happens if you passage them over time. Do both of the components continue to proliferate? How do we know that both of the components are really malignant?

Dr. Chung:

In the rat prostate, we have cultured fibroblasts and epithelial cells from the carcinosarcoma, and then innoculated the epithelial cells back into animals without the fibroblasts. The epithelial cells remain nontumorigenic (Chung, et al. Int.J.Cancer 43:1179-1187,1989). So here there is a micro-environmental change around the epithelial cells, which allows the fibroblast to stimulate epithelial growth.

Dr. Matusik:

Do the stromal cells do the same thing?

Dr. Chung:

The stromal cells remain tumorigenic.

Dr. Thompson:

Are the Nb-1 fibroblasts tumorigenic and are they androgen dependent?

Dr. Chung:

Nb-1 cells are both tumorigenic and androgen sensitive. The same thing applies to rUGM cells. They are androgen sensitive both *in vivo* and *in vitro*. Dr. Mike Freeman's poster describes the gene expression and the hormonal sensitivity of these cell lines.

Dr. Thompson:

I know you showed alu-probing and so forth. Are there any differences in the ratio of epithelial to either Nb-1 cells or 3T3 cells when you grow them *in vivo*? In other words, are there more Nb-1 cells under androgen stimulated conditions?

Dr. Chung:

Yes. We have not done a detailed quantitative analysis. I should stress the fact that innoculation of a nontumorigenic fibroblast with a nontumorigenic epithelial cell actually forms a tumor. When you look at the tumor, at least for the 3T3 cells and rUGM cells, it is nearly completely carcinoma, based on morphology and DNA dot blot. Therefore, we felt that fibroblasts, even though they are not there, are contributing probably immensely to the acceleration of epithelial cell growth. Unfortunately, we do not know the exact mechanisms involved.

POLYAMINE REQUIREMENT OF PROSTATE CANCER CELL PROLIFERATION

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INTRODUCTION

The name "polyamines" is commonly used to refer to the naturally occurring compounds putrescine (Pu), spermidine (Spd) and spermine (Spm). The structural formulas of these substances are shown in Fig. 1. As indicated by their names, Spd and Spm occur in high concentrations in seminal fluid. The formation of crystalline material, now known to consist of spermine phosphate, in human seminal plasma was reported as early as in the 17th century by Van Leeuwenhoek. Following the elucidation of its chemical nature, about 200 years later, the biochemical mechanisms of action of spermine (and other polyamines) in semen have been studied extensively. Production and physiology of seminal polyamines have been the subject of a number of reviews [1-3].

Polyamines in semen are largely derived from prostatic secretion. In rats high polyamine concentrations were found in the ventral and the dorsolateral prostate. These concentrations are known to be under the control of androgens [4]. In contrast, the anterior prostate (or coagulating gland) contains much lower levels of polyamines [2,5,6]. Seminal vesicle secretions contribute little or no polyamines to the semen [2,5]. The function of seminal plasma polyamines in reproductive physiology is still unclear. It has been postulated that polyamines may serve to regulate semen coagulation [2,6,7]. Polyamines are covalently incorporated into seminal proteins by the action of transglutaminase [7,8] and may thereby influence the transglutaminase-mediated protein cross linking reactions involved in clot formation.

The function of intracellular polyamines is not yet well understood either. Polyamines are abundantly present in virtually all mammalian cells. As polyamines, being protonated under physiological conditions, will behave as polycations, they are expected to interact readily with the negatively charged moieties of nucleic acids, proteins and phospholipids. In fact, such interactions and the resulting effects on various biochemical reactions have been demonstrated in numerous studies employing cell free systems. The biological significance of these effects is difficult to prove, however. Nevertheless, there is quite circumstantial evidence that polyamines somehow play a role in the process of cell proliferation (for reviews see refs. 9-15).

Many studies showed that the rate of polyamine synthesis is rapidly increased in response to various growth promoting stimuli. Furthermore, compounds that block the biosynthesis of polyamines were shown to have a profound inhibitory effect on cell proliferation in a large variety of experimental systems. These findings have raised interest in the possibilities for application of polyamine synthesis inhibition in cancer treatment. However, in contrast to the results obtained with experimental tumor models, clinical trials using polyamine synthesis inhibitors have not shown a significant antitumor activity of such agents so far [16,17].

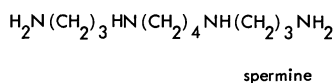
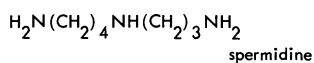
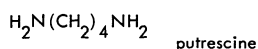


Figure 1: Structures of the natural polyamines.

In this overview the results from our experiments concerning the effects of polyamines and polyamine synthesis inhibitors on the proliferation of human prostate cancer cells will be presented and possible reasons for the failure of polyamine inhibition to stop cell growth *in vivo* will be discussed.

POLYAMINE BIOSYNTHESIS AND INTERCONVERSION

Polyamines are the products of exquisitely regulated biosynthetic pathways [18], as schematically depicted in Fig. 2. In mammalian cells decarboxylation of ornithine, catalyzed by the enzyme ornithine decarboxylase (ODC), provides the only route for the de novo synthesis of Pu. The subsequent coupling of two propylamine moieties, catalyzed by the enzymes spermidine synthase and spermine synthase, results in the consecutive formation of Spd and Spm. Decarboxylated S-adenosylmethionine (dcSAM), produced by the action of the Pu-activated enzyme S-adenosyl-methionine (SAMDC), serves as the aminopropyl group donor in these aminopropyltransferase reactions; another product of these reactions is 5'-methylthioadenosine (MTA).

Although the reactions catalyzed by Spd synthase and Spm synthase are effectively irreversible, it is well established that conversion of Spm into Spd and Spd into Pu may occur via the polyamine interconversion pathway [19]. This pathway involves acetylation, catalyzed by Spd/Spm N1-acetyltransferase (SAT) [20], followed by oxidation, catalyzed by (FAD-dependent) polyamine oxidase (PAO) [21].

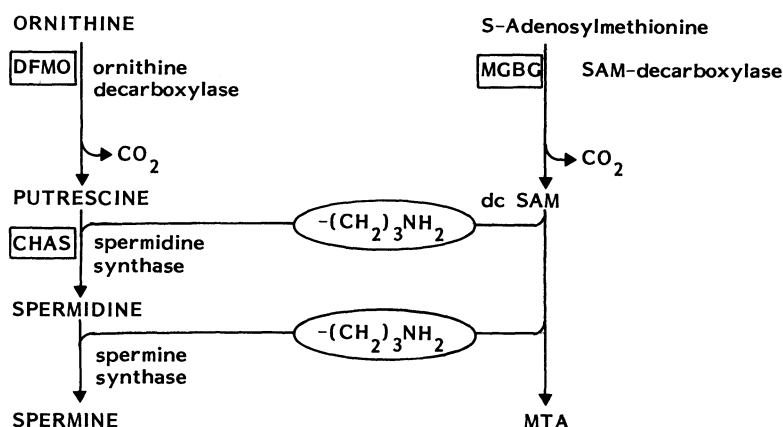


Figure 2: Biochemical pathways and inhibitors of polyamine biosynthesis. Abbreviations used: FMO, DL- α -difluoromethylornithine; MGBG, methylglyoxal bis(guanyldrazone); CHAS, cyclohexylammonium sulfate; MTA, 5'-methylthioadenosine.

Acetylation of polyamines is generally considered to be a crucial step in their catabolism and excretion. It may be assumed that release of polyamines from anionic sites and their transport across the cell membrane will be facilitated by the reduction of net positive charges which is accomplished by acetylation. In fact, in humans polyamines are excreted almost exclusively as the monoacetylated compounds (for review see ref. 22). Pu, Spd and Spm are converted to the N1-monoacetylated forms by the highly inducible, cytosolic enzyme SAT [18,22]. A nuclear enzyme, Spd N8-acetyltransferase, is responsible for the conversion of Spd (which is an asymmetric molecule, in contrast to Pu and Spm) into N8-acetyl-Spd. Acetylated polyamines do usually not accumulate intracellularly because the N1-acetylated forms (except for acetyl-Pu) are readily oxidized by PAO whereas N8-acetyl-Spd may be deacetylated by a not yet well characterized deacetylase to regenerate Spd. Under certain pathological conditions, however, including cancers [23-25], increased levels of acetylated polyamines have been detected.

The introduction of specific polyamine synthesis inhibitors has been a major breakthrough in polyamine research. Among these inhibitors DL-alpha-difluoromethylornithine (DFMO), a highly specific, enzyme-activated irreversible inhibitor of ODC [26], has been most widely applied. Exposure of cells or tissues to DFMO results in a considerable reduction of the cellular Pu and Spd content. At the same time the concentration of dcSAM, which is low under normal conditions, increases tremendously due to the unavailability of suitable acceptor molecules [27]. In humans increased urinary excretion of dcSAM has been observed during DFMO-treatment [28]. At the present time a variety of inhibitors for each of the enzymes involved in polyamine synthesis and metabolism has been described (for review see ref. 27). In our studies we mainly used DFMO, in addition to methylglyoxal bis(guanyldrazone) (MGBG), an inhibitor of SAMDC [29], and cyclohexylamine (CHAS), an inhibitor of Spd synthase [30].

INDUCTION OF ODC-ACTIVITY IN CULTURED PROSTATE CANCER CELLS

Stimulation of cell proliferation is known to be associated with an increased rate of polyamine biosynthesis. We have studied the kinetics of the induction of ODC-activity in cultured human prostate cell lines stimulated to proliferate after serum deprivation. Cells were cultured for at least 24 hours in culture medium without serum and then (partly) synchronized by the addition of fresh medium containing 10% fetal calf serum (FCS). At various time periods (0-8 hours) after stimulation by FCS the ODC-activity was determined in situ by quantitation of the amount of radioactive CO₂ released during incubation with ¹⁴C-labelled L-ornithine [31]. At the same time DNA-synthesis was monitored in parallel flasks by measurement of the incorporation of ³H-labelled thymidine into trichloroacetic acid precipitable material [32]. As shown in Fig. 3 for PC-3 cells, ODC-activity transiently increased following serum stimulation, reaching a maximum after four hours and showing a rapid decline thereafter. The increase of ODC-activity more or less parallels the stimulation of DNA-synthesis, which showed a peak at six hours after serum addition (Fig. 3). These results may suggest that the cellular requirements for polyamines during DNA-synthesis in the S-phase of the cell cycle are accomplished by increasing the rate of polyamine biosynthesis.

EFFECTS OF POLYAMINE SYNTHESIS INHIBITION ON CELL GROWTH

DFMO, an inhibitor of ODC-activity, has been widely applied to study the effects of polyamines on the growth of a large variety of normal and neoplastic cells. Treatment of cells in culture with DFMO usually leads to a rapid and almost complete disappearance of intracellular Pu as well as a substantial reduction of the Spd content. Table 1 shows the polyamine levels in PC-3 cells after four days exposure to 1 mM DFMO. Similar results were obtained with other cells lines. Whereas Pu concentrations fell to barely detectable levels within 24 hours already and Spd levels declined in a time-dependent fashion (80-90% reduction after 4 days in PC-3 cells), Spm concentrations remained constant for a considerable period of time or even showed an initial rise during the first two days of treatment. Such an effect on Spm levels should be explained by the DFMO-induced accumulation of dcSAM, which will promote the conversion of the remaining Pu and Spd into Spm.

DFMO-treatment of cultured cells has generally shown striking inhibitory effects on cell growth [9,10,13-15]. In this respect prostate cancer cell lines behave quite similar to most of the other DFMO-treated cell lines reported in the literature. Since DFMO inhibits the synthesis rather than affecting the already existing polyamines, the onset of growth inhibition requires a lag period -equal to about 1 or 2 doubling time periods- which should allow for the depletion of cellular polyamine pools. Polyamine depletion apparently affects the ability of the cells to traverse the cell cycle. It is still a matter of debate, however, whether polyamine-deficient tumor cells are blocked at a specific site of the cell cycle. It has been reported that transformed cells, in contrast to normal cells, may continue cell cycle traverse or accumulate in the S-phase in response to DFMO-treatment [34]. This result was not confirmed in other studies [35,36], however,

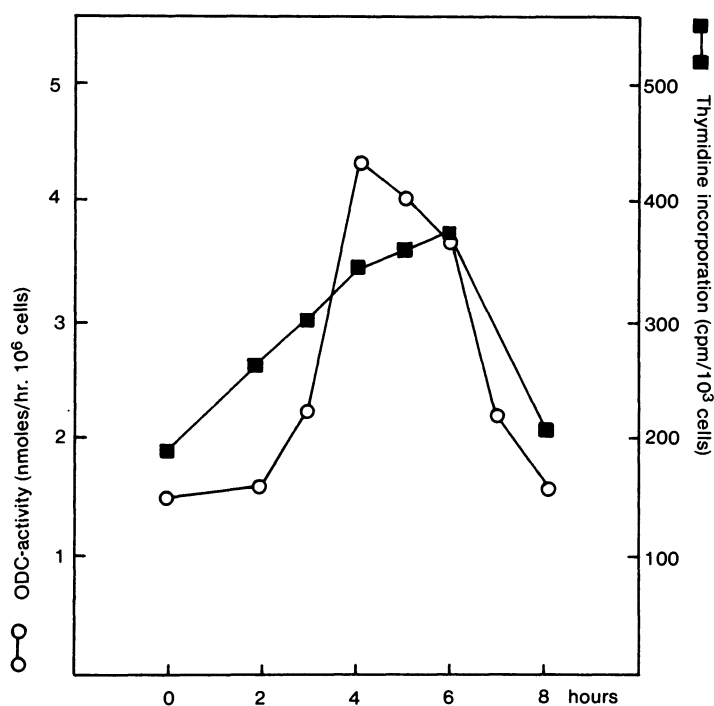


Figure 3: Induction of ODC-activity and DNA-synthesis in PC-3 cells following serum stimulation. Cells were serum starved for 24 hours. At various times after the addition of 10% FCS ODC activity was determined and expressed as nmoles ^{14}C -CO₂ liberated/hr. million cells. In parallel flasks the incorporation of ^3H -thymidine was determined. Data represent the average results from at least 2 flasks.

Table 1: Intracellular polyamine levels in untreated PC-3 cells and in PC-3 cells cultured for 4 days in the presence of 1 mM DFMO. Polyamine concentrations were determined as described before [33]

	<u>Polyamine levels (nmol/10⁶ cells)</u>	
	<u>PC-3 control</u>	<u>DFMO-treated</u>
putrescine	0.43 ± 0.09	0.04 ± 0.04
spermidine	1.92 ± 0.26	0.21 ± 0.05
spermine	2.54 ± 0.25	2.38 ± 0.58

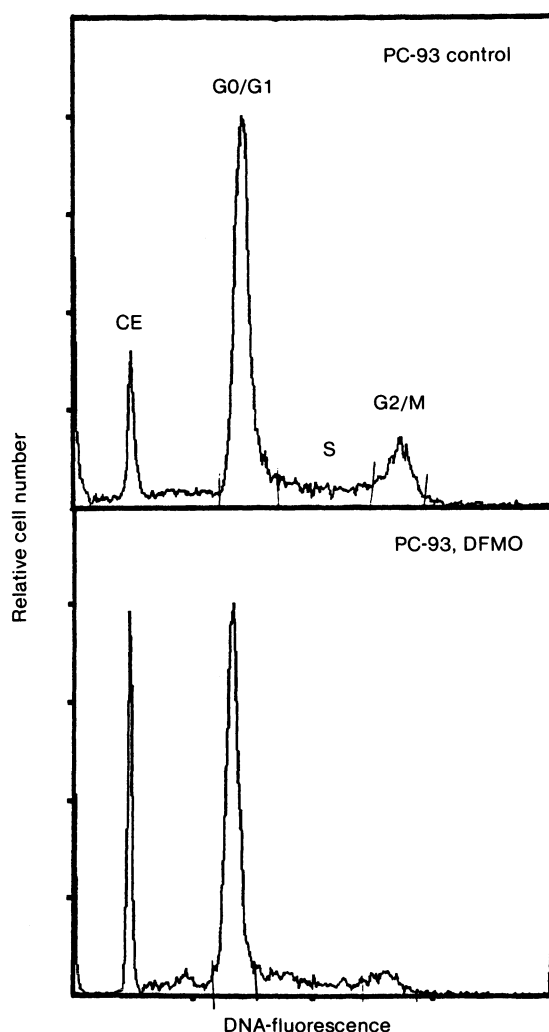


Figure 4: DNA histograms of control and DFMO-treated PC-93 cells. Cells were exposed to 1 mM DFMO for 4 days. CE, chicken erythrocytes.

and in fact it was demonstrated that the cells from four out of five human cancer cell lines accumulated in the G0/G1 cell phase fraction [36]. In the prostate cancer cell lines examined in our study (PC-3, PC-93 and LNCaP) DFMO-treatment resulted in a decrease of the S-phase fraction, disappearance of cells from the G2/M fraction and, consequently an increased percentage of cells in the G0/G1 fraction (Fig. 4). These observations suggest that adequate levels of polyamines are required for normal cell cycle progression of these prostate cancer cells.

The effects of polyamine synthesis inhibition on hormone-induced cell proliferation were studied using the androgen-responsive cell line LNCaP (Fig. 5). In the absence of steroid hormones, e.g. by culturing the cells in a medium supplemented with 10% charcoal-treated FCS, little or no increase in cell number was observed during a 5 day culture period (Fig. 5, first two bars). The addition of the nonmetabolizable androgen R1881 at the optimal concentration of 0.1 nM [32] resulted in growth as reflected by the increased cell numbers. This stimulation of growth was prevented completely, however, in the presence of 1 mM DFMO (R+D), unless Pu was added as well (R+D+P). These results clearly demonstrated that, also in

the case of androgen-induced growth, appropriate levels of polyamines are required to support cell proliferation. On the other hand, as shown by the lack of a stimulatory effect of Pu alone, polyamines are not sufficient to support growth in these cells, which rules out the possibility that hormone-induced ODC activation is a major factor in androgen regulated growth of prostate cancer cells.

REVERSAL OF DFMO-INDUCED GROWTH INHIBITION

As shown above, the growth inhibitory effects of DFMO can be blocked in the presence of exogenous polyamines. Likewise, DFMO-induced inhibition can be reversed by the addition of polyamines, even after an appreciable time of exposure to DFMO. Fig. 6 shows that after discontinuation of the treatment, cell proliferation is resumed and proceeds at a normal rate fairly soon. The lag phase observed in this type of experiments is dependent on the treatment period and can be shortened by the addition of polyamines (not shown). Such observations have raised the question whether the failure of DFMO-treatment to inhibit cell proliferation *in vivo* should be attributed to the uptake of exogenous polyamines. In addition it has not been established clearly yet which of the polyamines is most critically involved in growth support.

To address these questions, PC-93 cells were partially depleted of polyamines by exposure to DFMO for 4 days and then cultured in the presence of different polyamines (free as well as acetylated compounds) at various concentrations for another 4 days. Growth was evaluated by use of the MTT-assay [32]. The results, expressed as percentage reversal, are shown in Table 2. All tested compounds, except for N1-acetyl-Pu, were able to restore growth of DFMO-treated PC-93 cells. Spd, Spm and N1-acetyl-Spm were most potent in this respect providing almost full restoration of growth at a concentration as low as 2 μ M. Clearly, Pu was much less effective than Spd. Slightly higher concentrations were required also for N8-acetyl-Spd. N1-acetyl-Spd and N1-acetyl-Spm, however, appeared to be almost as effective as the parental Spd and Spm in this system.

To study the intracellular events leading to polyamine mediated restoration of growth, DFMO-treated PC-93 cells were cultured under the same conditions that were previously shown to allow for

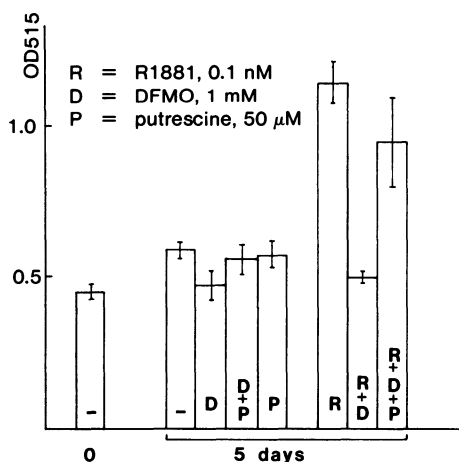


Figure 5: Effect of polyamines on the growth of LNCaP cells *in vitro*. Cell growth was determined by use of the MTT-assay [32] and expressed as the absorbance read at 515 nm. Data represent the mean $\pm$ S.D. from 8 wells per group. Cells were cultured in medium containing 10% charcoal-treated FCS for 5 days, without further additions (-) or in the presence of DFMO (D), R1881 (R) and/or Pu (P).

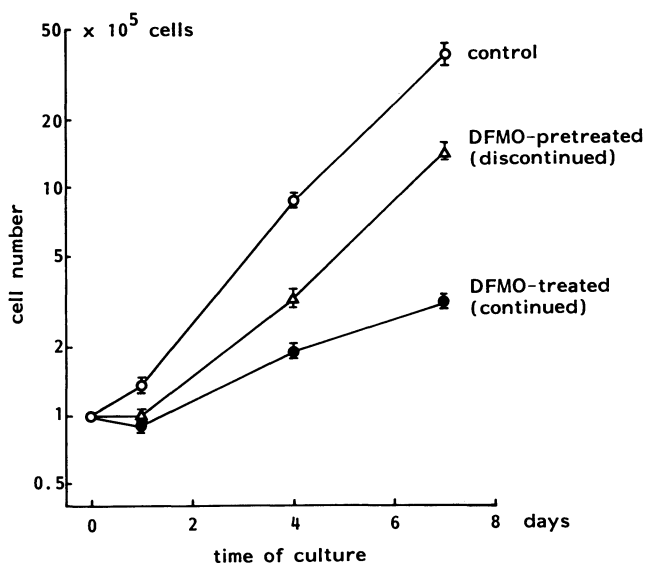


Figure 6: Growth curves of PC-93 cells in the presence and absence of DFMO. Except for the controls, cells were exposed to 1 mM DFMO for 2 days prior to plating.

Table 2: Reversal of DFMO-induced growth inhibition by free and acetylated polyamines

Polyamine conc:	Percentage Growth Reversal		
	2 μ M	5 μ M	10 μ M
Pu	10 $\pm$ 2	27 $\pm$ 4	87 $\pm$ 5
Spd	92 $\pm$ 6	95 $\pm$ 5	100 $\pm$ 7
Spm	96 $\pm$ 7	105 $\pm$ 9	103 $\pm$ 10
N1-Ac-Pu	0 $\pm$ 3	0 $\pm$ 4	3 $\pm$ 3
N1-Ac-Spd	71 $\pm$ 6	94 $\pm$ 6	97 $\pm$ 5
N8-Ac-Spd	29 $\pm$ 6	53 $\pm$ 5	79 $\pm$ 11
N1-Ac-Spm	87 $\pm$ 7	95 $\pm$ 8	100 $\pm$ 7

PC-93 cells, pretreated with 1 mM DFMO for 4 days, were cultured in the presence of 1 mM DFMO, 0.1 mM aminoguanidine (to prevent polyamine degradation and formation of toxic compounds in the presence of serum amine oxidases [37]), and 2, 5 or 10 μ M polyamines. Growth was evaluated by the MTT-assay [32]. The difference between untreated cells and cells exposed to DFMO alone during the entire experiment was defined as 100% reversal.

maximal rates of cell proliferation. Intracellular polyamine concentrations of these cells were determined and compared to the levels observed in control cells and in cells treated with DFMO continuously. Surprisingly, most of the added compounds were recovered from the cells in relatively low concentrations. For instance, Pu levels of cells cultured in the presence of 10 μ M Pu were found to be as low as in DFMO only treated cells. Spd levels in these cells, however, were largely restored to the normal control level. In fact, reversal of DFMO-induced inhibition of growth appeared to be associated with recompensation of intracellular Spd levels in all situations tested. This result implicates that polyamines, including the acetylated compounds, are extensively metabolized and ultimately converted into Spd. This result furthermore suggests that Spd may play a key role in the regulation of cell proliferation.

POLYAMINE UPTAKE AND INTERCONVERSION, A WAY TO ESCAPE FROM DFMO-INDUCED GROWTH INHIBITION?

The results presented above clearly showed that polyamine-depleted cells are capable taking up exogenous polyamines from the external environment and utilizing them for maintenance of cell proliferation. The existence of a specific polyamine transport system -although not yet well characterized at the molecular level- has been demonstrated in a number of cells and tissue types [38-41], including the rat prostate [42] and tumors derived from it [43]. It is of considerable practical interest that polyamine uptake was reported to be stimulated in polyamine-depleted cells [38,42,43]. Thus, polyamine uptake may provide an effective means to the cells to replenish intracellular polyamine pools even when biosynthesis is completely blocked. Uptake from environmental sources may well proceed even faster than resumed synthesis after discontinuation of treatment with polyamine inhibitors.

In order to study the uptake of polyamines by DFMO-treated PC-93 cells in a more direct way, we recently analyzed the intracellular polyamine content during exposure to various polyamines in a time course fashion (0-24 hours). The preliminary results from such studies indicated that intracellular concentrations of most of the added polyamines, including Pu and N1-acetyl-Spd, increased with time of incubation, reaching a maximum after 6-8 hours. When Pu was administered, Spd levels started to rise concomitantly and exceeded Pu levels after incubation times longer than 4-5 hours. In agreement with the reported energy-dependence of the transport process [39], Pu uptake, as well as its conversion to Spd, was almost completely abolished if the cells were incubated at low temperature (4 °C). Uptake of N1-acetyl-Spd was also shown to be temperature sensitive, suggesting that this compound is transported by the same mechanism. This result seems to be at variance with earlier reports in which the structural requirements of potential substrates for the polyamine transport system in L1210 were described [44]; from these studies it was concluded that primary amine groups of Spd, N1 as well as N8, are the critical determinants in that respect. Possibly, substrate specificity may not be identical in different cell types. Since acetylated polyamines are abundantly present in the circulation, our findings may have considerable impact with regard to the possibility that tumor cells may escape from DFMO-induced inhibition simply by uptake of these compounds from the environment.

The results from polyamine content analyses after 'long term' exposure to the various polyamine compounds clearly indicated that active metabolism, ultimately leading to the formation of spermidine, did take place in PC-93 cells. This can be achieved by means of the polyamine interconversion pathway, involving the enzymes Spd/Spm acetyltransferase (SAT) and polyamine oxidase (PAO), as schematically shown in Fig. 7. Interestingly, acetyl-Pu, a compound that cannot be converted to Spd, was indeed shown to be ineffective with respect to the restoration of growth, in contrast to N1-acetyl-Spd and N1-acetyl-Spm, both known to be readily converted by PAO. The conversion of N8-acetyl-Spd to Spd is less obvious; it is not well established yet whether this compound can be oxidized by prostatic PAO or whether it is deacetylated directly as has been shown to occur in liver [22].

CONCLUDING REMARKS

The presence of exogenous polyamines, either in the free or in the acetylated form, allows cells to proliferate at a normal rate even when the endogenous *de novo* formation is blocked by polyamine synthesis inhibitors such as DFMO. Since essentially all types of tumor cells were shown to be sensitive to DFMO when tested *in vitro*, the observed failures to respond *in vivo* may well be ascribed to the availability of polyamines from environmental sources in the *in vivo* situation. Application of polyamine inhibition for therapeutic purposes should therefore not be restricted to inhibition of biosynthesis alone, but should also prevent uptake and/or intracellular utilization of polyamines and their derivatives. Better knowledge of the properties of the polyamine-specific transport system may assist in designing specific transport inhibitors that will

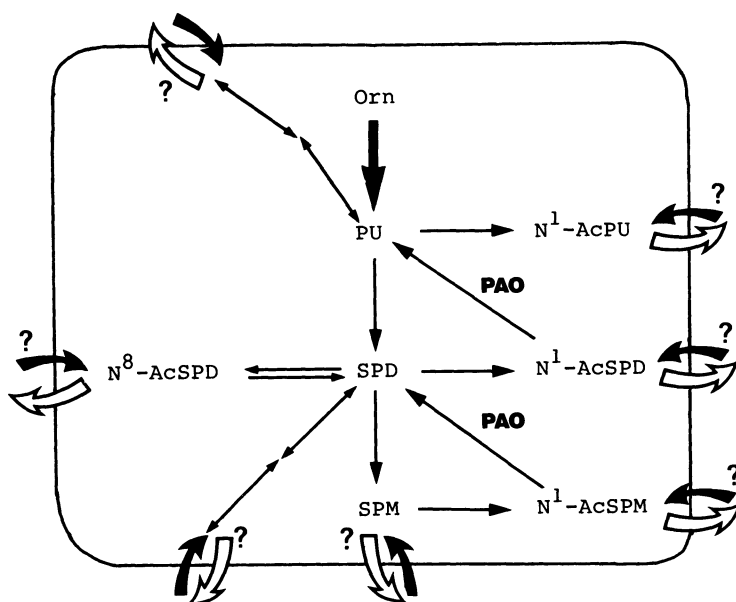


Figure 7: Schematic diagram showing the major pathways of polyamine biosynthesis, interconversion and uptake.

block the entry of exogenous polyamines into the cells. Studies on the further characterization of the uptake system are currently underway. Another possibility would be prevention of the utilization of polyamines once they taken up by the cells. The results from our studies suggest that especially the formation of Spd should be blocked in order to inhibit cell proliferation. This might be achieved, at least partly, by the use of specific inhibitors of PAO, an approach that was already shown to be successful in some murine tumors [45]. Preliminary studies using PC-93 cells also demonstrated that stimulation by N¹-acetyl-Spd was abolished in the presence of such an inhibitor. When these results can be substantiated further, a renewed approach based on interference with biosynthesis as well as uptake and/or interconversion may provide improved possibilities for therapeutic application of polyamine inhibition.

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DISCUSSION

Dr. Coffey:

Heparin binds the basic fibroblast growth factor in the extracellular matrix, and polyamines at times of growth are in millimolar concentrations. That is a fierce concentration. Is there a possibility that these basic polyamines could be releasing basic fibroblast growth factors from its sequestry to acidic extracellular matrix?

Dr. Romijn:

No.

Dr. Coffey:

Dr. Story, do you think that's possible? For instance, if you add polyamine to the cells, will it release basic fibroblast growth factor so that you do not have to treat it with heparinase? In other words, a positive ion exchange system?

Dr. Story:

When I return to the lab I will see.

Dr. Romijn:

I think that polyamine will bind to anything with negative charges. On the other hand, not all the polyamines that we are measuring are available as pools of free polyamines. Apparently, polyamines are well compartmentalized. I can't really say whether it's doing what you suggested.

Dr. Miller:

The concentrations of polyamines in prostatic secretions and in semen will reach millimolar concentrations. Have you looked to see if the cells are adding polyamines to the media also? What's the contribution of the cells? Do they actually secrete polyamines into the media?

Dr. Romijn:

Very little. We have not only measured the intracellular concentrations, but we have also measured the extracellular concentrations. We have some fetal calf serum in our medium, but we measure the

polyamine levels also in the fetal calf serum. Our impression is that it may vary quite a lot between one batch and another. The batches we have used for these experiments contain fairly low concentrations of polyamines. My impression is that the cells did not add to that to a measurable extent.

Dr. Heston:

Regarding the cells in which you are measuring the extracellular polyamine content, are they confluent or are they still growing? As you know, as the cells are growing, they are also very active in their uptake. Therefore, you might have cells secreting polyamines that are then rapidly taking them back up into the cell.

Dr. Romijn:

We used subconfluent cultures in all cases.

Dr. Heston:

With regard to serum oxidase that's present in fetal calf serum, how are you protecting against that?

Dr. Romijn:

We have added aminoguanidine in all cases. If we delete the aminoguanidine, there is growth inhibition with 10 micromolar spermine, for instance. You can not do the experiment without aminoguanidine.

Dr. Heston:

In terms of therapy, by inhibiting polyamine synthesis, or blocking polyamine transport, do you feel you would be producing a cytotoxic event to the cell, or do you think that it would be a cytostatic event?

Dr. Romijn:

I think that the data we have so far indicate that polyamine depletion leads to cytostatic events. I can not predict what happens over the long term, i.e. how long we have to deplete cells before it becomes really toxic. But I did not say that polyamine therapy should be a monotherapy. You could well combine it with for instance the substance that you have used. Also, in that case, it is an advantage if one also depletes *in vivo*.

Dr. Coffey:

You know that this replaces calcium. It is one of the most powerful positive molecules and I wonder if it could activate the calcium dependent DNase. We've always looked at these things in terms of cell growth, but is there any evidence of that in cell death or activating the calcium dependent DNA system?

Dr. John Isaacs:

One of the questions is whether the calcium magnesium dependent nuclease doesn't simply need calcium as an allosteric co-factor for its activity. If it's an allosteric regulator, then it's possible that polyamines could also be a regulator and that may be one of the explanations for why cell cycle specific agents that block cells in S seem to fragment their DNA.

HEPARIN-BINDING (FIBROBLAST) GROWTH FACTOR/RECEPTOR GENE EXPRESSION IN THE PROSTATE

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ABSTRACT

Heparin-binding (fibroblast) growth factors (HBGF) play both autocrine and paracrine roles in growth of normal and tumor prostate epithelial and mesenchymal cells. In the rat, expression of HBGF-1 dominates in normal tissue and slow-growing Dunning prostate tumor tissues. Both HBGF-1 and HBGF-2 are expressed in fast-growing, malignant variants of the Dunning tumor. Expression of HBGF-1 occurs in young normal prostate epithelial cells and disappears with age. Slow-growing Dunning tumor mesenchymal cells, not epithelial cells, constitutively express HBGF-1. HBGF requirement and receptor phenotype is altered in tumor epithelial cells. In contrast to IGF-1 and the Flg/Bek/Cek gene product, a candidate HBGF receptor, neither HBGF-1 nor HBGF-2 expression in normal and tumor tissues increases during androgen-stimulated growth. Flg/Bek/Cek is expressed in young prostates, during androgen-stimulated growth of adult prostate, constitutively in tumors, and appears limited to the stromal cell fraction of the slow-growing tumors. Expression is elevated in the fast-growing, highly malignant tumors. Normal and slow-growing tumor epithelial cells have receptor sites for HBGF that may differ from Flg/Bek/Cek. Similar to rat prostate tissue, HBGF-1 expression dominates in young human prostate tissue. However, both HBGF-1 and HBGF-2 mRNA as well as the Flg/Bek/Cek gene are detectable in adult human prostate tissue. These differences in rat and human tissue may reflect differences in the epithelial:stromal cell ratio in prostates of the two species.

INTRODUCTION

Advances in serum-free, defined cell culture methods have resulted in methods for selection and maintenance of both epithelial and mesenchymal cells from primary normal prostate and prostate tumors (1-3). A comprehensive, rigorous analysis of the growth requirements has revealed specific differences in requirement for androgen-independent growth of normal and tumor-derived cells (4,5). A member of the insulin-like growth factor (IGF), epidermal growth factor (EGF) and heparin-binding (fibroblast) growth factor (HBGF) families are required for normal epithelial cell proliferation at all cell densities (2-8). Cholera toxin also exerts a required mitogenic stimulus (1,2). Normal mesenchymal cells do not require the cholera toxin stimulus, but also require an IGF, EGF-like factor and an HBGF (unpublished results). Similar to most normal mesenchymal cells, normal prostate mesenchymal cells also respond to platelet-derived growth factor (PDGF) (unpublished results). Transforming growth factor type beta (TGF- β) exerts a powerful inhibitory effect on normal prostate epithelial cells (5) with little effect on normal mesenchymal cells (unpublished results). Epithelial cells from the slow-growing, androgen-responsive, relatively non-malignant Dunning R3327PAP tumor exhibit remarkable alterations in growth factor responsiveness (2,4). Similar to normal cells, proliferation is stringently dependent on IGF, however, the cells are independent on cholera toxin and require either EGF or an HBGF (4). At high cell densities, the epithelial cells are independent on both EGF or HBGF. TGF- β only inhibits tumor epithelial cell proliferation when the cells are stimulated by EGF (5). The presence of an HBGF appears to override the inhibition by TGF- β (5). The presence of HBGF in the local tumor environment may be the avenue by which tumor epithelial cells escape inhibition by TGF- β .

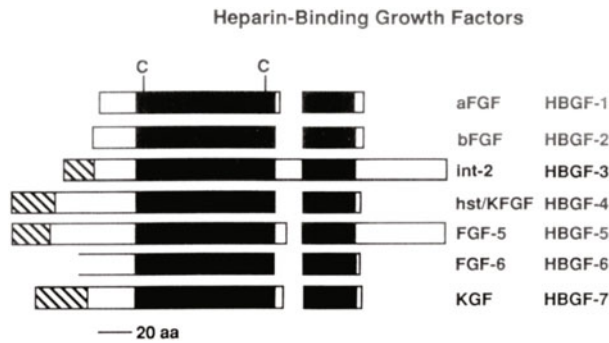


Figure 1: Topological relationships of the heparin-binding (fibroblast) growth factor family (adapted from ref. 10). Regional homology among the seven members of the family are indicated in black. Both the suggested HBGF number and common acronym is indicated. HBGF-3,4,5 and 7 exhibit candidate secretory signals indicated by the cross-hatches. The 5'-end of HBGF-6 has not been determined. All seven polypeptides exhibit homology at 19% of the residues and 28% if conservative substitutions are discounted. Conserved cysteine residues are indicated.

The current report summarizes the expression of the HBGF-1 (acidic fibroblast growth factor) and HBGF-2 (basic fibroblast growth factor) genes and HBGF receptor sites in normal and tumor prostate tissues.

RESULTS AND DISCUSSION

Heparin-binding Growth Factors (HBGF) in Rat Prostate

The HBGF family (for recent review, see ref. 9) currently consists of seven different gene products that exhibit a 30% to 55% amino acid sequence homology among family members (Fig. 1). HBGF-1

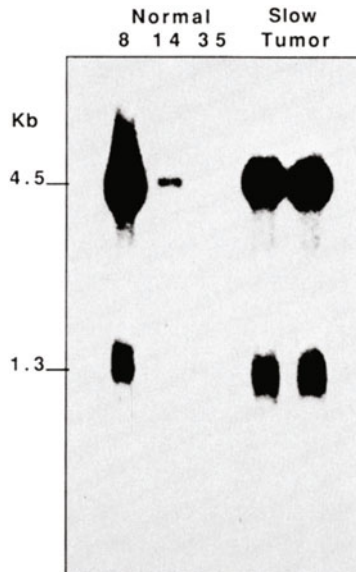


Figure 2. Expression of HBGF-1 mRNA in normal rat prostate and the Dunning R3327PAP tumor. Each lane contained 4 μ g poly(A⁺)RNA. See ref. 6 for methods. HBGF-2 mRNA could not be detected on the same blots.

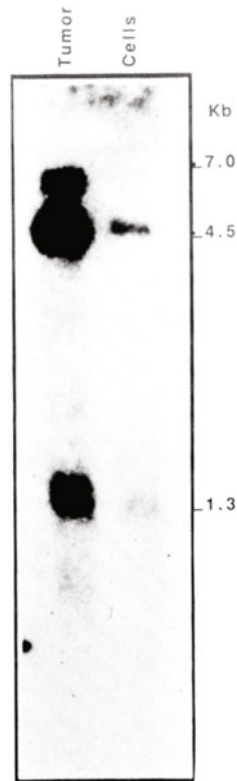


Figure 3: Expression of HBGF-1 and HBGF-2 in Dunning R3327AT3 tissue and cultured cells. The blots were first hybridized to [^{32} P]HBGF-2 cDNA followed by the HBGF-1 cDNA. The band at 7.0 is that of HBGF-2 and bands at 4.5 and 1.3 kB are HBGF-1 transcripts.

(acidic fibroblast growth factor) and HBGF-2 (basic fibroblast growth factor), the most widely distributed members of the family in tissues, are characterized by lack of a conventional secretory signal sequence. The structure of the other five members of the family have been deduced from cDNA that was cloned from various specialized cell or tissue sources and each exhibit a putative secretory signal sequence at the aminotermminus (9). Although the precise regulation of synthesis, secretion and metabolism of different members of the HBGF family is not clear, the affinity of the polypeptides for heparin-like glycosaminoglycans in the extracellular environment distinguishes the growth factor family and probably plays a major role in physiological function of the factors (9).

An age-dependent expression of specifically HBGF-1 mRNA characterizes normal rat prostate tissue (Fig. 2). HBGF-1 mRNA transcripts at 4.5 and 1.3 kilobases were abundantly expressed in 8 week-old animals, declined dramatically by 14 weeks and were undetectable in prostates of 35 week-old animals. HBGF-2 mRNA transcripts were undetectable in the same blots. HBGF-1 expression appeared to be a characteristic of the epithelial cells from normal young prostate and was undetectable in cultured normal mesenchymal cells (Fig. 3). These experiments do not preclude the qualitative presence of HBGF-2 in prostate tissue and cells. HBGF-2 transcripts at 1.8 Kb are detectable when extremely high slot loads of poly(A⁺)RNA were analyzed in separate experiments. However, the weak HBGF-2 signals are at least 5 to 10 times less than the positive samples for HBGF-1. The weak HBGF-2 signal precluded quantitative analysis of HBGF-2 transcript levels among different tissues and cells.

Both the slow-growing, androgen-responsive, low malignant Dunning R3327PAP tumor and the rapidly-growing, androgen-independent, highly metastatic Dunning R3327AT3 tumor exhibited constitutively high levels of HBGF-1 mRNA expression (Fig. 3). Significant expression of HBGF-2 mRNA (7.0

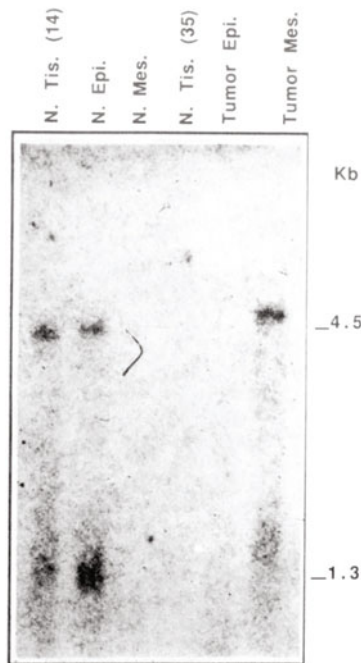


Figure 4: Expression of HBGF-1 mRNA in cultured cells from normal prostate and the R3327PAP tumor. No HBGF-2 mRNA could be detected under similar hybridization conditions.

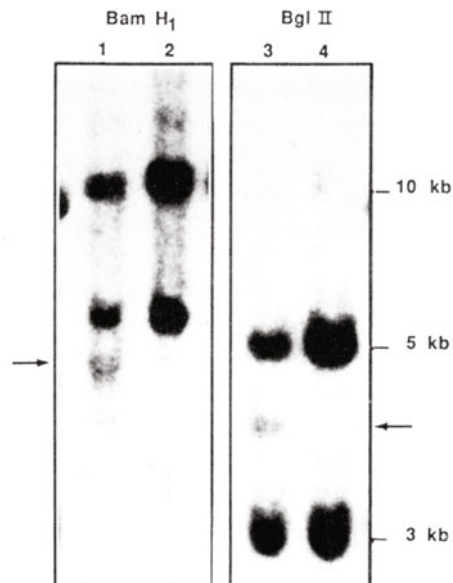


Figure 5: Southern restriction enzyme fragmentation analysis of R3327PAP tumor cell genomic DNA with HBGF-1 cDNA. Abnormal bands are indicated. Lanes 1 and 3 are DNA from tumor-derived mesenchymal cells and lanes 2 and 4 are from epithelial cells. The epithelial cell pattern is identical to DNA from normal Copenhagen rat liver, the Dunning R3327PAP and AT3 tumor tissue, and cultured AT3 tumor-derived cells.

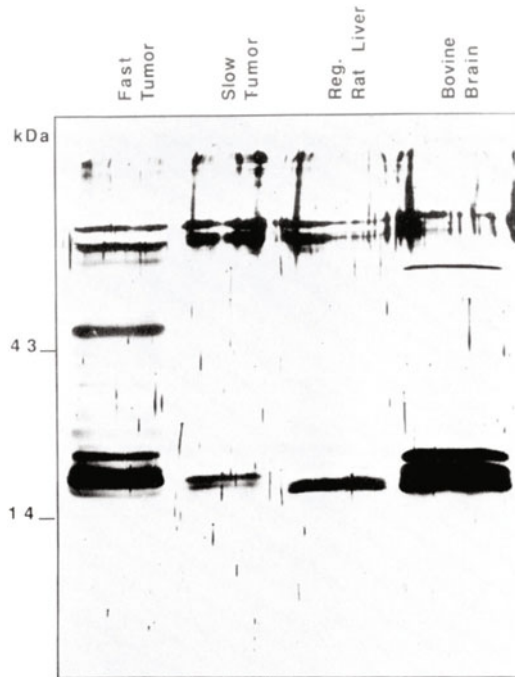


Figure 6: Western blot immunochemical analysis of HBGF-1-like antigen in the R3327PAP and R3327AT3 tumor tissues. Tissue extracts were enriched by heparin-agarose chromatography (8), electrophoresed, electroblotted and reacted with a polyclonal antibody raised against native bovine brain HBGF-1 which exhibits less than 10% cross-reactivity with HBGF-2. A 99% specific polyclonal antibody against amino acid residues 60-98 of bovine HBGF-1 yielded the same band pattern below the common artifacts in the upper gel. Bands were specifically reduced by excess synthetic peptide antigen. The R3327 tumor exhibits the major 16 kDa antigen similar to regenerating rat liver (20) and bovine brain (8). Bovine brain exhibits native 18 kDa HBGF-1 with acetyl-ala amino terminus and the truncated 16 kDa species beginning at asn₂₁ (8). The R3327AT3 extracts exhibit major additional antigens at 22 kDa and 52 kDa in addition to several other minor cross-reactive bands.

kilobases) relative to HBGF-1 was only apparent in the R3327AT3 tumor. Surprisingly, specifically the R3327PAP tumor-derived mesenchymal cells appeared to be the origin of the HBGF-1 transcripts (Fig. 4). No significant HBGF-1 expression was apparent in the R3327PAP tumor-derived epithelial cells. These results were surprising since the R3327PAP tumor is generally considered to be a well-differentiated adenocarcinoma whose abnormal properties are largely determined by abnormal epithelial cells. These results, together with the fact that the R3327PAP tumor routinely gives rise to immortal epithelial and mesenchymal cell lines in culture relative to normal tissue (4), suggest that the tumor might be an "adenocarcinoma" in respect to HBGF-1 expression. Abnormal bands in the Southern hybridization pattern of genomic HBGF-1 DNA were consistent with the abnormal expression of the HBGF-1 gene in specifically the tumor mesenchymal cells (Fig. 5). The results suggest that constitutive, abnormal expression of HBGF-1 by the tumor mesenchymal cells may support tumor epithelial and mesenchymal cell fractions by paracrine and autocrine mechanisms, respectively. Although the highly malignant R3327AT3 tumor tissue *in vivo* expresses high levels of both HBGF-1 and HBGF-2 mRNA, the single clonogenic cell type which emerges from the tumor in culture (6) expresses much reduced levels of both transcripts. HBGF-2 transcripts were barely detectable in the cultured cells (Fig. 3). Yet tumor tissue which results from the cultured cells implanted back into the host animals exhibited consistently high level expression of both genes. This suggests a yet undescribed host mechanism that supports high level expression of both genes or that HBGF-2 gene expression in particular is induced in host tissue by presence of the tumor. The AT3 tumor tissue also exhibits considerable heterogeneity in antigens that cross-react with specific antibody against bovine brain HBGF-1 (Fig. 6). This was in contrast to HBGF-1

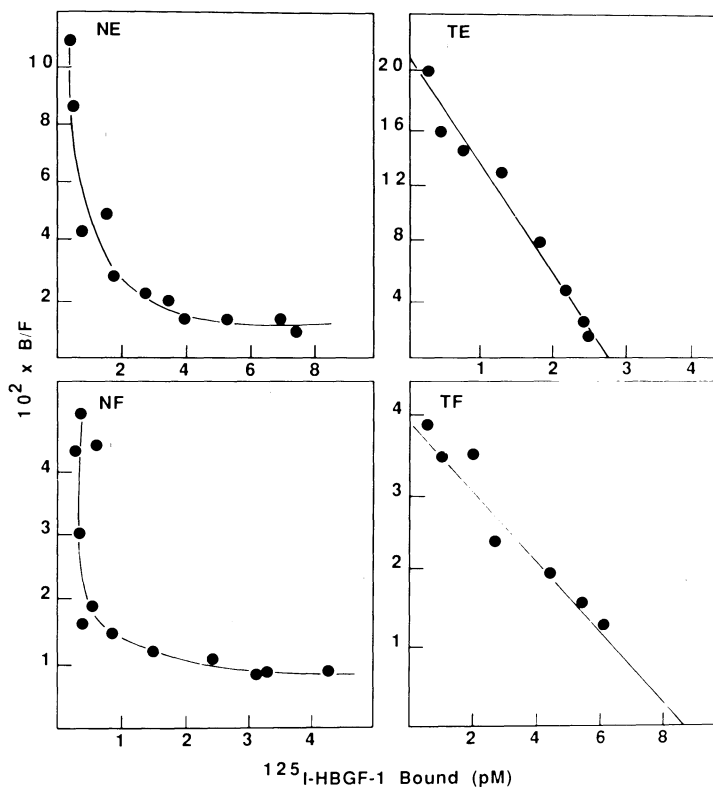


Figure 7: Scatchard plot of specific [^{125} I]HBGF-1 detergent extractable binding to prostate cells. NE, normal epithelial cells; TE, R3327PAP epithelial cells; NF, mesenchymal (fibroblast-like) cells from normal tissue; TF, mesenchymal cells from the PAP tumor. Reprinted from ref. 6.

antigen expressed by both young normal prostate tissue (not shown) and the R3327PAP tumor tissue (Fig. 6). Additional Western blot analysis not shown here using anti-HBGF-2 confirmed the presence of authentic 16 kDa HBGF-2 antigen in the AT3 tumor tissue extracts. The high-level expression of both HBGF-1 and HBGF-2 genes associated with the single, highly clonogenic cell type that composes the R3327AT3 tumor *in vivo* may explain its autonomy and extremely metastatic character.

The HBGF Receptor in Prostate Cells

Similar to most cell types, normal and tumor-derived prostate epithelial and mesenchymal cells exhibit abundant levels of heparin-like binding sites for HBGF on the pericellular matrix (6). By careful analysis and proper equilibrium binding conditions, the association of HBGF with non-receptor, heparin-like sites can be distinguished from high-affinity membrane-bound receptor sites (6-11). Scatchard analysis indicated that both normal- and tumor-derived epithelial and mesenchymal cells displayed similar numbers of total HBGF receptor sites that have an apparent mean molecular mass of 130 kDa (Fig. 7-9). Normal cells exhibited curvilinear Scatchard plots while the tumor-derived cells exhibited simple linear plots (Fig. 7,8). It is unclear whether the curvilinear plots indicate negative cooperative site-site interaction of a single receptor class or that the normal cells possess multiple types of binding sites relative to the tumor cells. R3327PAP-derived epithelial cells and R3327AT3 cells exhibited a reduced ED for HBGF for proliferation that is 20% of that for normal epithelial and mesenchymal cells and PAP tumor mesenchymal cells (4-6). These results may suggest that fractional occupancy of HBGF receptors is sufficient to support tumor epithelial cell proliferation relative to normal cells and tumor mesenchymal cells. Both normal and tumor cells internalize receptor-bound [^{125} I]HBGF-1 at equal rates (6), however, normal epithelial cells appeared to metabolize receptor-internalized HBGF-1 faster than tumor epithelial

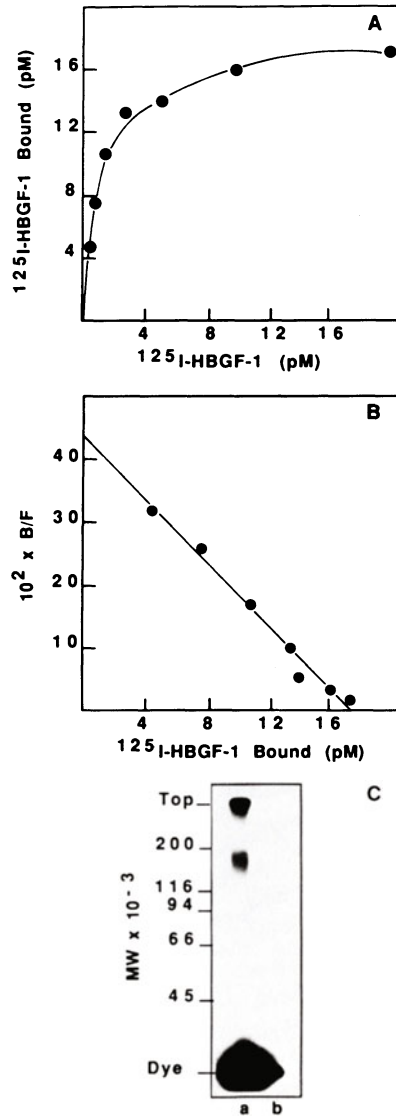


Figure 8 A-C: HBGF receptor characteristics of R3327AT3-derived cells. A. specific [¹²⁵I]HBGF-1 binding. B. Scatchard plot. C. SDS-PAGE analyses of [¹²⁵I]HBGF-1 covalently cross-linked to the cells.

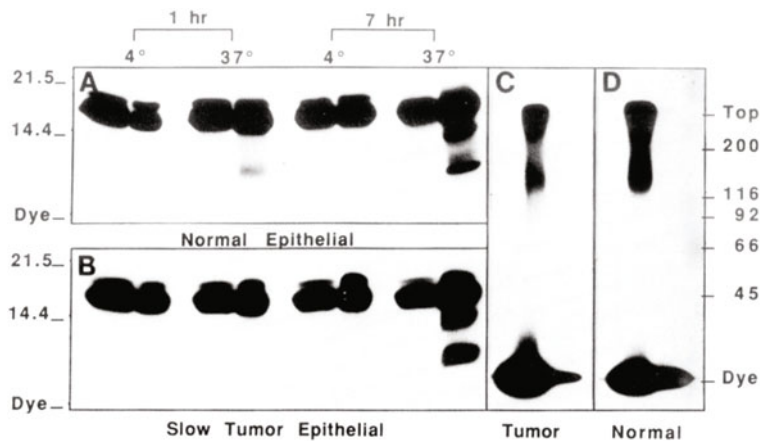


Figure 9: HBGF receptor characteristics and metabolism in normal and R3327PAP tumor epithelial cells. A,B. Receptor-bound [^{125} I]HBGF-1 was incubated at the indicated times and temperatures and then analyzed by SDS-PAGE and autoradiography. Each pair of lanes indicates medium ligand versus cell-associated ligand. C,D. Covalent affinity cross-linking of [^{125} I]HBGF-1. Reprinted from ref. 6.

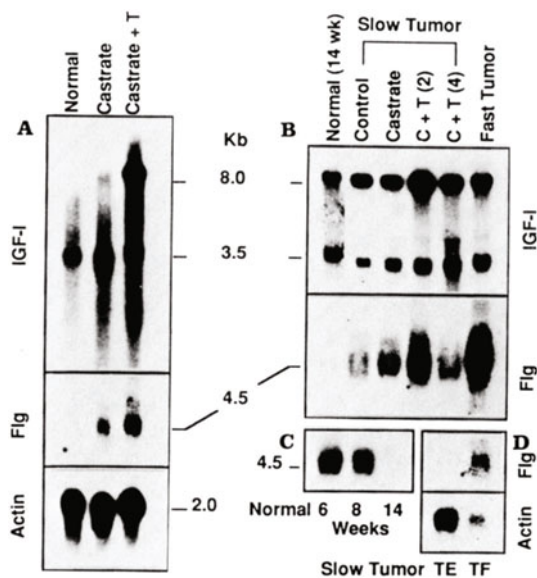


Figure 10 A-D: Comparative expression of insulin-like growth factor type one (IGF-1) and Flg/Bek/Cek to HBGF-1 and HBGF-2 in prostate. A. IGF-1 and Flg/Bek/Cek mRNA in normal (25 weeks), castrated and androgen-treated prostates. Animals were castrated for 14 days and then treated with daily doses of testosterone for 2 days. The same blot was probed with cDNA for rat IGF-1, Flg/Bek/Cek cloned from an R3327AT3 cDNA library and actin. B. Animals bearing the R3327PAP tumor for 60 days were castrated for 14 days and then treated with testosterone daily for 2 and 4 days before excision of tumor tissue for analysis. The two frames are the same blot. C. Normal rat prostate poly(A⁺)RNA was probed with the rat Flg/Bek/Cek cDNA. D. Poly(A⁺)RNA from R3327PAP epithelial (TE) and mesenchymal cells (TF) probed with rat Flg/Bek/Cek cDNA. The same blots were probed with actin cDNA.

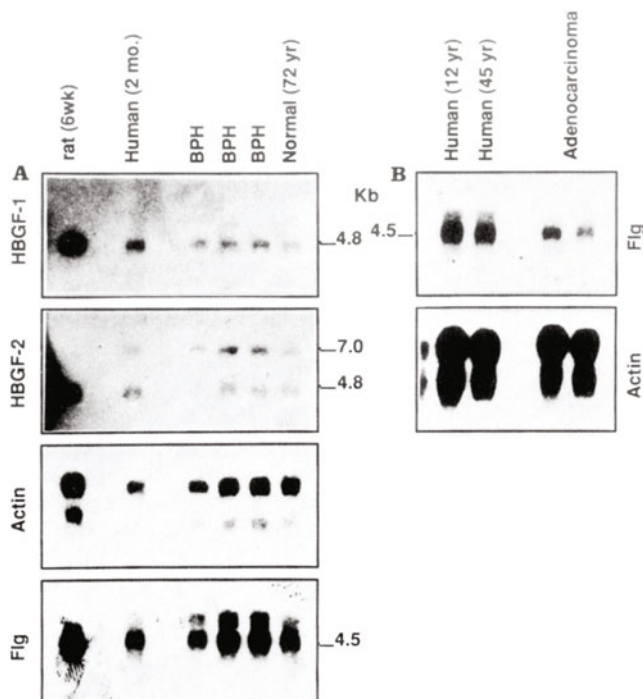


Figure 11 A & B: Expression of HBGF-1, HBGF-2 and *Flg/Bek/Cek* mRNA in human prostate. The indicated human prostate samples were obtained at autopsy (normal at 6 hr post-mortem) or surgery (BPH and adenocarcinoma). A. The four frames are from the same blot. The blot was first probed with HBGF-1 cDNA (upper frame) and then 7 days later with HBGF-2 cDNA without deprobing the blot (second frame). The 4.8 kB band is assumed to be HBGF-1 and the 7 kB band HBGF-2. The same blot was probed several months later with human *Flg/Bek/Cek* cDNA (fourth frame) and actin (third frame) after decay of the radioactive HBGF-1 and HBGF-2 bands. B. *Flg/Bek/Cek* gene expression of normal human prostate and surgically- removed well-differentiated, slow-growing adenocarcinoma tissue.

cells (Fig. 9). Internalized HBGF-1 was metabolized extremely slowly relative to other growth factors and appeared as discrete 10 and 13 kDa peptides inside the cells (Fig. 9). It is unclear whether this remarkable stability plays a functional role in HBGF metabolism and whether metabolism of the HBGF-1 ligand parallels metabolism of the HBGF receptor.

Hormonal-regulation of growth factor/receptor gene expression

Neither the HBGF-1 nor HBGF-2 mRNA levels appeared to be significantly affected by androgen in normal prostate, the R3327PAP tumor, or the cells derived from respective tissue (results not shown). This is in contrast to IGF-1 transcripts which were remarkably increased by testosterone therapy in the prostate of castrated animals and which transiently rose in the R3327PAP tumor after testosterone treatment of castrated animals bearing the tumor (Fig. 10). The *Flg/Bek/Cek* gene product, which has recently been proposed as a candidate for the HBGF receptor (13-16), is also increased by testosterone treatment of both normal animals and the R3327PAP tumor (Fig. 10). *Flg/Bek/Cek* gene expression appears similar to HBGF-1 gene expression in the normal and R3327PAP tumor and derived cells. Expression appears age-dependent in normal prostate and limited to the mesenchymal cells from the PAP tumors (Fig. 10). The *Flg/Bek/Cek* gene is overexpressed in the R3327AT3 tumors (Fig. 10) which facilitated ease of cloning the rat gene from an AT3 tumor cDNA library. These results suggest that, if the *Flg/Bek/Cek* gene product is the HBGF receptor in mesenchymal cells, then the epithelial cell HBGF receptor may be a different gene product.

The extremely low level of both HBGF-1 and HBGF-2 mRNA and antigen in normal, regenerating prostate tissue is also puzzling because an HBGF is assumed to be a requirement for both epithelial and mesenchymal cells during the regeneration process. Since both normal epithelial and mesenchymal cells in culture required an HBGF and exhibit HBGF receptors, another member of the HBGF gene family (Fig. 1) may be at play during androgen-stimulated normal prostate regeneration.

HBGF expression in human prostate

HBGF-2 has been isolated and sequenced from human benign hyperplastic prostate tissue (BPH) (17) and the HBGF-2 mRNA transcripts detected (18). There has been little comparative study of expression of HBGF-1 and HBGF-2 in human tissues and comparison to the data from model rat prostate models. In our hands, the expression of HBGF-1 and HBGF-2 in a limited number of human tissues so far analyzed can be summarized as follows: both HBGF-1 and HBGF-2 appeared to be co-expressed at equal levels in adult normal prostate and BPH tissue (Fig. 11). These results differed quantitatively from rat tissues which exhibited predominately HBGF-1 expression in normal prostates of only young animals. However, similar to rat tissues, HBGF-1 mRNA expression in young human prostate predominated and exceeded that of mature individuals. Expression of the Flg/Bek/Cek gene product (candidate HBGF receptor) in mature human prostate tissues also greatly exceeded that in mature rat prostate (Fig. 11). Neither HBGF-1, HBGF-2 nor the Flg/Bek/Cek mRNA expression appeared elevated in a limited number of surgically- removed, slow-growing, non-metastatic, well-differentiated human adenocarcinomas that have been analyzed (Fig. 11).

The collective and largely quantitative differences in gene expression between rat and human tissues may simply reflect the quantitative differences in the epithelial-to-mesenchymal cell ratio in the rat and human tissues. The epithelial to stromal cell ratio has been estimated at 5 to 1 in mature rat prostate and close to 1 to 1 in the human (19). The Dunning R3327PAP tumor also exhibits a high epithelial:stromal cell ratio. This idea is supported by the fact that both HBGF and Flg/Bek/Cek gene products appear to be of stromal origin in prostate tissues with well-defined epithelium and stroma except in the case of HBGF-1 expression in epithelial cells from young normal prostates. It is also noteworthy that the 3.5 kB transcript IGF-1-like transcript and the Flg/Bek/Cek gene appear to increase in normal rat tissue after castration, a condition which enhances stromal:epithelial cell ratio (Fig. 10). IGF-1 transcripts at 8, 3.5, and 1 kB are associated with specifically the mesenchymal cell fraction of various prostate tissues (our unpublished results).

CONCLUSIONS

Clearly, the HBGF family and receptors are candidates for a major role in developmental biology of the prostate, androgen-dependent maintenance and regeneration of prostate, prostatic hyperplasia, and the progression of prostatic tumors to life-threatening stages of malignancy. The expression of specific members of the HBGF gene family appears developmentally-regulated and cell-type dependent. Clarification of which members of the HBGF family are at play and the mechanism of their expression in various prostate tissues and cells needs complete comparative study. The nature of the heterogeneity of the HBGF receptor in various prostate cells and tissues awaits complete molecular characterization and reconstitution experiments using molecular and immunochemical techniques. Whether members of the HBGF family and their receptors are rate-limiting, causal factors in prostatic disease and can be of utility for diagnosis and targets for therapy also awaits rigorous investigation.

ACKNOWLEDGEMENTS

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DISCUSSION

Dr. Story:

As you probably know, Stephen Jacobs and I published a paper not too long ago in which we suggested that the predominant growth factor in the rat prostate was basic fibroblast growth factor(BFGF), not heparin binding growth factor 1 (acidic FGF). Are your studies confined strictly to the signal for heparin binding growth factor 1?

Dr. McKeehan:

We've done both. In our hands the presence of the androgen- HBGF 1&2 correlate exactly to the signal. We've used antibody immuno assay and on western blot after extraction of tissue they correlate with the presence of the signal. We haven't done immunohistochemistry adequately, although so far that all correlates. In summary, we believe that there are some quantitative differences in signal intensity and amount of androgen in rodent versus human tissues. But on the average, when we begin to look at overall expression and the cell type of origin, the HBGF 1 and HBGF 2 are co-expressed and the HBGF 1 expression seems to be age dependent in human tissues and in rat tissue. However, the HBGF 1 and HBGF 2 relationship is possibly a little bit different. That is, HBGF 2 clearly is higher in the human tissue compared to the rat tissues as we've measured it. But quantitatively, they are coexpressed, and the androgen is there equal roughly to the mRNA signal. The way that I interpret these differences right now is that it may reflect cellularity of the two tissues. That is, I understand from others that in the rat there is a higher epithelial/stromal ratio than in most human tissues. It is more like 5 or 10 to 1 in the rat, depending on the lobe and in the human it's more like 1:1. We now believe that the mesenchymal cell, at least in mature prostate and in the tumors, is the main cell of origin of both members of the HBGF family, at least in our hands; it's also the origin of the flag gene, which is now the candidate of HBGF receptor. These differences may reflect the difference in relative epithelial and mesenchymal cellularity of these glands. That is how we are manipulating those two cell types and which one is growing in relation to the other. During castration there is obviously a change in ratio to cellularity as well as the growth rates of the two cell compartments.

Dr. Thompson:

Is it your belief that there are marked differences in the biological activity of acidic and basic FGF? Are there specific receptors or should we consider them together?

Dr. McKeehan:

It's an open question at the receptor level until we sort through the receptor. It's very likely, in my opinion, that there's a receptor family, just like we see in other growth factors, i.e. the IGF family and now there's two PDGF receptors with varying specificity. We don't know that. We'll have to clone them and get the epithelial cell and the mesenchymal cell receptors (both high and low affinity classes). We have to determine if flag is the true binding site, or is really a signal transmittor of the true binding site. There are a lot of possibilities that we are sorting through now in the field. In answer to your question directly, this is very difficult but one must go through it step by step. First of all, we know that both of these factors are driven and their stability and lifetime in the environment is affected dramatically by heparin and sulphate pyridial glycan of the extracellular matrix. We now know that the HBGF 1 & 2 vary in a specific sensitivity cut site in the C terminus to thrombin and plasmin. Therefore, HBGF 1 is much more labile in the presence of thrombin, plasmin and many other proteases in the microenvironment where these proteases are at play. We've now shown this in our laboratory in preliminary results, and this may explain why HBGF 1 androgen is much less stable than HBGF 2 in a variety of tissues, as historically we just extract and try to isolate these two different proteins. We have shown now that you can use inhibitors of plasmin and thrombin that will prolong the life even in an endogenous cell culture system of HBGF 1 androgen. These are generally antiplasmins and antithrombins and that's a whole other story in itself. This can dramatically affect the lifetime and activities of these factors in a bio-assay depending on a whole set of conditions, i.e. extracellular matrix, heparin, heparin and sulphate around the cell, plasmin and thrombin and antiprotease balance, which will give you an apparent difference in lifetime and bioactivity of the two factors and which one might interpret as specificity. We believe so far that the two factors compete absolutely equally with the same binding site that they cross-link. It comes up as a 130Kd band by the type of analysis we've done.

Dr. Sun.:

You said that one cell line is keratin-negative, but when you put it *in vivo*, it becomes keratin positive. Is this a clonal population? Can you rule out the possibility that the keratin-positive cells belong to a different population that is selected *in vivo*?

Dr. McKeehan:

These are primary cultures from the tumor. We very seldom go culture- tumor- culture- tumor, where we would get a lot of selection. Also, I might mention that this correlates dramatically with gene expression in culture versus in the animal. Clearly this tumor is expressing much higher levels of endogenous HBGF 1 & 2 and the flag gene when it's back in the animal compared to culture. So, in answer to your question directly, I don't believe we are selecting a clonal cell line. I think we are at least going back into the animal with the major population in the culture. We've done that over and over.

CHARACTERIZATION AND PARTIAL PURIFICATION OF A NON-HEPARIN-BINDING PROSTATE GROWTH FACTOR FROM CANCEROUS HUMAN PROSTATE

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ABSTRACT

A polypeptide growth factor has been isolated from human prostatic tissue (PrGF) obtained at the time of radical prostatectomy. Following extraction in 1.0 M acetic acid and column chromatography, preliminary characterization shows this trypsin-sensitive mitogen to be stable to acidic and reducing conditions and only minimally heat inactivated. It has an apparent molecular weight of 22kD, approximate pI of 7.5, and does not bind to heparin-sepharose in 0.15 M NaCl. In contrast to EGF and bFGF, PrGF stimulates uptake of thymidine by quiescent NRK-52E kidney epithelial cells. To further delineate which factors stimulate this cell line, a separate preparation was made by extraction in high ionic strength, neutral pH, with protease inhibitors (9) and separated into heparin-binding and non-heparin-binding fractions. Only the fraction which did not bind to heparin showed mitogenic activity for the 52E cell line, and this activity was stable to acid conditions suggesting the presence of a mitogen similar to that obtained in the acidic extract. Using a separate fibroblast cell line which is stimulated equally by EGF, FGF, and PrGF the stimulatory effect of EGF but not PrGF was abrogated in a dose-dependent fashion by the addition of anti-EGF. Based on this preliminary characterization, our prostatic growth factor appears to be distinct from EGF and the heparin binding class of growth factors (FGF).

INTRODUCTION

Increasing evidence suggests that polypeptide growth factors, rather than androgens, directly regulate proliferation of prostate epithelium (1). In serum free cell culture cholera toxin, insulin, glucocorticoids, epidermal growth factor (EGF), and acidic fibroblast growth factor (aFGF) have been shown to be necessary for maintenance and proliferation of prostate epithelial cells (2). Transforming growth factor-beta (TGF- β) has been shown to be inhibitory to cell growth (3), an effect that can be overcome by the addition of heparin binding growth factors (4). Osteoblast stimulating factor, EGF, and EGF-like factors as well as aFGF, and basic fibroblast growth factor (bFGF), have been isolated from human and/or rat prostatic tissue (5,6,7).

Most investigators involved in the isolation of growth factors from the prostate have postulated the existence in the prostate of one or more mitogens that have not yet been isolated and identified, particularly in the non-heparin-binding fractions of prostate extracts (8,9). We report the partial purification and characterization of a mitogenic protein (PrGF) derived from tissue obtained at the time of radical prostatectomy for adenocarcinoma of the prostate which does not show affinity for heparin.

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MATERIALS AND METHODS

Tissues and Preparation of Tissue Extracts

Prostate tissue was obtained at open surgery for adenocarcinoma of the prostate. The pathologist present in the operating room selected areas of cancer and these were excised and frozen, stored at -70°C until use.

Two methods of homogenization were used. In an acidic extraction, 100 g lots of prostate were homogenized in 300 cc of 1.0 M acetic acid, cleared by centrifugation and dialyzed against 10 mM ammonium acetate. In a neutral extraction, 100 g lots of prostate were homogenized in 200 cc of 50 mM Tris with 1.55 M NaCl, pH 7.6 containing EDTA (10mM), phenylmethanesulfonyl fluoride (1mM), N-Tosyl-L-phenylalanine chloromethyl ketone (0.3 mM), N-ethylmaleimide (0.05 mM), soybean trypsin inhibitor (10 mg/ml), pepstatin A (4µM), and leupeptin (1mg/L). This was cleared by centrifugation and filtration and the pellet from an ammonium sulfate cut (25%-75%) suspended in and dialyzed against 50 mM Tris with 0.15 M NaCl pH 7.6. All steps were carried out at 4°C.

Assay of Mitogenesis

Either NRK-49F (ATCC CRL 1570) or NRK-52E (ATCC CRL 1571) were plated in 48-well culture plates at a density of 1×10^4 cells/well in DMEM supplemented with 10% fetal calf serum (FCS). After 48 hours the cells were inspected, washed with PBS and lcc of DMEM supplemented with 0.04% FCS was added to each well. The following day mitogens were added and 24 hours later 2.5 µCi ^3H -thymidine was added and incubated for 24 hours. Cells were then washed with PBS/BSA and treated with 10% TCA for 30 min. on ice. DNA was solubilized with lcc of 0.5N NaOH and ^3H -THD incorporation into DNA determined by liquid scintillation. The positive control in each experiment was the amount of ^3H -thymidine incorporation in cells treated with DMEM and 10% FCS. The negative controls were the amount of ^3H -thymidine incorporation induced by DMEM/0.04% FCS.

SP-Sephadex C-25 Cation Exchange Chromatography

SP-Sephadex was prepared as described by Pharmacia. The prostatic extract was applied to a column (4.5 cm x 60 cm) pre-equilibrated with ammonium acetate buffer, pH 5.5. Bound protein was eluted with an ammonium acetate gradient (0.01 - 1.0 M). Two-ml fractions were collected and assayed for growth factor activity.

Affi-Gel Blue DEAE Anion Exchange Chromatography

Pooled growth factor activity from the SP-Sephadex column was dialyzed against 10 mM ammonium acetate, pH 7.0. The sample was applied to an affigel blue DEAE column (3 cm x 19 cm) pre-equilibrated with ammonium acetate buffer, pH 7.0. Bound protein was eluted with an ammonium acetate gradient (0.01 - 1.0 M) and 1.0 ml samples were collected. Samples were assayed for PrGF activity. Active fractions were pooled and lyophilized.

TSK 300 SW HPLC

The TSK 300 column (7.5 mm x 30 cm) was equilibrated with 10mM ammonium acetate buffer, pH 7.0. The column was prestandardized with molecular weight standards. Approximately 5.0 mg of the sample was dissolved in 1.0 ml of 10 mM ammonium acetate buffer and separated on the TSK 300 column at a flow rate of 0.5 ml/min. (0.5 ml of sample applied on each run). Fractions (0.5 ml) were collected and assayed for PrGF activity.

Isoelectric Focusing

IEF was performed in 4% polyacrylamide tube gels containing 2% ampholyte (pH 3.5 - 10) with 0.01 M aminodiacetic acid at the anode and 0.01 M ethylenediamine at the cathode. Thirty microliters of sample in 10 mM ammonium acetate buffer was applied and run at 500 volts for 14 hours at room temperature. The pH gradient was determined after soaking 5 mm gel slices in 1.0 ml of HPLC grade water for 1 hour with constant stirring. PrGF activity was recovered from 5 mm gel slices by simple diffusion. The gel was added to 0.5 ml of 10 mM ammonium acetate buffer pH 7.0 containing 0.15 M NaCl and incubated at 4°C for 10 hours. Each gel slice was assayed for growth factor as described.

To verify the pI determination, IEF was performed in the presence of 9 M urea with an equilibration time of 15 hours (7,500 volt hours) as described by O'Farrell (10). Briefly, IEF was performed in 5% polyacrylamide containing 2% ampholyte (pH 3.5 - 10) with 10mM phosphoric acid as the anode buffer and 10 mM histidine as the cathode buffer.

Heparin Sepharose Column Chromatography

Heparin and BSA were linked to cyanogen bromide activated Sepharose-4-B as outlined by manufacturers instructions (Sigma). The BSA column served as a non-specific binding control. Columns (1 x 5 cm) were prepared and equilibrated with Tris buffer (0.01 M Tris HCL, pH 7.4). One ml of the PrGF preparation was added to each column and the column was washed with Tris until no protein was detected in the effluent. Bound protein was released by washing with Tris containing 3.0 M NaCl. Samples were dialyzed and precolumn, nonbinding effluent and binding fractions from both BSA and heparin columns were assayed for growth factor activity.

Physiochemical Characteristics

Stability to boiling was determined by placing a tube containing partially purified (post anion exchange) PrGF in boiling water for twenty minutes. The sample was then rapidly chilled by ice water bath. Susceptibility to reduction was determined by incubating partially purified growth factor with 50 mM DTT for one hour. The sample was dialyzed against 0.01 M ammonium acetate buffer, pH 7.0 and assayed for growth factor activity. Acid stability was checked by acidification to pH 4.5 with 0.1 N HCl, reacting overnight at 4°C and titrating to pH 7.6 with 0.1 N NaOH. Samples were then assayed. The effect of protease digestion (trypsin; 100 micrograms/ml) also was determined for the partially purified PrGF. Twenty-five micrograms of the PrGF preparation was incubated with trypsin for one hour at room temperature. The reaction was stopped by the addition of trypsin inhibitor.

Susceptibility of PrGF to Antibodies to EGF

Partially purified PrGF or pure EGF was incubated with anti-EGF antibody (R&D) and then assayed for mitogenesis at varying concentrations of antibody.

RESULTS

Purification

Following extraction in acidic conditions, activity was eluted from SP Sephadex C-25 cation exchange column at an ammonium acetate concentration of 0.31 - 0.6 M (Figure 1). The active fractions were pooled and applied to DEAE with activity being recovered from the non-binding flow through (Figure 2). This was applied to gel filtration column and activity recovered in the MW range of 18 - 40 kD (Figure 3). Active fractions were pooled and concentrated on centricon (10 kD pore size) and SDS-PAGE of this material revealed two bands (Figure 4). The sample was subjected to nondenaturing isoelectric focusing and growth factor activity eluted at a pI of approximately 7.5, which matched the position of a major protein band on stained gels. The isoelectric focusing gel was subjected to a second dimension SDS-PAGE and the protein band correlating with growth factor activity produced a single band under reducing conditions with a MW of approximately 22kD (Figure 5), suggesting that the activity had been localized to a single protein.

Characterization

Characterization of partially purified PrGF as described in methods section revealed that PrGF is sensitive to trypsin digestion confirming that it is a protein. As well, it was shown to be stable to acid treatment (PH 4.5 overnight), and reduction with DTT. It showed a minor reduction in activity following boiling for 20 minutes. When separated by affinity to heparin, only the non-heparin-binding fraction retained mitogenic activity (Figure 6).

Differential Cell Line Stimulation

PrGF stimulated ³H-thymidine uptake by quiescent NRK 52-E kidney epithelial cells (Figure 7). In contrast, pure EGF in a dose range of 25 - 400 ng, and pure bFGF in a dose range of 3 - 800 ng failed to stimulate ³H-thymidine uptake by these cells (Figure 8,9). All 3 factors (EGF, bFGF, and PrGF) were able to stimulate ³H-thymidine uptake in the fibroblast cell line (NRK 49-F) (Figure 10). Preincubation of EGF

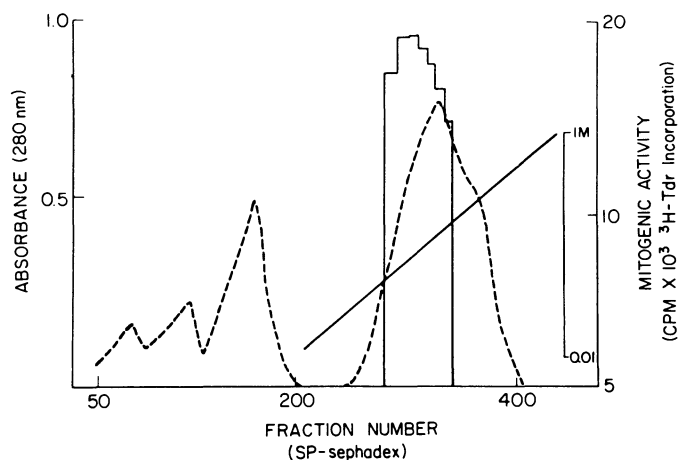


Figure 1: Cation exchange chromatography of prostate homogenate. The column was equilibrated with 10mM ammonium acetate (pH5.5) The binding protein was eluted with a linear ammonium acetate gradient (0.01 - 1.0M).

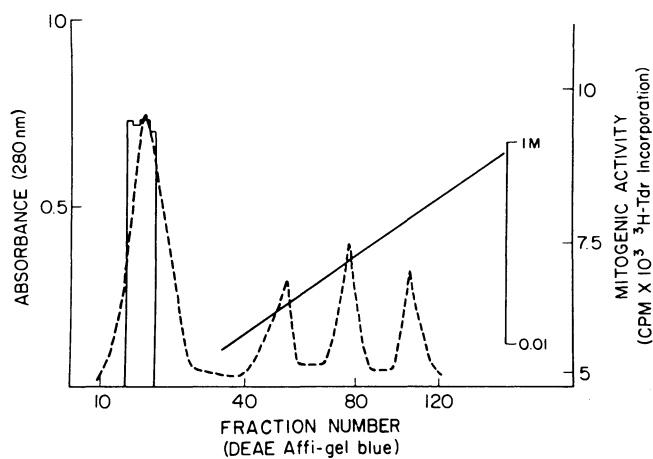


Figure 2: Anion exchange chromatography of the active fractions from the cation exchange column. The column was equilibrated with 10mM ammonium acetate (pH 7.0). The sample pH was adjusted to 7.0 with ammonium hydroxide. Bound protein was eluted with an ammonium acetate gradient (0.01 - 1.0 M).

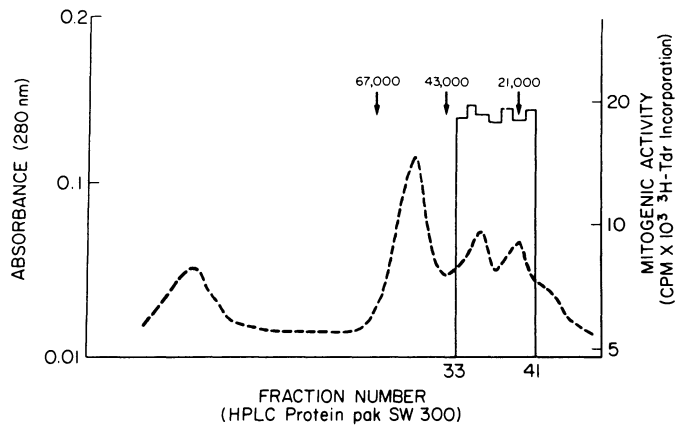


Figure 3: Molecular sieving of the active fractions from the anion exchange column. HPLC TSK 300 column was used. The column was equilibrated with mM ammonium acetate buffer, pH 5.5 containing 0.2 M NaCl. The column was eluted under isocratic conditions at a flow rate of 0.5 ml/min. of 0.5 ml/min.

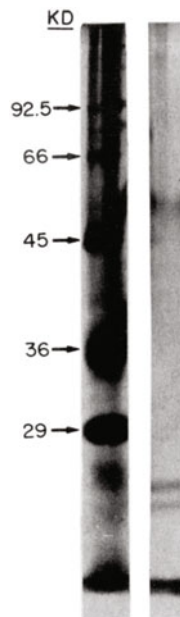


Figure 4: SDS-PAGE of Centricon concentrated PrGF. Separating gel contained 12% acrylamide. The gel was run a a constant voltage and silver stain. Lane 1, molecular weight standards; Lane 2, PrGF preparation.

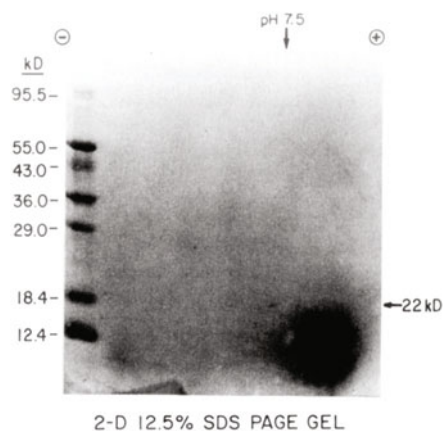
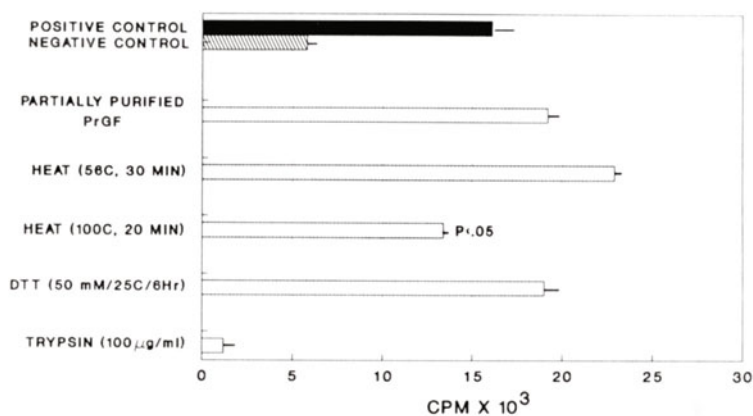


Figure 5: Second dimension SDS of proteins in isoelectric focusing gel. Separating gel contained 12.5% acrylamide. IEF was performed by the method of O'Farrell (29) as described in Materials and Methods.



- ACID STABLE:
 - 1) Extracted in 1.0M acetic acid, pH 2.3
 - 2) Incubated 24 hr at 4C (pH 2.7 after homogenization)
- DOES NOT BIND TO HEPARIN SEPHAROSE
- MITOGENIC FOR NRK-52E EPITHELIAL CELLS
 - 1) bFGF is not mitogenic
 - 2) EGF is not mitogenic
- MITOGENIC ACTIVITY NOT REDUCED BY ANTI-EGF
- MOLECULAR WEIGHT 22 kD
- pI 7.5
- COMPLETE INACTIVATION BY TRYPSIN DIGESTION
- STABLE TO REDUCTION
- ACTIVITY PARTIALLY REDUCED BY HEAT
 - 1) stable 56C, 30 min
 - 2) partial inactivation 100C, 20 min

Figure 6: PrGF Characteristics. Physical and chemical characteristics of partially purified PrGF (following anion exchange).

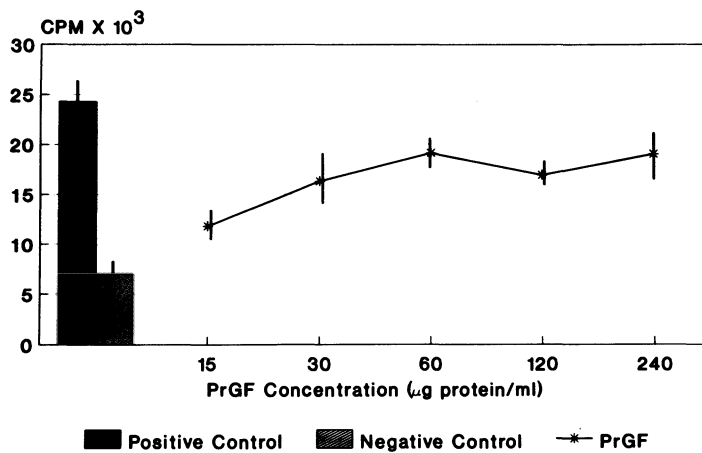


Figure 7: Dose response PrGF; NRK-52E. PrGF is mitogenic for the NRK-52E rat kidney epithelial cell line, as measured by the incorporation of ³H-thymidine by quiescent cells.

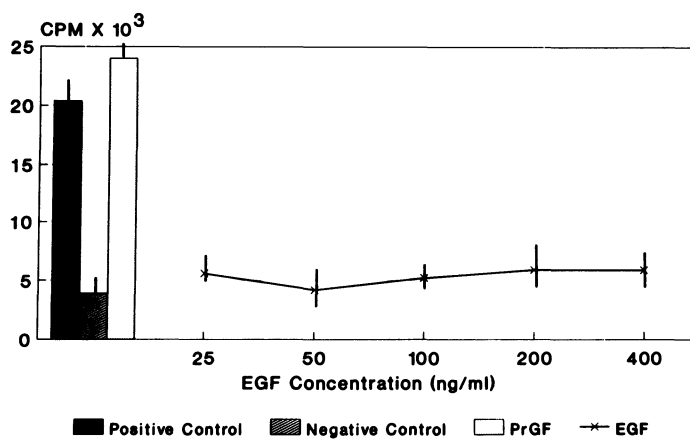


Figure 8: Dose response EGF; NRK-52E. Epidermal growth factor is unable to stimulate ³H-thymidine incorporation in the NRK-52E cell line.

with anti-EGF abrogated the EGF mediated stimulation of fibroblasts in a dose dependent fashion, while no diminution of PrGF mediated stimulation was noted following preincubation with the same antibody (Figure 11).

Mitogenic activity of prostate growth factor extracted at neutral pH

When tissue was homogenized at pH 7.6 in Tris buffer as described in materials and methods, the resultant extract stimulated the epithelial cell line. When this extract was separated into heparin-binding (FGF family) and non-heparin-binding fractions, only the non-heparin-binding component retained mitogenic activity for the epithelial cell line (Figure 12), while both the heparin bound and unbound fractions were mitogenic for the fibroblast cell line (Figure 13). The activity found in the non-heparin-binding fraction of this neutral extract was not reduced by acidification to pH 4.5 overnight as described (Figure 14).

DISCUSSION

An increasing amount of evidence suggests that excessive synthesis or an altered version of polypeptide growth factors or of their receptors may result in the transformation of recipient cells (22). The presence in prostatic tissue of genomic DNA and mRNA transcripts which encode for growth factors as well as the factors themselves has been demonstrated. Nerve growth factor (NGF) has been found in the prostate of the guinea pig, bull and rabbit but not mouse, rat, hamster or human (21,28). Basic fibroblast growth factor (bFGF) (9), selective osteoblastic mitogens (7,14), EGF-like activity (25), as well as other mitogens (12,13,23) have been isolated from human prostate. Leutinizing hormone releasing hormone (LHRH) receptors (29), transforming growth factor-type beta ($TGF\beta$) receptors (19), EGF receptor (29), insulin like growth factor (IGF-I) receptors (17), acidic fibroblast growth factor (aFGF), and bFGF receptors (30) all have been demonstrated in human or rat prostate tissue or prostate cell lines. Messenger RNA or genomic DNA for α FGF (30), bFGF (11) osteoblast stimulating factor (27) and platelet derived growth factors 1 & 2 (PDGRI, PDGF2) (26) have been demonstrated from various prostatic sources. Prostatic epithelial cells or tumor cell lines have been shown to be stimulated by cholera toxin, insulin, bFGF, glucocorticoids, EGF, aFGF (2), as well as $TGF\alpha$ (17), and other as yet unidentified mitogens (16,23).

Many interactions between growth factors, androgens, and their receptors have been noted. The $TGF\beta$ receptors in the rat ventral prostate have been shown to increase in number following castration, suggesting the possible role of $TGF\beta$ in the mechanism of castration induced cell death (19). Serum and $TGF\beta$ but not EGF have been shown to increase the transcription of the bFGF gene in human fibroblasts (18). A decrease in androgen level has been shown to cause an increase in the number of EGF receptors, as well as the expression of the c-myc gene in cultured prostate epithelial cells (17). Heparin-binding growth factors have been shown to attenuate the $TGF\beta$ induced inhibition of rat prostate tumor epithelial cell growth (8).

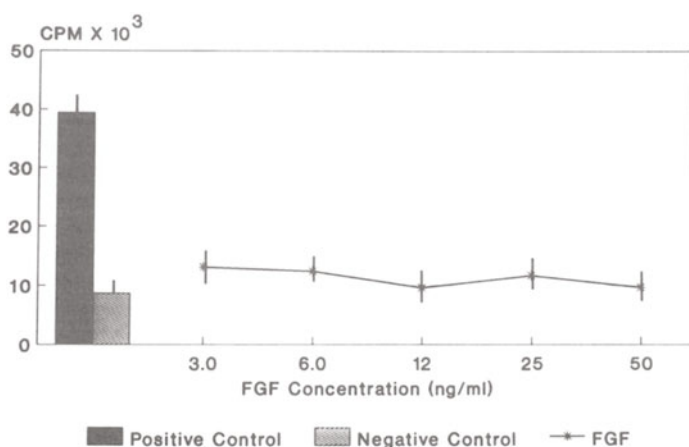


Figure 9: Dose Response FGF; NRK-52E. Basic fibroblast growth factor is unable to stimulate ³H-thymidine incorporation in the NRK-52E cell line (tested to concentration of 800 ng/cc - data not shown.)

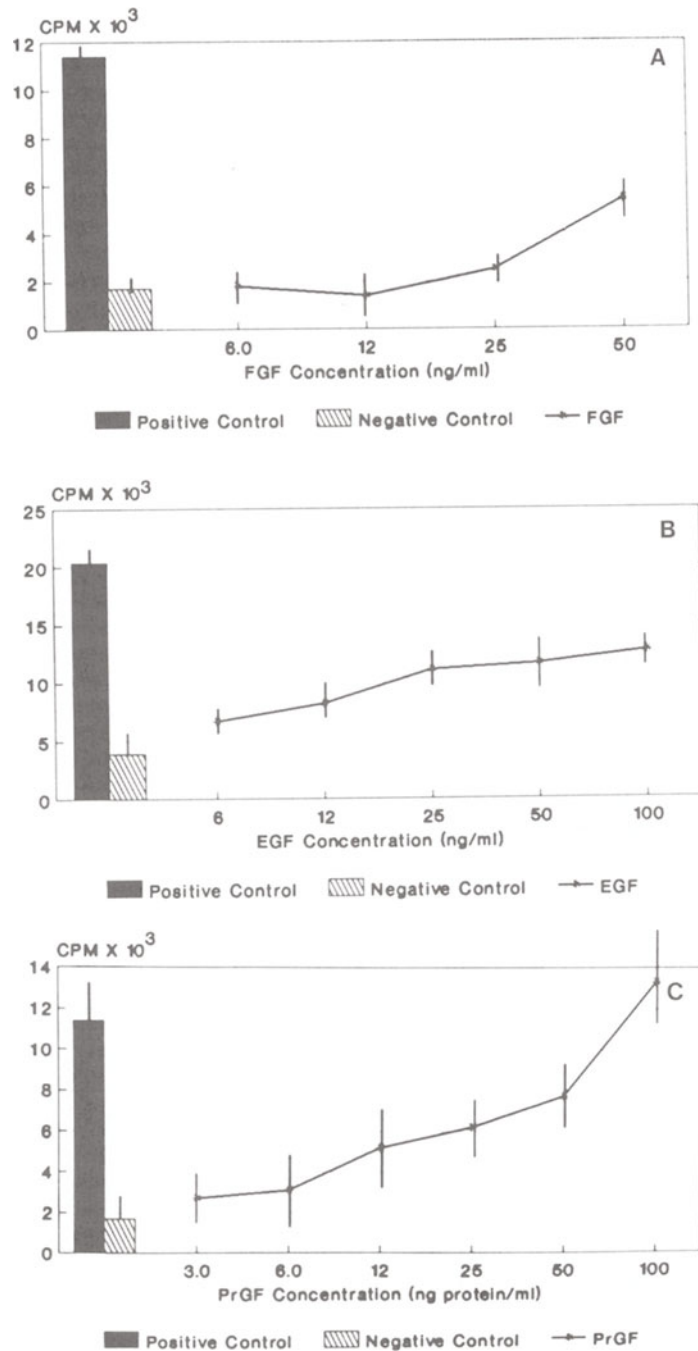


Figure 10: A. Dose Response FGF, NRK 49F. B. Does Response EGF, NRK-49F. C. Dose Response PrGF, NRK 49F. All three factors tested are capable of stimulating DNA synthesis in the NRK-49F fibroblast cell line.

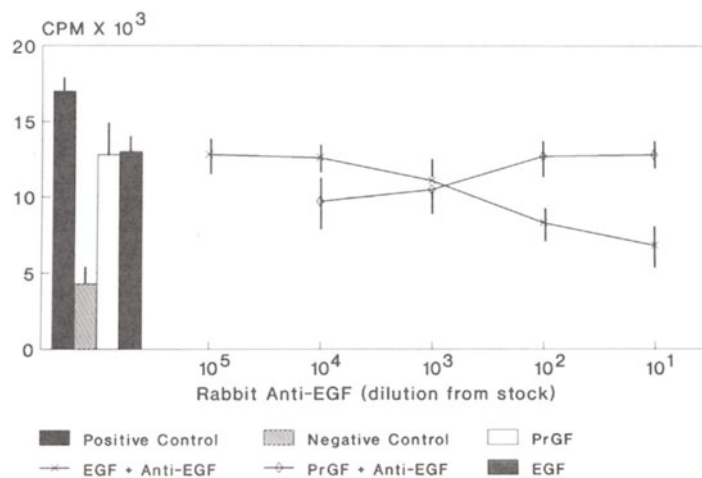


Figure 11: Effect of Anti-EGF on PrGF. Preincubation of EGF with anti-EGF reduces mitogenicity for the fibroblast cell line while having no effect on PrGF's mitogenicity.

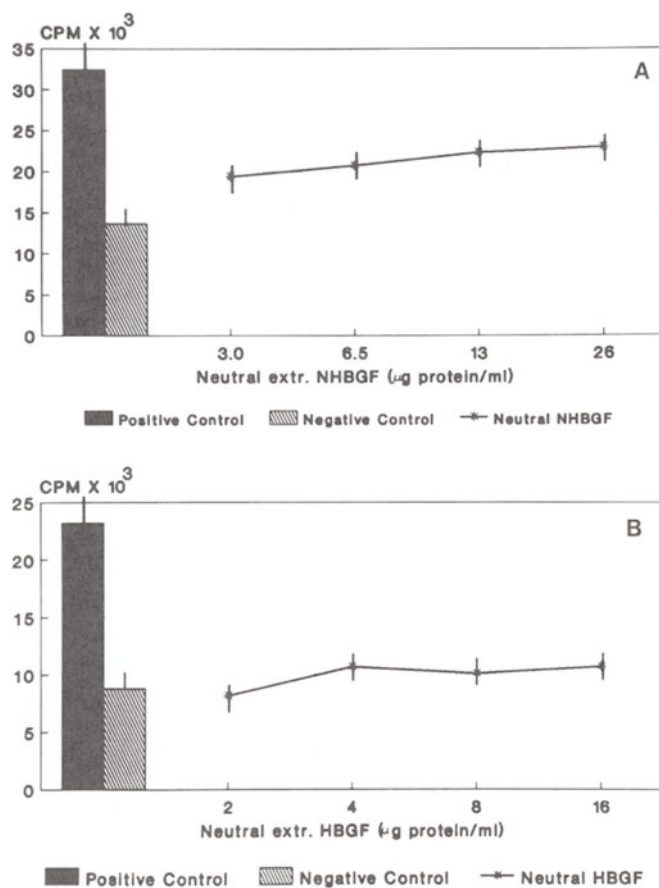


Figure 12: A. Dose Response NHBGF-Neutral Extract; NRK 52E. B. Dose Response HBGF-Neutral Extract; NRK 52E. When prostate tissue is homogenized at neutral pH (after Story) only the non-heparin-binding fraction is able to stimulate the epithelial cell line.

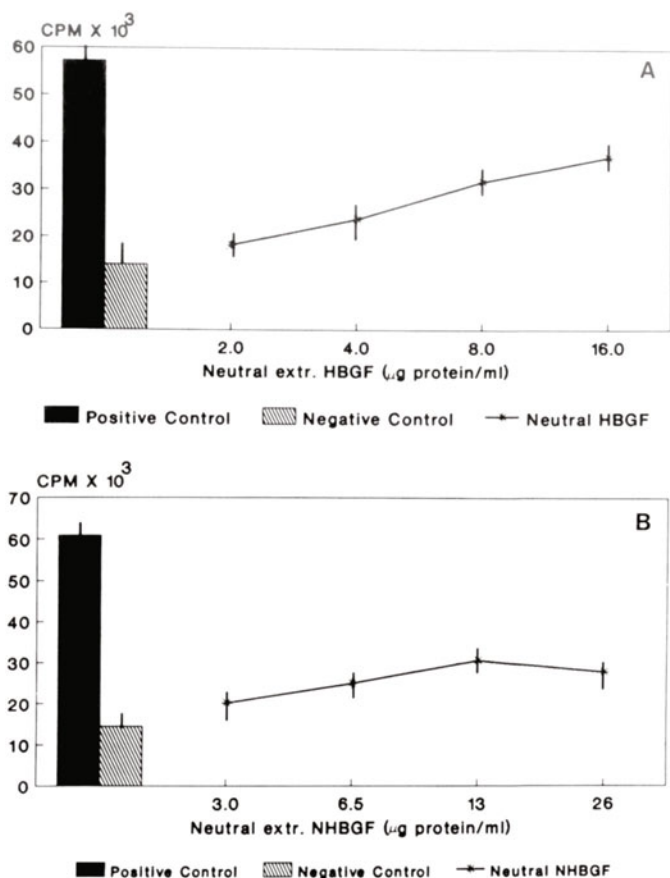


Figure 13: A. Dose Response HBGF-Neutral Extract; NRK 49F
 B. Dose Response NHBGF-Neutral Extract; NRK 49F
 When prostate tissue is homogenized at neutral pH (after Story)
 both the non-heparin-binding and the heparin-binding fractions
 are able to stimulate the fibroblast cell line.

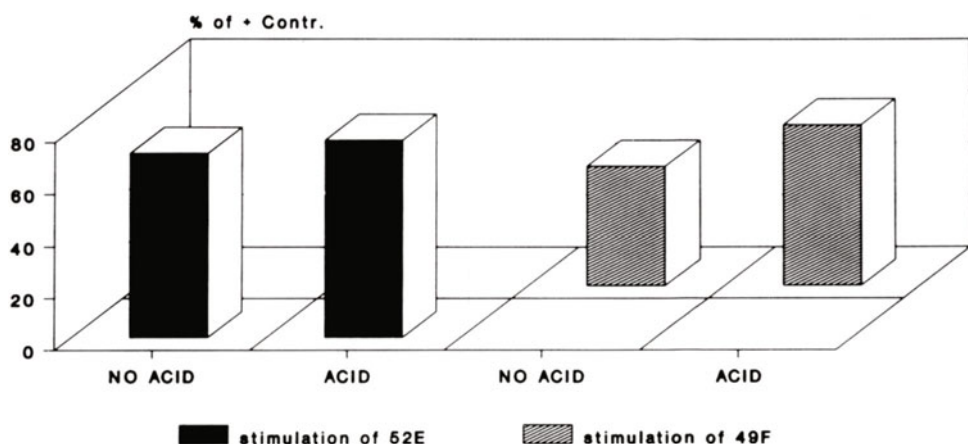


Figure 14: Acid stability of NHBGF from neutral extract (After Story). Thus when the cancerous tissue has been extracted under neutral conditions, and depleted of bFGF and other HBGF activity by passing over heparin sepharose, the remaining activity for NRK 52E epithelial cells is acid stable suggesting the presence of the same growth factor seen in acid extract.

Significant differences in the amount or activity of growth factors, their receptors, and androgens have been noted between cancerous and noncancerous prostatic tissue. LHRH receptors were found in all samples of human prostate cancer, but no normal prostates and only one of 13 BPH samples examined by Fekete (29), while all samples showed the presence of EGF receptors. Davies et al showed that the increase in EGF receptors and c-myc expression correlated with deteriorating differentiation of cultured prostatic epithelial cells (17), and were significantly higher in human carcinoma specimens than BPH (16). Mansson showed that differences in growth rates and degree of malignancy in prostatic cell lines were associated with different patterns of FGF gene expression (30).

Recent findings in related fields include the finding that transformation of cells by avian erythroblastosis virus (AEV) may result in part from the inappropriate acquisition of a truncated form of the EGF receptor (20) which (as previously mentioned) has been shown to be present in normal, hyperplastic, and neoplastic human prostate (29). Human fibroblasts have been shown to produce four different mRNA's which hybridize with cDNA for bFGF, and serum or TGF β increases the transcription of the bFGF gene but EGF does not (18).

A current consensus view of how growth factors actually interact *in vivo* in the human prostate would include a prominent role of bFGF as a growth stimulating factor, perhaps as an intermediary in injury induced hyperplasia which would require subsequent androgen interaction (31), or as the factor which would allow tumors to overcome the tonic inhibition of TGF β on prostatic epithelial cells (33). It is a widely held belief that fibroblasts mediate epithelial hormonal responses (32), possibly by elaboration of growth factors. TGF α is proposed as an autocrine regulator in prostate cancer (34,35), and TGF β has been proposed as a factor necessary for detachment of cells as a first step in metastasis (36), and as an intermediary in castration induced death of androgen dependent epithelial cells (19).

Initial interest in the biological role of growth factors in the prostate was stimulated by the isolation from human prostatic tissue of bFGF by Story (9) and Nishi (37). Subsequently other investigations have shown this factor to be produced in the prostate, and stimulatory to prostatic cells. Despite the extensive investigation of prostate growth factors, only FGF and EGF have been isolated from human prostate tissue. Each time the total quantity of mitogen in a preparation is calculated, the amount of mitogenic activity attributable to EGF and FGF fails to account for the total mitogen content.

Both Story and Nishi have postulated the existence of an as yet undescribed mitogen in the non-heparin-binding fraction of prostate homogenates. Using an extraction method initially developed at our institution (15) for isolation of mitogens from rat prostate, we have isolated and characterized a polypeptide growth factor from human prostates removed for adenocarcinoma (PrGF) which appears to be distinct from bFGF, and EGF, and does not bind to heparin. PrGF differs from EGF in MW, pI, stability to reduction, partial heat lability, effect of inhibitory antibodies and pattern of cell line stimulation. PrGF differs from bFGF in MW, pI, predominant heat stability, its low affinity for heparin, and pattern of cell line stimulation. Continued processing of new tissue and further purification will generate a quantity of pure product sufficient for amino acid analysis and sequencing, at which time the degree of homology to known growth factors will be discerned.

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DISCUSSION

Dr. Barrack:

I would like to know more about the source of tissue that you used. Can you tell us how homogeneous it was, for example what percentage of the cells were actually prostate cancer epithelial cells and what the stage of cancer was? How do you know that your growth factor is not coming from stroma?

Dr. Petros:

We haven't attempted to separate the epithelium from the stroma and it's been from open operations not from transurethral operations, that have been done almost exclusively for carcinoma, but the tissue is definitely heterogeneous. It contains areas of normal as well as some hyperplastic tissue, but we believe that it is predominantly carcinoma, but it is absolutely a mixture.

Dr. Matuo:

Even your tissues are heterogeneous, and I am very much interested in your non-HBGF. I have one suggestion and I have one question. My suggestion is, before further purification, to finish the competition assay using A431 cells. Is the acid treatment necessary or not? Have you ever tried to check the activity without acid treatment?

Dr. Petros:

Yes, we have, and what we found was that when the tissue was extracted under neutral conditions and depleted of the heparin binding factors, that it still had activity and that activity was acid stable. We have been able to find activity in both preparations that have been treated with acid and those that have not.

Dr. Matuo:

Is it not activated by acid treatment?

Dr. Petros:

It does not appear to be.

PANEL DISCUSSION

Growth Factors - 1

Panelists: Donald Coffey, David Bilhartz, Michael Freeman, John Petros and Wytse van Weerden

Question from the floor:

Has anyone, particularly Dr. Ratliff, tried to localize whether these growth factors come from epithelial or stromal cells? Also, has anyone examined the central zone of the prostate that forms most of the BPH in comparison to the outer zone of the prostate in which most cancer would develop?

Dr. Ratliff:

We haven't looked at that at all. All of the tissue that we have extracted has come from open prostatectomies. The majority of that tissue has been from prostate cancer.

Dr. Petros:

Dr. Chung, your system of separating cells and then putting them back together and seeing tumorigenesis is fascinating. Have any subcellular fractions been tried? For instance, have just membranes been isolated and tried? Have these been able to induce tumorigenesis? Have the nucleotides or any of the subcellular parts been able to do it? You showed that laminae didn't.

Dr. Chung:

We have tried all components in the Matrigel. In addition, we have tried a number of other extracellular matrices. The results show that collagen 4, laminin, and heparin sulphate will induce epithelial tumor growth, whereas collagen 1 and 3 fail to do it. Also, we can reconstitute Matrigel based on their chemical composition analyzed, and it does just the same job as Matrigel.

Dr. Petros:

What about cell membranes?

Dr. Chung:

We have not done the experiment.

Dr. van Weerden:

Dr. Chung, you cultured these epithelial and fibroblast cells, put them together, and then transplanted them in nude mice. What happens when you transplant them separately in both sides of the nude mice, so you keep them separate *in vivo*? What happens to the epithelial cells?

Dr. Chung:

We have tried that too, but the tumorigenic fibroblast cells do not simulate the non-tumorigenic cells to grow. We have obtained no evidence of cross circulation that can support the growth of epithelial cells. In this respect it's unlike MCF7 cells.

Dr. Freeman:

Dr. Romijn, regarding the role of polyamines and serum amine oxidases in cell death, I've read that serum amine oxidases in fetal calf serum can perhaps be mutagens by changing the polyamine degradation pathways and inducing cytotoxic agents. As a result, the traditional approach to cell culture, where cells are grown in serum, might induce genetic instability because the cells are growing in a mutagenic environment. You had talked mostly about polyamine requirements for growth, but what about the possibility

that these pathways might be mutagenic? Might they influence genetic instability, cell death and regression in culture?

Dr. Romijn:

Personally, I consider the effect of serum as a cell culture artifact. The point is that not all types of serum contain those aminoxidases that produce toxic side products and those products are most probably toxic aldehydes. For instance, in horse serum there is no aminoxidase that produces those toxic side products.

Dr. Freeman:

I've also read that cells cultured in horse serum don't show genetic instability, at least to the same degree as cells cultured in fetal calf serum.

Dr. Romijn:

Cells simply die because of excess of aldehydes and I'm not aware of any mutations induced in this way.

Dr. Petros:

Dr. McKeehan, the changes that are seen in the tumors taken out of the organism and put into culture include differences in keratin expression, as well as the expression of mRNA transcripts for heparin binding growth factors. You said that that can be reversed when you put it back into the animal. Have you been able to find things that tend to reverse either the phenotypic expression or the genetic expression in culture? In other words, do co-inoculations with other cell lines or any specific serum or other agents make that revert back to the natural wild type?

Dr. McKeehan:

Not really. The data I have shown seems to be fairly independent of extreme variations in cell cycle, manipulation of the medium with at least serum, and the growth factors as a whole. We haven't yet really dissected every individual growth factor out, like Dr. Story alluded to the effects of TGF beta. We'll probably do that to see if there's an interplay at the level of gene expression and on to the receptor, which may be the other level. In answer to the original question about the tumorigenicity of these cells, we first of all take the individual epithelial cells or mesenchymal cells from a slow growing tumor, and neither one in our hands so far is tumorigenic (i.e. the isolated cells). However, dissociated cell suspensions from the primary tumor, if we put them right back, develop tumor, but if we take the isolated cells, at any dose we've tried so far, we do not get a good tumor. Of course, these experiments are frustrating, since it's a slow growing tumor. It takes 150 days anyway. However, we have some indicator that if we mix them back together, even after being in culture past dissociating with collagenase, as Dr. Chung might suggest, we might start to get a tumor. We haven't done this because these experiments take so long. We're jumping on to transfection experiments in each of these cell types with the growth factor genes themselves. We're assuming that growth factor gene expression is limiting, and it's also a time factor. We've got some interesting results with the difference in HBGF 1 and HBGF 2. If we take HBGF 2, in particular, the sense constructions and new transfections with the individual epithelial cells or fibroblasts (we're using soft agar to start), the tumorigenicity falls. That expression of HBGF 2, in particular, is making these cells grow remarkably in soft agar, and they wouldn't before, neither the mesenchymal or epithelial cell, and we would expect them to make tumors, just like we would expect the AT3 cells already doing that. It has the capability somehow of tremendous over expression of both HBGF 1 and HBGF 2, in particular. So, this looks very dramatic and interesting that neither of these cells grow in soft agar, but if we get over expression and put in HBGF 2, which they didn't express in the first place, they really grow well in soft agar. We're also doing the antisense experiments going to the AT3 for both gene products, as well as the HBGF 1 with the mesenchymal cells from the slow tumor. We've failed so far to get antisense using neomycin resisting approaches of the HBGF 1 type gene, which may or may not be significant. So we've switched now to inducible promoter protocols where we can get the gene in. We'll be doing those experiments correlating soft agar and tumor. So it looks like it may be purely a correlate of over expression of these two growth factor genes.

Dr. Catalona:

Dr. McKeehan, I would like to raise a point about methodology, particularly since you are looking at mRNA of the growth factors. I was collaborating with one of our colleagues who wanted to look at vitamin B12 production in human seminal vesicles. For some reason, human seminal vesicles are very high in vitamin B12. He wanted to look at the mRNA probe, and he asked if we could get him some human seminal vesicles at the time of radical prostatectomy. We did this over a period of several weeks, and it

turned out that unless one has the blood supply intact, right up until the point that the prostate is removed and the blood supply is clamped, and the seminal vesicles are put immediately into nitrogen, that the ribonuclease's just destroyed the message very quickly. I'm wondering whether the differences in the message that you saw in the human system and the animal system may be related to tissue procurement? How was the human prostate procured for these studies?

Dr. McKeehan:

That clearly could be a possibility. The human tissue in our experiment is very limited and we depend on our clinical collaborations. With the normal human tissue, most of it was from very early autopsy. So, the lower levels could actually be lower quantitative levels in these tissues. The BPH was surgically removed and processed immediately, as were the andocarcinomas.

Dr. Coffey:

Let me just comment on that. This is a terribly important question. Earlier you heard Dr. Isaacs talk about the expression of the *ras* gene you're correlating with things. Well first you can look at the message but there can always be a difference in that. Then you can look at the protein expression. If you look at immunohistochemistry it is not very quantitative. But even if you look at the P21 protein, that has very little relationship to what it's really doing *in vivo*, because for it to be effective it must be post translated and modified to have a posttranslational mystery to be added to it, before it works in the membrane. We don't know those things on growth factors, but it is almost certain that it's going to have similar complications. So, when we relate these things, it is going to be a little hard, but we have to do what we can at each step and just explain how we've done it.

Dr. Ratliff:

I want to note for general discussion that we spent a lot of energy and time in identifying the factors from these organs, and now are just beginning to look at expression. But, when you think about the biology of the system, are these factors associated with initiation of the disease process or are they simply a byproduct of the disease process itself?

Dr. Thompson:

You know, I think you need an *in vivo* system to put it in directly, like a transgenic or the system I work with where you put genes into normal tissue without perturbing the biology. I think that's always going to be a question. You're always going to be wondering what these things are doing. Yes, they are there, they are compartmentalized, but what can they really do *in vivo*?

Dr. Miller:

If you look at how carcinomas arise in human tissues, a cervical carcinoma for example, the epithelial cells undergo the process of neoplastic transformation away from the stroma. They're separated by a basement membrane. So, I look at the stromal interaction as being something that may occur after cells have actually undergone neoplastic transformation. The same may be true in the prostate. If you see the recent descriptions of dysplasia in the prostate, it appears to be a pure epithelial sort of phenomenon.

Dr. Coffey:

Do you ever see fibroblasts around an epithelial adenocarcinoma give any abnormality? In other words, if you look at the fibroblasts in the vicinity of an adenocarcinoma, do you see any abnormality?

Dr. Miller:

Sometimes. It is infrequent enough, however, that we would comment on that diagnostically. For example, some carcinomas will induce a change in the stroma that so mimics a sarcoma that we would refer to it as atypia in the stroma itself. Endometrial carcinomas are a good example of that, or head and neck cancers. But that is not something that is tremendously common.

Dr. Catalona:

Could you comment for a minute on lesion in the bladder that occurs following inflammation or trauma, that's sort of a pseudosarcoma.

Dr. Miller:

Yes, it is the so called post-surgical spindle cell neoplasm. This lesion is a stromal proliferation. Actually, this is a complex topic; let me explain why. The problem here is that you have a cell that looks stromal, proliferates, can become highly invasive, does not metastasize, and expresses muscle markers as well as immunohistochemically detectable keratins. So, we're really not sure exactly what it is. It seems to

be a self perpetuating stromal lesion that is non metastatic. It is similar to the so called desmoid tumors, which have been referred to as non metastatic sarcomas. They're tumors which are proliferative, autoregulatory, but do not metastasize.

Dr. John Isaacs:

I would like to pose a pragmatic question to the whole group working on growth factors. It seems to me that the clinically relevant issue is why does prostatic cancer grows in bone? Why are we spending so much energy trying to find a factor that makes other cells grow? One of the things that would be useful is to look at what's in bone that allows prostatic cancer cells to grow, because that seems to be one of the unique characteristics of prostatic cancer. Is there any experimental reason why one can't extract factors from demineralized bone, specifically using human prostatic cells as the test system?

Dr. Linehan:

It seems to me that it's very likely that a molecular abnormality, such as a recessive oncogene, could be the primary initiating event in cancer. I think that is likely to be the case in colon cancer, i.e. Vogelstein's work (chromosome 5), and kidney cancer (chromosome 3). You have the deletions and you also have linkage if that's where the disease is; the same thing with retinoblastoma. It seems to me more likely that growth factors, abnormalities and all these things that we've been talking about are probably "secondary events". If you put retinoblastoma back into retinoblastoma back into sarcoma back into lung cancer, they sort of untransform. If you put chromosome 11 back into Wilm's tumor and now there's some data that if you put chromosome 3 back into kidney, they will untransform. The problem is that it's not clear if that's ever going to be a therapeutic approach one can aim at. As for bone, people have shown that TGF beta has a significant inhibition of lymphocytic proliferation, and maybe not a regulatory function for the body's immune system. What is the greatest single source of TGF beta in the human body? Bone. So, it's conceivable, for example, that prostate cancer goes to bone, something like TGF beta, EGF and PDGF induces lytic reaction in the bone. This could release beta from the bone, which may or may not affect growth of prostate cancer, but it could affect the body's immune response to prostate going to bone.

Dr. Coffey:

Dr. Collette Freeman held a conference in Annapolis with about 12 leading people in metastasis and some outside people in other disciplines. What happens is that we oversimplify this metastasis story. First of all, the cells must get there. That means they need to have motility or filtration. But then they have to dock, and there's all sort of special signals for docking these cells at the site they are going to metastasize to. But, if they just docked, they would never get into the tissue. So, not only do they have to dock, they have to be released from the dock, and then they have to go into that tissue. Then, in many cases they have to destroy the normal tissue to take over and have their own growth factors for that area to make them grow. As we begin to break into this field, and get into all the factors, it's not overly complicated but we must not oversimplify. One of the questions that came up was whether some of the melanoma models might not be a special type of case a little different from some of the solid metastasis that we see. I just want to stress that it goes a little bit beyond simply growth factors and things for metastasis to the bone.

Dr. Matuo:

As you know, there is no leader sequence in HPGF 1 & 2. This is a very good opportunity to discuss the sequence and mechanism of HBGF 1 & 2. Where is the localization of the receptor?

Dr. Coffey:

I certainly don't know, but I'll take a shot at it. One of the things that came out of the Gordon Research Conference that was dynamite was from Lawrence's lab. The message for actin is transported to the leading edge of the cell where it's needed for motility. Some of the myosins are transported the other ways. In other words, the mRNA is coming out of the nucleus on specific tracts. The message goes to a specific site to be translated. It's not just diffusing out the ribosomes or leader sequences. But how this thing is really doing all that remains to be worked out. It is a great frontier of biology at the moment. So there is great specificity to where these things go, or are put together. Dr. Bill Isaacs has looked at some cotranslational things that happen and had some papers in the Journal of Cell Biology on that. So, it's going to take a little while before we break in to the compartmentalization. It is my belief, and there's a lot of evidence for this, that a lot of these particulate particles are travelling along very special railways that are lined up on the cytoskeleton. And the inside of the cell is a highly chemo-mechanical dynamic. You can see these little particles go out and back on the railroad tracks. They come in and out on the nucleus, and the cell motility in the internal organization of the cell is going to be very very important. How much of this is keratins setting that up and actin and myosin and tubulin is going to be a very interesting to find out.

Dr. Freeman:

I have a question related to the cytoskeleton in transformed cells. Is there a good model or is there any strong evidence that mutations in cytoskeletal proteins will alter the transformed properties of cells? In other words, can actin perhaps serve as an oncogene or something similar to that?

Dr. Coffey:

Yes. There have been several studies showing that actin mutations are powerful in transformation events. When you see changes in the cytoskeleton, the microtubules, the nucleus, and the whole cell structure, you think that something very fundamental must be involved. Actin would be a good candidate in this case. Otto Varberg said very clearly and we tend to forget it, that tissue culture is a carcinogen. Let me repeat that. Tissue culture is a carcinogen. The minute you put cells in a tissue culture, you get genetic instability. How much you get is dependent on the cells and what they're touching. But just touching plastic starts genetic instability. So, this is a big problem, i.e. when you put something in a cell and it causes a transformation sometimes. As to whether it's primary or secondary is hard to know. A lot of the times we put oncogenes in, and you get a tumor, and then when you get that tumor out it wasn't that oncogene that was being expressed. But I think actin is one of the prime candidates. I think tropomyosin isoform, whether its mutated or not, is another prime candidate for some of this. But that is all at the very beginning stages.

TRANSFORMING GROWTH FACTOR α : A POTENTIAL AUTOCRINE GROWTH REGULATOR IN PROSTATIC CARCINOMA

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INTRODUCTION

Since the classical observation of Huggins and Hodges in 1941 [1], the efficacy of androgen-ablative therapy has been well established in the management of patients with advanced prostate cancer. However, this type of therapy remains palliative in nature. Although, initially patients with stage D disease are expected to experience beneficial responses to androgen ablation, the vast majority of them eventually will face progression of the disease despite the continuous androgen-depleted state [2]. This phenomenon is attributed to the presence in the primary and metastatic sites of an androgen-insensitive prostate cancer cell subset which is able to proliferate in the absence of any androgenic support [3]. Patients with androgen-refractory disease (stage D3) have a dismal prognosis with a mean survival period of less than one year [4], for existing therapeutic regimens are not effective for this group of patients [5]. Therefore, it is important to study the mechanisms through which these androgen-insensitive cells are able to escape androgen dependency and become autonomous in cell proliferation.

The present report summarizes results of our studies on the androgen-independent human prostate cancer cell line, PC3, in an attempt to elucidate the mechanism of its autonomous growth. The PC3 cell line was originally derived from a bony metastatic site of prostatic adenocarcinoma [6]. These cells are rapidly proliferating, poorly differentiated, and androgen-independent.

To demonstrate the presence of a possible autocrine growth regulator which provides a growth advantage for PC3 cells, the overall strategy in the present study is to generate the conditioned medium from these cells and, then, to explore the growth promoting properties in the conditioned medium.

Preparation of PC3 Cell Conditioned Medium

The PC3 cell line was obtained from the tumor depository, Frederick Cancer Research Facility, National Cancer Institute, Frederick, Maryland. They were routinely maintained in RPMI-1640 tissue culture medium containing 10% fetal bovine serum and penicillin (100 U/ml) streptomycin (100 ug/ml). For the purpose of collection of conditioned media, PC3 cells were grown to 75-85% confluence in 150 cm² tissue culture flasks (Corning Glassworks, Corning, NY). Conditioned media were collected by washing cells twice with phosphate buffered saline and incubating the cells in RPMI-1640 medium containing ITS (insulin, 6 ug/ml; transferrin, 6 ug/ml; selenous acid, 6 ng/ml) and penicillin/streptomycin for 48 hours. The resulting conditioned medium was aspirated from the flask, centrifuged (400 X g for 10 minutes), filtered through a 0.45 um membrane, dialyzed against distilled water at 4°C with 3.5kD cutoff and, finally, lyophilized for future use.

Effect of PC3 Conditioned Media

Conditioned media (CM) harvested from PC3 cells cultured in serum-free media were able to stimulate proliferation of PC3 cells as well as for two other human prostate cancer cell lines, DU145 and LNCaP (Table 1). In addition, the growth of epithelial cells harvested from fresh prostate cancer tissues was also enhanced by PC3 conditioned media (Table 2). Subjecting the conditioned media to the boiling water-temperature for 30 minutes abolished this mitogenic activity. These results indicated that PC3 cells synthesize and secrete factors that may act through autocrine and/or paracrine mechanisms to promote PC3 cell proliferation. Fractioning PC3 cell conditioned media by HPLC using a gel filtration column showed that the greatest mitogenic activity was eluted at a volume indicative of ~6kD molecular weight (Fig. 1). A similar effect of PC3 cell conditioned media was observed by Eaton et al [7]. However, these authors noted that a mitogenic activity in PC3 cell conditioned media was associated with the -20-30kD fraction. In the present study, the 20-30kD fraction contained a weaker mitogenic activity than that in the ~6kD fraction.

Evidence for TGF- α in PC3 cells

TGF- α is a 5.6kD polypeptide that is derived from a 160 amino acid transmembrane precursor of 17-19kD molecular weight [8,9]. It has been isolated from the conditioned media of human and murine transformed cells and has been regarded as an autocrine growth regulator [10,12]. In the present study, immunoblot analysis of proteins separated by SDS-PAGE electrophoresis using polyclonal anti-TGF- α (Peninsula Laboratory) showed the presence of immunoreactive bands in conditioned media from PC3, DU145, and LNCaP cultures with the same electromobility as that of the purified TGF- α (Fig. 2). Analysis of PC3 cells and PC3 tumors grown in nude mice demonstrated a 17-19kD immunoreactive band upon immunoblotting with anti-TGF- α . These data indicate that PC3 cells and solid tumors synthesize the 17-19kD precursor and secrete the fully processed 5.6kD TGF- α into the conditioned media.

Cells (1×10^4 /well) were plated in 24-well plates and allowed to adhere for 16-18 hours. Media was then changed to serum free RPMI-1640 or WAJC 404 containing ITS+ alone or ITS+ and PC3CM. Cultures were incubated for 48 hours and were pulse labelled with ^3H -thymidine (1 uCi/well) for the final 24 hours of incubation. Cultures were washed (3X), cells were lysed and ^3H -thymidine incorporation was evaluated as an index of cellular proliferation. Fold increase in ^3H -thymidine incorporation was calculated as follows:

Table 1: Effect of PC3 conditioned medium (PC3CM) on benign and malignant prostate epithelial cell proliferation *in vitro*

Cell line	Fold increase in ^3H -thymidine incorporation		
	10 ug/ml PC3CM	100 ug/ml PC3CM	1000 ug/ml PC3CM
PC3	3.49 + 0.02*	2.85 + 0.11*	8.84 + 0.68*
DU145	3.67 + 0.06*	3.58 + 0.15*	5.51 + 0.25*
LNCaP	2.44 + 0.42*	1.72 + 0.16*	3.27 + 0.49*
Fresh Prostatic Carcinoma	1.68 + 0.15*	1.62 + 0.09*	1.49 + 0.07*
BPH Epithelia	1.07 + 0.09*	0.92 + 0.06	0.98 + 0.10

N = 8/group

* P < 0.05 compared to serum free negative control

Table 2: Effects of PC3 conditioned medium on benign and malignant prostate epithelial cell growth *in vitro*

Cell line	Cells/flask x 10 ⁴		
	Basal Media	Complete Media	PC3CM
PC3	49.7 + 3.9	98.4 + 11.7*	84.6 + 7.1*
DU145	19.6 + 1.7	50.7 + 3.8*	34.7 + 1.9*
LNCaP	6.0 + 1.2	80.0 + 8.3*	23.3 + 3.7
BPH Epithelia	9.3 + 2.4	78.0 + 11.2*	5.3 + 3.1

N = 6/group

* P < 0.05 compared to basal media control

$$\text{Fold increase} = \frac{\text{experimental (dpm)} - \text{serum-free control (dpm)}}{\text{serum-free control}}$$

Cells (1 x 10⁵/flask) were plated in 25²cm flasks and allowed to adhere overnight. Media was then changed to include basal media consisting of RPMI-1640 with ITS+ for the established cancer lines or WAJC 404 consisting of ITS+, cholera toxin (10 ng/ml) and prolactin (10 ng/ml) for BPH epithelial cells, complete RPMI-1640 with 10% FBS for the cancer lines and complete WAJC 404 for BPH or basal media containing 100 ug/ml of PC3CM. Cultures were incubated for 6 days with media being replaced on days 2 and 4. Cells were counted on day 6 by detaching with trypsin and counting with a hemacytometer.

Role of EGF and EGF Receptor

Because EGF is structurally and functionally similar to TGF- α [13,14] and the two growth factors share the same receptor [15,16], the present study also examined EGF and EGF receptor in prostate cancer cells. In an immunoprecipitation experiment using anti-human EGF, the presence of an im-

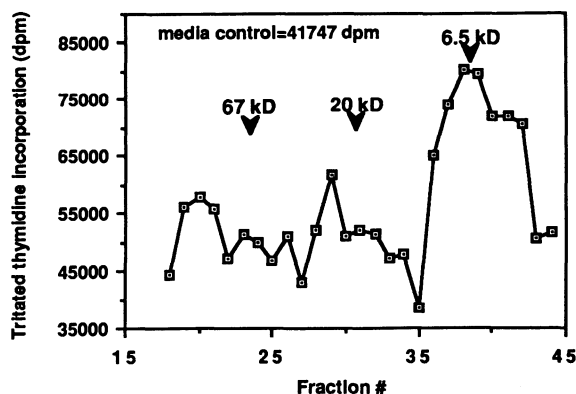


Figure 1: HPLC fractionation of PC3(Conditioned Medium). PC3 CM (50 mg/ml) was loaded onto a Biosil TSK-125 column and eluted using PBS at a flow rate of 1 ml/min. Fractions were collected at 1 ml intervals and tested for mitogenic activity against PC3 cells using a ³H-thymidine incorporation assay. Protein content in each fraction was determined using a UV detector.

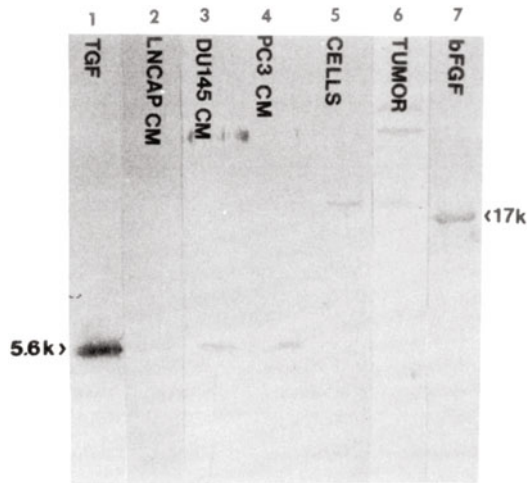


Figure 2: Immunoblot analysis of TGF- α in prostatic cell lines. PC3 and DU145 tumor homogenates as well as cell lysates and conditioned medium from established prostate cancer cell lines were prepared as outlined in Materials and Methods. Proteins were separated by SDS-PAGE electrophoresis (15% acrylamide) and TGF- α was detected by immunoblotting with rabbit anti-rat TGF- α . (1) purified TGF- α positive control (5.6kD); (2) LNCaP CM; (3) DU145 CM; (4) PC3 CM; (5) PC3 cell lysate; (6) PC3 tumor homogenate; (7) bFGF control (17kD).

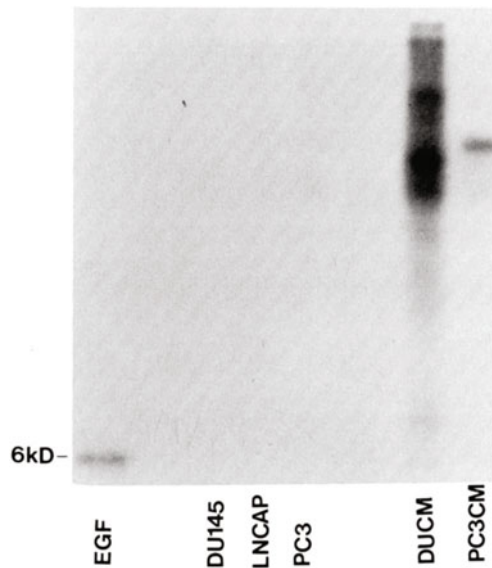


Figure 3: Immunoprecipitation analysis of EGF in prostate cell lines. Cells were labeled with ^{35}S -methionine for 24 hours and harvested from 150 cm² flasks, lysed, and incubated with rabbit anti human EGF followed by addition of protein A sepharose beads. Conditioned media were also harvested following ^{35}S - methionine labeling and immunoprecipitated with ant-EGF. Proteins were eluted from beads using Laemmli buffer and separated on a 10% polyacrylamide gel. Proteins were detected by fluorography. (1) purified EGF positive control (6kD); (2) DU145 cells; (3) LNCaP cells; (4) PC3 cells; (5) DU145 conditioned media; (b) PC3 conditioned media.

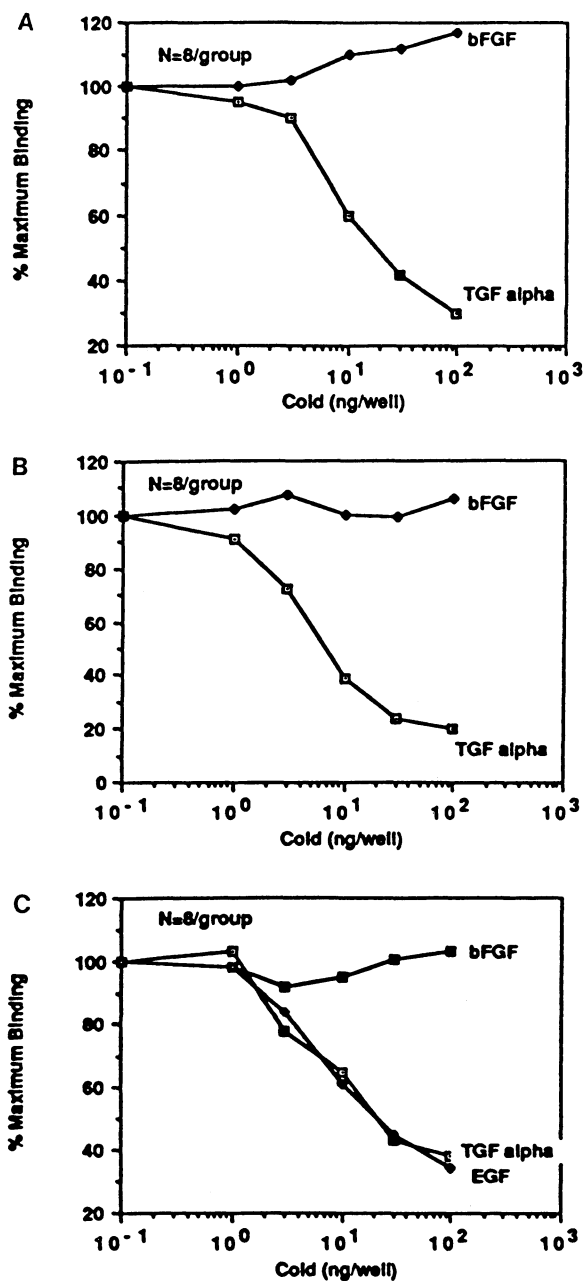


Figure 4: EGF receptor analysis on three types of prostate cell: (A) PC3; (B) DU 145; (C) BPH. In radio-receptor studies, cells (1×10^5 /well) were plated in 24 well plates and allowed to adhere over night. Cultures were washed (3X) with cold binding buffer and 1 ng/ml of ^{125}I -TGF- α was added to each well followed by addition of non-radiolabeled TGF- α , EGF, or bFGF at half log dilutions (0-100 ng/well). Cultures were incubated (4°C) for 4 hours, washed (3X) with cold binding buffer, and lysed. Radioligand binding was measured using a gamma counter.

munoprecipitable band with the same electromobility as that of purified EGF (6kD) was observed in the conditioned media of DU145 cells but not in PC3 and LNCaP cells (Fig. 3). Fowler et al [17] recently reported the presence of immunoreactive EGF in human prostate cancer tissues in high frequencies (68%), while only 6% of BPH tissues studied had EGF. These data suggest that EGF may also be an important growth factor in some prostate cancers. However, as indicated in the present study, PC3 cells contain TGF- α but not EGF.

EGF receptor in prostate epithelial cells was determined by using ^{125}I -TGF- α and competing with non-radioactive TGF- α , EGF, or basic FGF. Specific binding was demonstrated in cultured BPH epithelial cells [18,19] as well as in PC3 and DU145 cells (Fig. 4). The presence of EGF receptor in benign and cancer specimens of the human prostate has been reported [7,20,21] and the 125kD and 170kD forms of EGF receptor molecules in DU145 cells were also reported by Lin and Clinton [22]. These findings demonstrate the presence of specific, high-affinity EGF/TGF- α receptor in benign and malignant prostate tissues and cell lines.

Effect of Exogenous EGF and TGF- α

EGF is mitogenic for isolated prostatic epithelial cells from the rat, dog, and human [7,23,24]. Results of our studies also indicate that addition of EGF in cultures of BPH epithelial cells and DU145 enhanced cell proliferation (Table 3). However, EGF had no mitogenic effect on PC3 cells. Similarly, exogenous TGF- α was mitogenic for BPH epithelial cells and for DU145 cells. Growth of PC3 cells was not further stimulated by TGF- α in high density cultures. But in low-density cultures, PC3 cell proliferation was stimulated by TGF- α . The observation that EGF and TGF- α had a minimum effect on PC3 cell cultures in these studies could be attributed to the possibility that PC3 cells were producing TGF- α as an autocrine growth regulator. Therefore, addition of these growth factors exogenously to cultures of PC3 cells had little or no growth-stimulating effect. The above possibility is supported by the observation that cultivation of PC3 cells in the presence of anti-TGF- α resulted in a 61% decrease ($P < 0.05$) in cell proliferation (Fig. 5). Addition of normal rabbit serum or anti-EGF did not significantly alter the rate of PC3 cell growth. Basic FGF and PDGF were not mitogenic for either benign or malignant human prostate epithelial cells. TGF- β , a protein structurally unrelated to TGF- α [25,26], was a potent growth inhibitor for isolated epithelial cells.

Table 3: Effect of Exogenous Defined Growth Factors on the Growth of Benign and Malignant Prostate Epithelial Cells *in vitro*

Growth Factor	Cells x 10 ⁴ /flask			
	PC3 Day 4	PC3 Day 6	DU145	EPH Epithelia
Day 0 Control	2.4 $\pm$ 0.6	2.4 $\pm$ 0.6	3.1 $\pm$ 0.7	11.3 $\pm$ 1.8
ITS+ control	27.0 $\pm$ 1.8	87.3 $\pm$ 6.6	32.0 $\pm$ 4.6	10.3 $\pm$ 1.7
10% FBS control	35.2 $\pm$ 2.8	125.3 $\pm$ 8.1*	189.0 $\pm$ 9.6*	120.0 $\pm$ 17.9*
TGF- α (10 ng/ml)	51.3 $\pm$ 7.0*	102.5 $\pm$ 2.4	61.3 $\pm$ 9.4*	64.0 $\pm$ 4.6*
EGF (10 ng/ml)	30.0 $\pm$ 2.1	93.3 $\pm$ 6.8	79.3 $\pm$ 2.9*	56.2 $\pm$ 9.1*
TGF- β (1 ng/ml)	9.9 $\pm$ 2.0*	10.0 $\pm$ 2.0*	17.3 $\pm$ 1.8*	10.7 $\pm$ 1.3
bFGF (10 ng/ml)	27.3 $\pm$ 6.1	88.0 $\pm$ 6.1	30.0 $\pm$ 5.8	16.0 $\pm$ 2.0
TGF- α + TGF- β				11.7 $\pm$ 0.7

N = 8-12/group

* P < 0.05 compared to ITS+ negative control

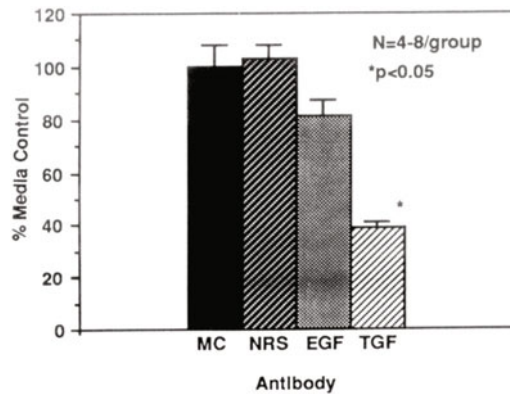


Figure 5: Inhibition of PC3 growth by anti-TGF- α . PC3 cells (1×10^4 /well) were plated in 24-well plates and incubated in media containing anti-TGF- α , anti-EGF, or normal rabbit serum. Control wells contained serum-free medium alone. Cultures were incubated for 6 days and media was changed on days 2 and 4. Cell counts were performed by detaching cells with 0.25% trypsin and counting with a hemacytometer.

CONCLUSION

Our results indicate that: 1) conditioned medium from androgen-independent prostate cancer cell line PC3 possesses growth-promoting activity for the established prostate cancer cell lines PC3, DU145, and LNCaP as well as freshly isolated epithelial cells from prostatic carcinoma specimens; 2) immunoreactive TGF- α is present in the conditioned medium of PC3, DU145, and LNCaP cells; 3) PC3 and DU145 cells possess EGF receptors; and 4) the growth of PC3 cells is marginally stimulated by exogenous TGF- α , but can be inhibited by addition of anti-TGF- α . These data demonstrate that TGF- α is an autocrine growth factor for the prostatic carcinoma line PC3 and may explain the escape of PC3 cells, and possibly other androgenindependent prostate cancer cells, from the characteristic requirement of androgen for proliferation.

Cells (1×10^5 / flask) were plated in 25² cm flasks and allowed to adhere overnight. The following day (day 0), plating media was replaced with RPMI-1640 media containing 10% FBS (positive control), ITS+ (negative control) or ITS+ plus growth factors. Cultures were incubated for 6 days with media being replaced on days 2 and 4. Cells were counted on day 6 by detaching with 0.25% trypsin and counting on a hemacytometer.

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DISCUSSION

Dr. Story:

When you check for growth of BPH derived epithelial cells, and particularly its response to exogenously added bFGF, was this done in the presence of bovine pituitary extract or bovine brain extract?

Dr. Lee:

No. We didn't put bovine pituitary or bovine brain extract in.

Dr. Story:

So, the cells were grown in the absence of this agent?

Dr. Lee:

That's right. Does anyone want to add something to that?

Dr. Sherwood:

The media that we utilized to test the responsiveness of BPH epithelial cells to growth factors in the studies that Dr. Lee just presented was the medium developed by Dr. McKeehan known as WJJC 404. This medium was supplemented with ITS (insulin, transferrin, selenous acid) and cholera toxin, but did not contain bovine pituitary or bovine brain extract.

Dr. Lee:

We repeatedly could not show basic FGF or any stimulatory factor in terms of epithelial cell proliferation.

Dr. Linehan:

Has anyone looked, for example, with another assay, like a radio-receptor assay, to look in conditioned media for transforming growth factor alpha? In their initial studies evaluating expression of the TGF α message in human renal cell carcinoma, Derynch, et al. demonstrated increased expression in tumor tissue, however they did not detect it in the conditioned media. We have heard from a number of groups that there is increased message for TGF α in human prostate carcinoma. Have you had the opportunity to evaluate by Western blot or by radio-receptor assay for the presence of TGF α in the media?

Dr. Lee:

No. We didn't do radio-receptor assays for TGF alpha. Western blot clearly demonstrates it's a specific antibody, that the presence of a single band in the condition media. This is 5.6 kilodaltons, and nothing else. Also, in the cell extracts and cell lysate, we could not detect 5.6 kD, but rather it's around 20kD.

Dr. Ratliff:

Your exogenous TGF alpha didn't stimulate very well. Do you think that it's the higher molecular weight form that is actually doing the activation, or is it the lower weight form?

Dr. Lee:

Do you mean didn't stimulate PC3 cells?

Dr. Ratliff:

Right.

Dr. Lee:

We sort of expected that. We had concerns similar to yours. Later on we considered the fact that PC3 cells are able to secrete TGF alpha, and if TGF alpha is considered an autocrine growth factor, then the PC3 cells were able to pull out a lot of it. If you put exogenous source of TGF alpha, its effect is not going to be very easily shown. As a matter of fact, we showed that when the cells in their early stage are at low density, then they form. But at high density we couldn't show it. Probably it is attributed to the presence of its endogenous source.

Dr. Ratliff:

What other tumors have been shown to secrete TGF alpha into the medium?

Dr. Lee:

Breast cancer cell line, MCF7, and a variety of tumors produce TGF alpha.

PROSTATIC GROWTH FACTORS (PrGFs)----FROM THE IDENTIFICATION OF PROBASIN TO THE ROLE OF PrGFs

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ABSTRACT

Rat prostates contain a unique androgen-regulated protein of 19-21 kilodaltons, "probasin" (*prostatic basic protein*), that is statistically related to members of alpha-2-microglobulin ligand carrier family. Probasin has low but distinct growth factor activity as determined by DNA synthesis stimulation with BALB/c 3T3 fibroblasts. With this as turning point, we have studied identification and significance of prostatic growth factors. Rat dorsolateral prostate contains heparin-binding growth factors (HBGFs or FGFs) and prostatic EGF-related mitogen (PEM); PEM is a member of the non-HBGF family that is the major growth factor in normal prostate but hardly detectable in prostate tumor tissues. In human pathologic prostates, HBGFs are major growth factors both in benign prostatic hypertrophy (BPH) tissues and in prostate cancer. The HBGF content in BPH tissues is higher than in prostate cancer, whereas osteoblast growth factor (OGF) as assayed by DNA synthesis stimulation using MC3T3-E1 osteoblasts is higher in prostate cancer. A metastatic cell line, AT-3, established from the Dunning rat prostatic carcinoma produces OGF, insulin-like growth factor II (IGF-II) and transforming growth factor beta (TGF- β). PEM and OGF, both of which have low affinity for heparin, are different from each other in stability to heat and acid treatments. Partially purified OGF contains IGF-II and TGF- β in addition to an acid labile polypeptide growth factor with a molecular weight of about 19 kilodaltons. Cell lines with high potency for metastasis (AT-3, MAT-LyLu and MAT-Lu), established from the Dunning rat prostatic carcinoma, can produce higher amount of IGF-II. TGF- β at a concentration as low as 0.05 ng/ml either stimulates attachment or detachment of AT-3 cells, depending on the kind of culture media. Detached AT-3 cells are able to grow in suspension. TGF- β stimulates cell growth of MC3T3-E1 osteoblasts.

In my hypothesis, i) angiogenesis of HBGFs and growth inhibition of TGF- β on epithelial cells might have the most critical influence on the latency of prostate cancer, ii) cell detachment stimulating activity of TGF- β has a possible role in the initial stage of metastasis, and iii) OGF, in addition to TGF- β and IGF-II, might promote the continuous formation of osteoblastic bony metastases.

Abbreviations used: HBGF-1, heparin-binding growth factor 1 (acidic FGF); FGF, fibroblast growth factor; HBGF-2, heparin-binding growth factor-2 (basic FGF); IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; TGF- β , transforming growth factor type β ; PrGF, prostatic growth factor; DLP, dorsolateral prostate; BPH, benign prostatic hypertrophy; PEM, prostatic EGF-related mitogen; OGF, osteoblast growth factor; CHAPS, 3-[(cholamido-propyl)dimethylammonio]-1-propane sulfonate.

Growth Factor Activity of "Probasin"

Probasin (prostatic basic protein) is a 19-21 kilodaltons, androgen-regulated protein that was originally isolated from the nuclei of rat dorsolateral prostate (1,2). Subsequently, we demonstrated that the majority of the probasin produced in the tissue was localized as a secretory protein by immunohistochemical analysis using monoclonal antibody (3). Purified probasin showed distinct growth factor activity at a concentration of 100 ng/ml as determined by DNA synthesis stimulation assay using BALB/c 3T3 fibroblasts (5).

We recently found that DLP extracts contain a 20-22 kilodalton heparin-binding protein (prostatic heparin-binding protein) with near identical chromatographic properties to HBGF-1, but only 0.2-1% of the mitogenic activity of HBGF-1. Sequence and immunochemical analyses demonstrated that the prostatic heparin-binding protein is identical to probasin (6). Immunofluorescent analysis using a specific monoclonal antibody against probasin suggests that probasin may have functions on reproductive physiology (5). Matusik et al. (7,8) have recently revealed that the DNA sequence of probasin has high homology to members of the alpha-2-microglobulin ligand carrier family, and that the localization of probasin to nuclei and secretory granules resulted from initiation of protein synthesis at different AUG codons; initiation at the first AUG codon produces a protein with a 14 amino acid signal peptide that is localized in secretory granules and initiation at the second AUG codon produces a protein lacking a signal peptide that is localized in nuclei.

Growth factors that we found in the prostate (PrGFs)

The clinical and pathological characteristics of BPH and prostate cancer have suggested the presence of certain local tissue factors. Pioneer work by Jacobs et al. (9,10) and subsequent studies by Story et al. (11-13) and Matuo et al. (5,14-21) demonstrated the presence of polypeptide growth factor(s) in normal and pathologic prostates. Prostatic growth factors (PrGFs) that have been found in the prostatic tissues and cultured prostatic cancer cells are summarized in Table 1 and reviewed below:

i) HBGF: Nishi et al. (14) first found a basic FGF-like growth factor in BPH tissues that can be classified as a member of the HBGFs family. This finding was subsequently confirmed by Story et al. (12,13). The abundance of HBGFs in prostate tumors is indicated not only by investigations at the protein level (15) but also by those at the nuclei acid level (22,23). Expression of mRNA for HBGFs was recently examined in rat and human prostates (Table 2); HBGF-1 is preferentially expressed in rat prostate, and HBGF-2 in human prostate. HBGF is a potent mitogen for mesoderm-derived cells (24,25). The androgen-independent proliferation of isolated epithelial cells from androgen-dependent rat prostate tumors and normal prostate of rat and human requires "prostatropin", which is identical to HBGF-1 except the first 14 N-terminal amino acids are missing (26,27). It has become clear that among the growth factors, HBGFs could be mitogenic factors controlling capillary proliferation during tumor progression (28). As expected from their sequence homologies, HBGF-1 has a biologic effect similar to that of HBGF-2, although HBGF-1 is 30- to 100-fold less potent (28).

ii) IGF-II: Although there are a few reports of IGF-II production by neo-plastic cells, a possible role of IGF-II in fetal growth suggests the production of IGF-II by rapidly growing cells. The production of IGF-II in prostate cancer was first reported in AT-3 cells, established from the Dunning rat prostatic

Table 1: Prostatic growth factors (PrGFs) and their possible functions

Growth factor	Tissues or cells	Possible function
FGF (HBGF)	Dunning rat tumor: aFGF AT-3 cells: bFGF BPH tissues: bFGF Human prostate cancer: bFGF	Angiogenesis
EGF-like GF	Normal rat prostate: EGF and EGF-like high molecular weight GF	Growth of normal epithelial cells
OGF	Prostate cancer (rat & human) AT-3 cells	Osteoblastic bony metastasis
IGF-II	AT-3 cells	Metstasis(?)
TGF- β	AT-3 cells	Metastasis

Table 2: Expression of HBGF mRNA in the prostate (22,23)

Prostate	HBGF-1 (aFGF)	HBGF-2 (bFGF)
Human prostate:		
BPH	-	++
Cancer	-	++
Rat prostate		
Normal*	++	-
AT-3 cells	++	-

*Normal rat of 6-8 weeks of age

adenocarcinoma, by radioimmunoassay using IGF-II monoclonal antibody (18). Although cross-reactivity of this antibody with human IGF-I is less than 3%, the possibility of IGF-I can not be ruled out. In vitro cell lines were established from biologically distinct in vivo Dunning R 3327 rat prostatic tumor sublines (29). Of five cell lines with different metastatic potency, AT-3, MAT-LyLu and MAT-Lu, all of which have higher potency for metastasis, can produce higher amount of IGF-II than AT-1 and AT-2, which are less potent for metastasis (Table 3). These results, together with its possible role in fetal growth (30, 31), suggest that IGF-II is a mitogen for prostatic cancer cells. Kato et al. (32) demonstrated that rat IGF-II stimulated proteoglycan synthesis in chondrocytes. Glycosaminoglycans existing in vivo as complexes of proteoglycans have been shown to affect a variety of biological functions such as attachment (33), motility (34, 35), differentiation (36, 37), proliferation (38-43) and angiogenesis (44). Therefore, IGF-II may contribute to tumor formation at the peripheral (or local) region of the prostate tumor and at the metastasizing site. In addition, Massague et al. (45) indicated that IGF-II stimulation is required for cellular transformation by TGF- β .

iii) TGF- β : Ikeda et al. (46) first found that PC-3 cells, established from human bone marrow metastasized by prostate cancer can produce TGF- β type 2. Two groups (47, 48) showed that cultured osteoblasts can respond mitogenically to exogenous TGF- β . By contrast, TGF- β is well known as a potent inhibitor of the proliferation of many cells in vitro, particularly of epithelial cells. Mckeehan and Adams (49) recently found that TGF- β inhibited EGF-dependent growth of epithelial cells derived from the Dunning prostatic carcinoma, and that HBGF-1 completely attenuated the inhibitory effect of TGF- β , suggesting that prostate tumor cell growth may escape the inhibitory effects of TGF- β if HBGF is at adequate levels. Kyprianou and Isaacs (50) found the presence of high affinity, low capacity receptors for TGF- β in the rat prostate; androgen deprivation by castration resulted in a significant increase in radioactive TGF- β binding to prostatic membranes, an effect that could be reversed by androgen administration to castrated animals. They have also found recently that steady state levels of TGF- β mRNA determined by Northern blot analysis was increased about 10-fold and maximally expressed by 4 days after castration (51). These results suggest a negative regulation of TGF- β and its receptor levels in the ventral prostate by androgens. The immunosuppressive effect of TGF- β indicates its role on malignant progression (52). We have recently found that AT-3 cells could produce TGF- β in a latent form (Table 4); colony formation of NRK-49F cells in soft agar was not stimulated by the sample when it was extracted at pH 7.5 (21). TGF- β at a concentration as low as 0.05 ng/ml either stimulated the attachment or detachment of AT-3 cells, depending on the culture media; TGF- β increased detachment about 13-fold in RPMI 1640, about 6-fold in DME and 4-fold in WJJC 404, whereas cell detachment was not stimulated in F-12 and α -MEM. Detached cells were able to grow in suspension. TGF- β at 0.1 ng/ml could also stimulate about 4-fold the growth of MC3T3-E1 osteoblasts established from mouse calvaria (21). These results suggest that TGF- β has functions not only on cell attachment but also on cell detachment, implying that TGF- β may be relevant to the first initial step of metastasis (21). There are several questions which remain unanswered: i) the biochemical details of cell detachment-stimulation with TGF- β , ii) expression of TGF- β mRNA and its regulation, iii) the activation mechanism and the regulation of the latent form of TGF- β , and iv) the type of TGF- β (beta 1 or 2).

iv) PEM: We first reported that the major growth factor in normal rat prostate is not a member of HBGFs (16), which is referred to as non-HBGF, but has properties similar to EGF, being referred to as PEM (20). PEM is a potent mitogen for prostatic epithelial cells in primary culture (19, 20). We have recently found that PEM is a mixture of EGF-like growth factor and unknown, acid stable growth factors by radioreceptor assays (unpublished data).

Table 3: Production of IGF-II by five cell lines established from the Dunning prostatic carcinoma of rat

Cell line	Metastatic Potency	Cell number (x 1/10 ⁶)	IOGF-II (ng)
AT-1	low	1.6	0
AT-2	low	2.6	2
AT-3	high	7.3	26
MAT-LyLu	high	11.0	17
MAT-Lu	high	3.4	10

v) OGF: Human prostate cancer is known to cause osteoblastic bony metastasis. The concept that a factor secreted by prostate cancer cells can induce an osteoblastic response was one of the clues that led to the study of OGF. A certain factor that is preferentially mitogenic for MC3T3-E1 osteoblasts was found in human prostate cancer tissues, but not in normal and benign hypertrophic prostates (17). OGF was purified from culture media conditioned by AT-3 cells in the presence of 0.1% CHAPS to minimize the loss of growth factor during purification (Fig. 1). Partially purified OGF is a more potent mitogen for MC3T3-E1 osteoblasts than BALB/c 3T3 fibroblasts (Fig. 2); two activity peaks were eluted from the molecular-sieve column, the major of which was fractionated at the position around 19,000 daltons. Most of the OGF activity (60-80%) is unstable against heat or acid treatment, and is not classified as a member of the HBGF family because OGF is not adsorbed on Heparin-5PW column even at low ionic strength (0.15 M NaCl). We also found that partially purified OGF contained IGF-II and TGF- β in addition to an acid labile factor. The acid labile active entity of OGF is now under investigation.

PrGFs relevant to latency and metastasis of prostate cancer

Tumor angiogenesis, the growth of new capillary blood vessels, is responsible for enabling tumors to enlarge in size. In addition to the angiogenic function of HBGFs, the finding that TGF- β has an inhibitory function on the growth of AT-3 cells (21) and normal prostate epithelial cells (49), and that the inhibitory function can be attenuated by HBGF-1, together with the finding that TGF- β has cell detachment stimulating activity for cultured prostate cells (21) allows us to speculate about the role of PrGFs on latency and metastasis of prostate cancer (Table 5).

The cells (29) were cultured in a 100-mm dish (Falcon 303) with 20 ml of Ham's F-12 medium containing 250 nM dexamethasone and 0.3% calf serum. The resulting culture media (40 ml) were lyophilized, followed by acid ethanol extraction (18). The extracts were lyophilized, reconstituted with 300 μ l of water, and subjected to IGF-II assay (18). Volume used for assay was 30 μ l for AT-1 and AT-2, and 10 μ l for other cell lines, respectively. Values were adjusted for 19 ng IGF-II derived from 40 ml of 0.3% calf serum. Cell numbers were calculated when the culture media were collected.

Table 4: Production of TGF- β by AT-3 cells: Stimulation of colony formulation of NRK-49F cells in the presence of EGF

Culture time (day)	Cell number (x 1/10 ⁶)	Colony number/dish	
		+ EGF	-EGF
2	0.3	140	3
		181	6
6	1.2	409	5
		390	4
10	1.9	363	3
		332	6

Colony numbers per dish in control experiment were 2 and 2, and those for TGF- β at 3 ng/ml were 454 and 435, respectively. EGF concentration was 5 ng/ml.

AT-3 culture medium
 ↓
 lyophilized
 ↓
 dissolved in 0.1% CHAPS
 ↓
 centrifuged
 ↓
 Supernatant
 ↓
 dialyzed against 10 mM Tris-HCl
 (pH 7.5)-0.15 M NaCl-0.1% CHAPS
 ↓
 HPLC on a Heparin-5PW column
 ↓
 Non-HBGF
 ↓
 dialyzed against 5 mM Tris-HCl
 (pH 7.5)-0.1% CHAPS
 ↓
 HPLC on a IEX-535K column
 ↓
 HPLC on a TSK G300SW column
 ↓
 Partially purified OGF

Figure 1: Partial purification of OGF from culture media conditioned by AT-3 cells. CHAPS, a non-cytotoxic zwitterionic detergent, was added in all the steps to minimize the loss of growth factor during purification (53).

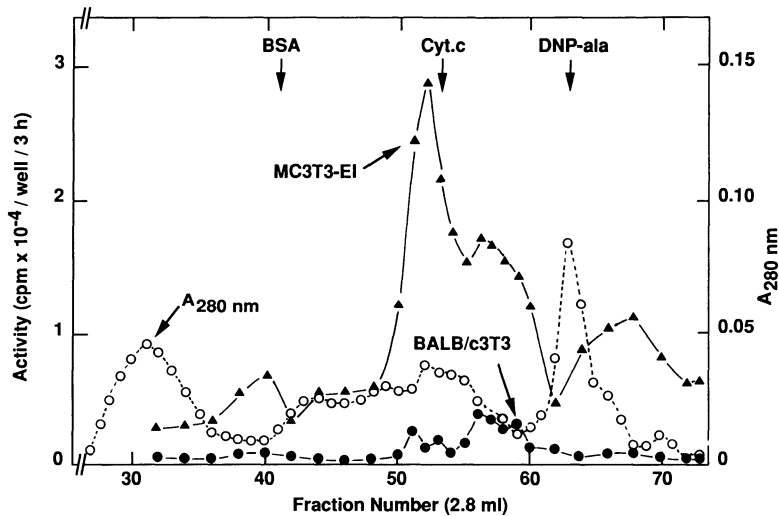


Figure 2: Molecular-sieve chromatography of OGF in AT-3 culture media on a TSK G3000SW column. Non-HBGF fractions obtained by Heparin-5PW HPLC (Figure 1) were dialyzed against 5 mM Tris-HCl (pH 7.5) containing 0.1% CHAPS. The dialyzed sample was subjected to HPLC using an IEX-535 column. Fractions that were not adsorbed at 0.15 M NaCl were collected, concentrated by ultrafiltration, and applied to a TSK G3000 SW column (21.5 mm ID x 60 cm). The resulting fractions were subjected to a DNA synthesis stimulation activity assay using BALB/c 3T3 fibroblasts and MC3T3-E1 osteoblasts (17). Molecular weight markers were BSA (bovine serum albumin; 67,000), cyt. c (cytochrome c; 12,000) and DNP-ala (dinitrophenyl alanine; 255).

Table 5: Hypothetical contributions of PrGFs to latency and metastasis of prostate cancer

State	PEM	HGBF	TGF- β	IGF-II	OGF
Normal prostate*	+++	+ -	++	-	-
Latent cancer	?	++	++	+-	+-
Clinical cancer-	+++	++	++	++	

i) Normal prostate: PEM is the major growth factor in normal prostates of adult rats. It is difficult to discuss major growth factors in normal human prostates because the definition of normal prostate should include age of the specimens in addition to pathological features (17). It is suggested, however, that non-HBGF, probably EGF-like growth factors, is predominant in normal young human prostates (54). HBGFs may not be needed for normal growth of adult prostate, although they may be responsible for androgen dependent growth and function of the prostate as a paracrine factor secreted from the stroma. TGF- β may inhibit overgrowth of normal prostatic epithelial cells. IGF-II and OGF might not be expressed in normal prostate.

ii) Latent cancer: In an early period of prostate cancer, the HBGF content is low but the TGF- β content high. Thus tumor angiogenesis and overgrowth of cancer cells are depressed. IGF-II and OGF might be hardly produced in latent cancer.

iii) Clinical cancer: By increasing the HBGFs content, tumor angiogenesis is stimulated and growth inhibitory effect of TGF- β is attenuated. Thus, tumors can enlarge even if TGF- β is produced. It is possible that TGF- β unresponsive cells increase and grow. At this stage, when HBGFs expression is too low to promote tumor angiogenesis or attenuate TGF- β function, the tumor can be localized in a latent form. Once tumor size increases due to high HBGFs content, the tumor can progress into clinical cancer. In parallel with tumor angiogenesis, TGF- β stimulates cell detachment of cancer cells. When the detached cells arrest on bone, OGF in addition to TGF- β and IGF-II can function as a potent mitogen and stimulate osteoblastic bony metastasis. For the clarification of in vivo functions of these four growth factors, expression of mRNAs and histological localization of PrGFs (stroma vs. epithelium) together with androgen receptors should be unveiled. We predict that there must be great a difference in the contents of HBGFs, IGF-II and OGF between latent cancer and clinical cancer and also between benign hypertrophic prostates and prostate cancer.

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DISCUSSION

Dr. Heston:

There was a report a while back where I think they had looked at the growth of prostatic epithelial cell lines in the presence of stroma derived from bone, and found that to be growth inhibitory. As the MC3T3 E1 cells are isolated from mouse, you don't see a problem here. I was curious if you've looked at any other bone derived elements in this system?

Dr. Matuo:

That is a very interesting question. We only looked at the MC3T3 E1 osteoblasts and of course we have to use another cell line, I think we have to have some extract from the bone, but we've never done that experiment.

Dr. Heston:

Have you identified a component in the HAM F12 that you think may be related to its effect of TGF beta in contrast to the RPMI media?

Dr. Matuo:

Can you repeat the question?

Dr. Heston:

Is there anything in the HAM medium that might be different from the RPMI medium?

Dr. Matuo:

The specific components in F12 are hypoxanthine and linoleic acid. In α -MEM, the specific component is ascorbic acid. We don't know the independent effects of these medium components.

Dr. Chung:

To test your hypothesis, have you tried to monitor the TGF- β mRNA in the highly metastatic line versus the low metastatic line? I think you could have the TGF- β which can be secondarily fibronectin and tenascin biosynthesis, both of which are known to affect cell attachment on the extracellular matrix. Have you considered these other possibilities as an alternative explanation of your hypothesis?

Dr. Matuo:

Yes, I think so. Concerning the expression of TGF- β mRNA, I think we should have that experiment. We coated dishes with fibronectin to see whether it helps attachment of AT3 cells. Fibronectin does not help to attach AT3 cells, but collagen does. Expression of TGF- β and fibronectin is controlled by TGF- β . And that is negatively regulated by androgen.

Dr. Chung:

In a recent report, it was observed that chick fibroblasts attachment to plastic dishes was facilitated by fibronectin, but inhibited by tenascin. So one of the possibilities is that TGF- β may make tenascin-like molecules which dissociate the fibronectin attachment. Therefore, cells will commit to metastasis, which is what you're saying. One of the other things I was thinking about is whether anybody has put TGF beta gene into the cell and observed where the cell will move, like what Dr. Coffey was commenting on this morning? I don't know whether this sort of experiment has been done. Do you know some literature on that?

Dr. Matuo:

No, I don't think we have. I think we should have mRNA expression using different metastatic property cell lines.

Dr. Story:

Just a point of clarification, did you indicate that the TGF- β -like material was present in BPH as well as prostate cancer, but that there was more in prostate cancer? Is that correct?

Dr. Matuo:

That is a little bit complicated. He said, "We found that OG activity is higher in prostatic cancer than BPH." But our OGF activity is a mixture of several growth factors. At least one is very acid stable. The second one is acid stable. In the acid stable growth factor fraction, we found IGF-II and TGF- β .

UROGENITAL SINUS DERIVED GROWTH INHIBITORY FACTOR

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INTRODUCTION

The phenotypic differentiation of several tissues including skin, eye, lung, mammary gland, and gut are regulated during organogenesis by tissue-tissue interactions (1-5). Likewise, stromal-epithelial interactions are of fundamental significance in the development of the prostate gland from the fetal urogenital sinus (6-8). In the urogenital sinus, such interactions rely on the stimulation by androgens (9), yet a more detailed understanding of specific mechanisms awaits further study. The rapidly growing field concerned with the study of growth factors have suggested that these factors function in cell and tissue differentiation as well as in cell proliferation. Indeed, with many systems it has become evident that factors and hormones which control or influence growth, are also regulatory to cytodifferentiation. A popular theory regarding the putative mechanisms associated with stromal-epithelial interactions is the suggestion that local-acting paracrine growth factors, secreted by the stroma, act to regulate epithelial growth and differentiation. In order to determine whether growth factors are secreted by the urogenital sinus, we cultured the urogenital sinus from day 18 fetal rats and studied the conditioned medium for growth factor activity. In order to maintain the tissue-tissue arrangement as *in vivo*, the urogenital sinus was cultured intact as organ cultures since reciprocal inductive influences between epithelium and stroma are likely to be important. The analysis of conditioned medium revealed that a potent growth inhibitory activity was evident (10). No growth stimulatory activity was observed (10). These results were somewhat of a surprise to us since during organogenesis, a process characterized by rapid growth, one would postulate that if any growth regulatory activity would be measurable, it would be stimulatory in nature. However, we must consider that the overall process of ductal and acinar morphogenesis involve both cell proliferation and a slowing of growth concurrent with the attainment of a cytodifferentiated phenotype. Accordingly, it is quite possible that both growth stimulatory and inhibitory signals are operable, which together determine the placement of a cell between the extremes of a proliferating stem cell and a quiescent differentiated cell. With this possibility in mind, we set out to characterize the growth inhibitory activity in urogenital sinus conditioned medium in order to understand its potential role in the morphogenesis of the prostate gland.

Production of Growth Inhibitory Activity by Urogenital Sinus Organ Cultures and Derived Cell Lines

Urogenital sinus was removed intact from day 18 fetal rat embryos. The organ was cultured in Dulbecco's minimum essential medium (DMEM) containing 5% calf serum, 5% Nu-serum (Collaborative Research), insulin (5 ug/ml), testosterone (0.5 ug/ml), penicillin (100 units/ml), and streptomycin (100 ug/ml). Organs were placed in 24-well culture dishes where they promptly attached (10). To assay for growth factor activity we used the rat bladder NBT-II epithelial cell line for several reasons. This cell line is easily handled and sub-passaged to generate large numbers of replicate cultures. In addition, studies by Cunha and associates have shown that rat bladder epithelium can be induced to assume a prostatic phenotype by urogenital sinus stroma (8). Accordingly, we examined the effects of urogenital sinus conditioned medium on the biological properties of NBT-II cells as an initial starting point in our studies. The initial collection of

medium conditioned for 48 hours by organ cultures showed growth inhibitory activity when used at a 30% (v/v) ratio (10). Control studies showed that this activity was endogenous to the conditioned medium and was not due to dilution or any other effects on the basal medium. The production of growth inhibitory activity continued until regression of organ explant at approximately one month of culture. A monolayer of fibroblastoid cells emanated from the explant periphery during the first week of culture and reached confluence in approximately 2 weeks. If the urogenital sinus organ culture was removed at the time of monolayer confluence, growth inhibitory activity was not detectable in the medium conditioned by monolayer alone. The monolayer of cells remained confluent and quiescent for approximately 3-4 weeks with undetectable growth inhibitory activity in the conditioned medium. At approximately 4 weeks of culture, multicellular foci of cells formed into three-dimensional spheroids which grew radially and eventually reach approximately 1-3 mm in diameter. The monolayer surface was observed to be peppered with such spheroids. At the point of visual identification of spheroids, growth inhibitory activity was first detected in the conditioned medium. The production of growth inhibitory activity continued for up to 9 months of continuous culture of the spheroids. The fibroblastoid monolayer which produced spheroids has been sub-passaged for 2 years now, and has been termed the U₄F continuous cell line. This cell line will predictably form spheroids several days post-confluence and will produce growth inhibitory activity only when in the spheroid configuration. Histological analysis of spheroids revealed them to be composed of a core of eosinophilic ground substance (extracellular matrix), surrounded by a 3-9 cell layer of fibroblastoid cells. The ability of the U₄F cells to produce growth inhibitory activity only when organized as spheroids suggested that they possibly require interaction with their own specific extracellular matrix for expression of the growth inhibitory activity. Of recent interest to us has been the appearance of a variant cell line, spontaneously derived from late passage U₄F cells. These cells proliferate to confluence but do not form typical spheroids. However, these cells produce growth inhibitory activity at confluence. These cells have been continuously sub-passaged and have been designated the U₄Fl cell line. These data suggest that U₄Fl cells have modulated their requirements for the production of growth inhibitory activity. They no longer require a spheroid formation. It is possible that they no longer require certain interactions with their own biomatrix. The elucidation of the specific requirements for the production of growth inhibitory activity by urogenital sinus in organ cultures and the derived cell lines awaits further study. Figure 1 shows an overall schematic summary of the production of growth inhibitory activity and derivation of cell lines by urogenital sinus organ cultures.

Properties of the Growth Inhibitory Activity

The conditioned medium from urogenital sinus or derived cell lines produced other effects besides growth inhibition when added to NBT-II cells. The growth inhibitory effects were characterized by an inhibition in the incorporation of [³H] thymidine and a decrease in cell number. A concentration of 30% (v/v) conditioned medium resulted in a 35-50% decrease in [³H] thymidine incorporation and a 40% decrease in cell number after 3 days exposure (10). Of additional interest was our observation of a significant change in the phenotypic morphology of NBT-II cells when exposed to conditioned medium. These cells normally assume a typical epithelioid morphology, characterized by islands of polygonal cells. When exposed to 30% (v/v) conditioned medium they became larger, more spread-out and stellar in shape with multiple lamellipodia and filopodia (10). Since conditioned medium induced changes in proliferation and morphology, we determined whether exposure to conditioned medium altered protein synthesis and secretion by NBT-II cells. Although there was no significant changes observed in the incorporation of [³⁵S] methionine into total

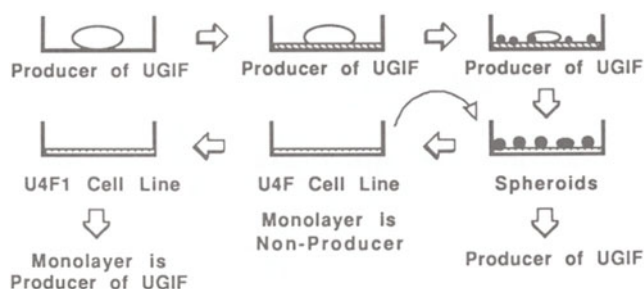


Figure 1: UGS Organ Culture Day 18 Fetal Rats. Production of UGIF by fetal rat urogenital sinus organ cultures and derived cell lines.

cellular protein, we did observe an 18.6% increase in secreted proteins by these cells (10). Studies at this point showed that urogenital sinus conditioned medium induced a decrease in cell proliferation, a change in phenotypic morphology, and an increase in the synthesis of secreted proteins. Each one of these events are identified as paralleling terminal differentiation (11). For many characterized cell systems the state of terminal differentiation is one of reduced proliferation or quiescence, concurrent with morphological alterations, and the expression of new protein (12). Accordingly, we deemed it possible that a factor, or set of factors produced by the urogenital sinus in organ culture might act to induce differentiation in responding cells, concurrent with a decreased proliferative state. Our data with NBT-II cells is consistent with such a suggestion.

Whenever it is proposed that putative growth inhibitory factors are responsible for an observed growth inhibition, it should be demonstrated that such a growth inhibition is not due to toxicity and is reversible in nature. To address such issues, we first asked the question of whether there was a linear cell response to increasing concentrations of conditioned medium. For these experiments, NBT-II cells were exposed to 0-30% (v/v) of conditioned medium and the effect on [³H] thymidine incorporation determined. Between the concentrations of 0 to 15% conditioned medium, the cell response was a direct and linear function of the concentration of the conditioned medium (13). Concentrations of conditioned medium from 15-30% did not produce a greater cell response. These data showed that the cell response was a linear and saturable function of the percent volume of conditioned medium. The fact that the response was saturable was of interest, since it indicated that cell response was probably not toxic, and that if factor(s) exist, they might be acting through saturable receptor sites. To study toxicity further, cells were exposed to maximal concentrations of conditioned medium (30%, v/v) for three days and assayed for cell viability through measuring the uptake of crystal violet dye and trypan blue dye exclusion. Both of these assays showed that cell viability was identical between control and treated cultures, on each of the three days of treatment. Cultures exposed to conditioned medium continued to proliferate, but did so at a slower rate (increase in population doubling time). Upon removal of conditioned medium, the cultures assumed a population doubling time identical to the non-treated control, and reached the same density at confluence as control. These experiments showed that cell response to conditioned medium was reversible.

The observations that cell response to conditioned medium was saturable, reversible, and non-toxic in nature also suggested that a putative growth factor or set of factors produced by urogenital sinus and derived cell lines was responsible for such activity. Studies were next directed to determine some of the physicochemical properties of the putative factor. Conditioned medium was exposed to heating (80 °C, 15 min.), acid (PH 3.0, 15 min.), and reducing agent (100 mM dithiothreitol, 100 mM B-mercaptoethanol, done separately). The activity was stable to both heat and acid treatment. Conversely, activity was diminished by exposure to reducing agents. These properties are similar to those reported for the transforming growth factors type beta (TGF-Bs) family (13). Since TGF-B1 is known to be growth inhibitory to many epithelial cells in monolayer culture and is secreted by many cell types (usually in an inactive form), experiments were designed to determine whether the activity in conditioned medium might be due to endogenous TGF-B. For these experiments, several cell lines known to be responsive to TGF-B1 were assayed for response to conditioned medium. In addition, a neutralizing affinity purified antibody to TGF-B1 (neutralizes TGF-B1, B2, and B1.2 activity) was tested for cross-reactivity with growth inhibitory activity. The first cell line tested was NBT-II, which was responsive to TGF-B1. Accordingly, the Mv-Lu-1 cell line (mink lung epithelial) was used owing to its well-characterized response to TGF-B1. Mv-Lu-1 cells were growth inhibited by both TGF-B1 and conditioned medium. The response to TGF-B1 was fully neutralized by antibody, whereas the response to conditioned medium was unaffected. Control studies showed that low concentrations of conditioned medium producing a minimal effect could not be neutralized by increasing concentrations of neutralizing antibody. The results were identical when A-549 cells (human lung epithelial) were tested. We tested NRK cells (normal rat kidney fibroblasts) since these cells are stimulated by TGF-B1 in the presence of epidermal growth factor (EGF) (13). NRK cells were stimulated by TGF-B1 and inhibited by urogenital sinus conditioned medium when cultured in medium containing EGF. The antibody predictably neutralized the effects of TGF-B1 and did not affect the response to conditioned medium. To confirm these results we determined the effects of conditioned medium using Y-79 cells. The Y-79 cell line was derived from a human retinoblastoma, and is devoid of TGF-B receptor complex (14). Accordingly, this cell line does not respond to the TGF-Bs tested to date. When treated with increasing concentrations of conditioned medium, Y-79 cells demonstrated a typical linear and saturable dose-response curve of inhibition of [³H] thymidine incorporation. These results were of significance, since they suggested that the putative factor(s) present in conditioned medium does not act through the TGF-B receptor complex. In summary, these studies together indicated that the growth activity in urogenital sinus conditioned medium was not due to any of the toxic cytokines or to the currently characterized TGF-Bs. The list of genes and proteins belonging to the TGF-B family is growing at a rapid pace and the possibility that the growth

inhibitory activity described here belongs to this family cannot be ruled out. Based on these observations we set out to purify the activity from conditioned medium in order to identify the factor(s) responsible for the growth inhibitory effect.

Purification of Urogenital Sinus Derived Growth Inhibitory Factor

In order to follow growth inhibitory activity through various steps of purification, a functional bio-assay was developed to quantitate activity in the form of units of inhibition. The assay was based on a mini-assay technique for [^3H] thymidine incorporation developed by our laboratory. The assay is highly accurate and has been validated as compared to the standard filter assays of [^3H] thymidine incorporation into TCA-precipitable DNA. The advantages of the mini-assay is that it allows for the ability to assay a large number of samples per assay (several hundred) in less than half the time of a standard filter assay. Accordingly, this procedure allows for the assay of over 300 samples per day with relative ease. The units of inhibition assay takes advantage of the linear and saturable dose response curves exhibited by the Mv-l-Lu cells upon exposure to increasing concentrations of conditioned medium. Mv-Lu-l cells were chosen since they represent the cell line with greatest responsiveness to the growth inhibitory activity in terms of maximal % inhibition (V_{max}) over all other tested cell lines to date. Partially purified activity from conditioned medium was used to generate standard curves to which samples of unknown activity were compared. The units assay is based on a computer analysis of unknowns vs. standard curve of UGIF activity, where 1/2 maximal response is assigned a value of 1 unit of activity. Linear regression analysis determines the units of inhibition activity for the unknowns (done in triplicate, with several concentrations). Units of activity can therefore be compared directly between different preparations and sample volumes. It must be pointed out that although this assay is a functional assay, it is not mono-specific, since any growth inhibitory factor will be assigned units of inhibition with this assay. Accordingly, additional assays such as the incorporation of [^{35}S] methionine into secreted proteins of PC-3 cells, typical cell morphological changes, and SDS-PAGE analysis of purification material at each step were also used to monitor activity at each step.

The elucidation of the basic physicochemical properties of the growth inhibitory activity in crude conditioned medium and the development of the units of inhibition assay were essential accomplishments for the formulation and development of a purification protocol. Various experiments were done to determine the elution profiles and yields of growth inhibitory activity with several chromatographic, ion exchange, electrophoretic, and HPLC procedures. In the final analysis, a 4 step procedure was considered most optimal in terms of simplicity, degree of purity, and apparent yield. Owing to the acid stability of the activity, several hundred ml of conditioned medium was processed first by dialysis with 1 M acetic acid, lyophilized and stored frozen until further use. The lyophilized material was resolubilized in 1 M acetic acid and applied to a preparative Bio-Gel P-200 gel filtration column equilibrated in 1 M acetic acid. The sample was eluted in 1 M acetic acid and aliquots of each fraction was assayed with the units of inhibition assay. A broad peak of bio-activity was observed eluting from the P-200 column with a corresponding molecular weight of 12-20 kiloDaltons. Bioactive fractions were collected, pooled and lyophilized. The sample was resolubilized in 50% formic acid and applied to a C-18 reverse phase HPLC column and eluted by a linear gradient of acetonitrile. Aliquots of each fraction were assayed for growth inhibition. A minor region of activity eluted isocratically, and a major, sharp peak of bio-activity was observed eluting at 43% acetonitrile. Upon analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) a predominant protein species of 12 kiloDaltons was observed consistent with the peak of bioactivity. The major peak of activity was found to be insufficiently pure at this point to utilize for amino acid sequence analysis. Accordingly, an additional step was inserted into the procedure. Owing again to the acid stability of the growth inhibitory activity, the pooled preparation from the P200 column was electrophoresed first through a preparative acid polyacrylamide gel (equilibrated in 1 M acetic acid) prior to application to the C-18 reverse phase column. Acid gels were chosen since SDS interfered with the bioassay, could not be fully removed from the electroeluted material, and since the SDS-denatured activity could not be re-natured. The utility of preparative SDS-PAGE for identification of activity was not feasible. Using acid gel electrophoresis, a prominent band was observed at 11-14 kDa (Mr confirmed by non-reducing SDS-PAGE). The protein was excised, electroeluted, dried and assayed positive for bioactivity. Control studies with other electroeluted proteins from the acid gels indicated that no other identifiable protein bands produced activity. The electroeluted material was chromatographed through the C-18 HPLC column and eluted with a linear gradient of acetonitrile. The elution profile indicated a protein species, eluting at 40-50% acetonitrile. Assay of fraction aliquots for growth inhibition showed bioactivity associated exclusively with the protein eluting at 43% acetonitrile. This peak represented a near-homogeneous preparation of a protein we have provisionally termed urogenital sinus derived growth inhibitory factor (UGIF) pending further identification. Whether this protein represents a novel growth regulatory factor awaits further purification and characterization of primary structure. Our analyses of the conditioned medium suggests that the UGIF protein is the sole

protein responsible for the growth inhibitory activity of the conditioned medium. Subsequent studies with purified UGIF protein have confirmed that this protein is responsible for the morphological response and effects on protein synthesis and secretion, as observed with the original conditioned medium.

Differential Cellular Response to UGIF

Since earlier studies had suggested that several variant cell lines may be responsive to the growth inhibitory activity in urogenital sinus conditioned medium, we decided to screen a number of cell lines with partially purified UGIF activity in order to determine the degree of cell selectivity in response. For these studies, partially purified UGIF (eluate from gel filtration chromatography) was added to replicate cultures in increasing concentrations and cultures assayed for inhibition of [³H] thymidine incorporation. Of eight different cell lines tested, several differences in reactivity were noted. Some cell lines such as Mv-Lu-1 were inhibited by over 80%, others were showed a low response (<20% maximal inhibition), and in the case of NIH 3T3 cells, no responsiveness at all. The responsive cells all demonstrated the typical dose-response curve with a linear and saturable function of the concentration of UGIF activity. There did not seem to be a selectivity of one particular cell type over another. Epithelial cell lines as well as fibroblast cells showed maximal saturable response anywhere from 20-80%. If any pattern could be drawn it was that epithelial cells seemed to be more sensitive as evidenced by requiring lower units of UGIF to produce 1/2 maximal response.

Two patterns of cell response were of interest. First, it was observed that v-Harvey-*ras* transformed NIH 3T3 cells were inhibited by over 30% with saturable concentrations of UGIF. In contrast, the non-infected, non-transformed NIH 3T3 cells which were the control cell line, showed no responsiveness to UGIF at any concentration tested. This observation is of significance since it suggests the possibility that UGIF may down-regulate the expression of the *ras* oncogene without affecting the normal mitotic control of the non-transformed cell. Studies to further characterize this effect of the UGIF activity are currently in progress. The second pattern of response was that observed between the two prostatic epithelial cell lines PC-3 and LNCaP. Both of these cell lines were derived from human prostatic adenocarcinomas. PC-3 was metastatic to bone, is androgen insensitive, and expresses no known prostate-specific proteins (15). In contrast, LNCaP was metastatic to lymph node, contains androgen receptors, is androgen responsive, and expresses prostatic acid phosphatase, a prostate specific protein (16,17). Accordingly, although both cell lines represent metastatic adenocarcinomas, the LNCaP cell line exhibits properties more consistent with the differentiated phenotype of normal prostatic epithelial cells, whereas the PC-3 cell line is anaplastic and is dedifferentiated as compared to normal. The PC-3 cell line showed a potent inhibition in growth when exposed to UGIF, whereas the LNCaP cell line showed a minimal response, at only higher concentrations of UGIF. In addition, UGIF induced a 50% increase in the secretion of newly synthesized proteins by PC-3 cells, yet showed no change over controls with LNCaP cells as shown with [³⁵S] methionine incorporation studies. Densitometric scanning of SDS-PAGE fluorographs of secreted proteins from PC-3 cells showed the selective induction of five protein species. Of these, a protein band was observed with a molecular weight of approximately 36 kiloDaltons, which was induced more than 3.4-fold when PC-3 cells were exposed to UGIF. This protein is of interest since prostate specific antigen (PSA) has a molecular weight of 34 kiloDaltons, is a secreted protein, and is a prostate-specific marker protein. Accordingly, studies are underway to determine whether the protein induced by UGIF represents an induction of PSA. These results suggest possibility that UGIF may be more effective with cells that have progressed away from the differentiated phenotype. Cells closer to the normal phenotype may be less responsive. These suggestions are highly speculative, however, provide the framework from which to study more closely the effects of UGIF activity on cell differentiation and its possible role *in vivo*.

Possible Roles of UGIF *In Vivo*

Urogenital sinus derived growth inhibitory factor was isolated from organ cultures of fetal tissue and derived cell lines. The possibility that UGIF activity represents an artifact of cell culture cannot be ruled out. A question can be asked as to what possible role a growth inhibitory factor may have during embryogenesis. To answer such a question one should consider the dynamic cellular events associated with the process of organogenesis. For example, in the urogenital sinus-prostate system of development, ductal and acinar morphogenesis requires a proliferation of presumed stem cells, which is induced and directed by the stroma in response to androgens. In this scenario, the stroma acts to induce cell proliferation, to form the framework of the eventual organ which will become the prostate. However, it is clear that stroma ultimately induces a functional phenotype, ie. a differentiated ductal and acinar network. It can be suggested that at some point the proliferation of epithelial stem cells slow, concurrent with an induction of cytodifferentiation, leading to the terminally differentiated prostatic phenotype. This phenotype is typified by rela-

tively quiescent epithelial cells. Accordingly, it is possible that prostatic morphogenesis involves inductive signals from stroma to induce stem cell proliferation, followed by inhibitory signals to induce cytodifferentiation and inhibition of cell proliferation. It is clear that several growth stimulatory factors and/or their receptors are associated with the prostate gland. These are most notably aFGF (18), bFGF (19), EGF (20,21), and TGF- β (22). Whether UGIF represents a paracrine acting factor partially responsible for growth cessation and induction of epithelial cell differentiation in the developing prostate remains speculative. However, if UGIF did have such a role, it might explain why UGIF is more active with less differentiated cells. It's possible that as a cell proceeds further to a state of commitment toward a differentiated phenotype, it loses responsiveness to UGIF since the stimulation to differentiate is no longer required. Along this line of thought, it is possible that as a cell proceeds to a state of terminal differentiation, it down-regulates receptors for UGIF or other factors which induce differentiation. This would explain why cells of less differentiation, typified by the anaplastic PC-3 and transformed *ras*-3T3 cells, respond to UGIF whereas cells showing some form of differentiation show a lessened response. It is possible that in disease states such as prostatic carcinoma, where cells progress to a less differentiated state, that such cells are re-programmed to express properties similar to embryonic stem cells. Hence, as part of the re-programming these cells may acquire the ability to respond to differentiation inducing factors as embryonic cells do.

Many non-transformed cell lines are known to assume a state of quiescence upon reaching confluence in monolayer culture. With several such cell lines it has been shown that tissue-specific proteins begin to be expressed, associated only with the confluent, quiescent state. The most referenced model system for the study of terminal differentiation *in vitro*, is the mesenchymal 3T3 stem cell model. These cells, upon confluence, differentiate into adipocytes as evidenced by the accumulation of lipid in the cytoplasm (23). The TAI cell line developed by Ringold and associates (24-26) reaches confluence, remains quiescent and undifferentiated for approximately 2-3 weeks, then differentiates into adipocytes. The TAI differentiation process is accelerated by glucocorticoids and indomethacin. Like TAI cells, the U4F cell line is mesenchymally derived from embryonic tissue. It is of interest to note that the time course of TAI cell differentiation after reaching confluence is almost identical to the time course of UGIF secretion by U4F spheroids after they reach confluence. In addition, we have shown that U4F cells show a typical dose-response to partially purified UGIF, suggesting that UGIF may act in some kind of autocrine loop. The morphological phenotype of the differentiated adipocytes is a stellar appearing cell with multiple filopodia extensions. A nearly identical morphology is noted by a number of cell types responding to UGIF (see discussion of NBT-II cell morphology). It may be possible that UGIF or a UGIF-like protein acts in an autocrine capacity to self-induce terminal differentiation of cells such as TAI cells upon a certain time period post-confluence. Through the use of cell models such as the PC-3 cell line and the 3T3 cell-adipocyte system, we can address speculative questions such as these using purified UGIF protein.

SUMMARY

The studies reviewed here have partially characterized a growth inhibitory activity of unknown identity, derived from the conditioned medium of fetal urogenital sinus organ and cell cultures. This activity affected the cellular properties of protein synthesis, secretion, and phenotypic morphology of several variant cell lines *in vitro*. The activity was not reversible in action. A selectivity of response was observed suggesting the possibility that transformed cells and less differentiated cells were more sensitive and responsive. Owing to the determination of several physicochemical properties, a protein of approximately 12 kiloDaltons has been purified to near homogeneity which elicits identical biological properties as observed with the crude conditioned medium. This protein has been provisionally termed UGIF (urogenital sinus derived growth inhibitory factor) pending further analysis. Whether the purified protein responsible for UGIF activity if novel, requires identification of amino acid sequence and further characterization. The availability of a purified protein will allow for the development of antibody and oligonucleotide probes. The availability of such probes will aid in the further study of this protein and its potential role *in vivo*.

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DISCUSSION

Dr. Smith:

One of the things that confuses me about the inhibitory effects is the fact that you see this difference with different cell types. I can understand how some cell types would not respond at all; some cell types would require a higher dose. Your data suggests that you get a plateauing effect so that you plateau out at 20% or 50%. Do you think that perhaps you've got heterogeneous cell populations, that may be some of the clones in that cell type are responsive and others are not? If you clone those cells, maybe 100% would respond? Have you tried that?

Dr. Rowley:

We haven't tried that, but the cell lines that we have used, such as mink lung epithelial cell lines, the A549's, this particular 3T3 cell line, were all clonal derivatives and they seem to respond. As for the other cell lines, we just don't know. The U4F cells and U4F1 cell lines are not clonal derived. They are heterogeneous, which may have something to do with spheroid formation versus confluency in terms of production of this factor. We just don't know. I might also add that this factor is produced not only in the medium with serum, but we've shown production of it now in defined medium, because we were concerned with possible inhibitory factors present in the serum. Also, we've labeled it with ^{35}S methionine labelled it, showing that it was a synthesized protein. So we have looked at that.

Dr. Lee:

This is a conceptual problem. I don't know if you feel strongly about it, where you have shown in sequence cell proliferation preceding cell differentiation. I thought from our experience that cell proliferation and cell differentiation are opposite events of the cell. In other words, if the cells are undergoing differentiation, proliferative activity would be diminished and vice versa.

Dr. Rowley:

That's exactly the same way I feel about this. Generally, one sees an inverse relationship between proliferation and differentiation. I think that is where growth stimulatory factors and inhibitory factors working together can offer to the tissue a state of high proliferation of stem cells and then acquiescing to a more differentiated phenotype, which may be what a factor such as this one is doing.

Dr. Tindall:

It would be very interesting if that 34kD band on the SDS gel did turn out to be PSA. David Bilhartz in our lab measured PSA immunoreactivity in PC3 cells. I believe that he found no reactivity.

Dr. Bilhartz:

That is correct. We found PSA in LNCaP cells but not in PC3 cells.

Dr. Rowley:

If you look at our control lane, there's basically nothing there. There's really not a band on the fluorograph and there's a very strong band after stimulating it with this factor.

GROWTH FACTOR ANTAGONISTS IN PROSTATIC CANCER: SURAMIN AND CYTOTOXIC POLYAMINES AS POTENTIAL THERAPY

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Prostatic Cancer

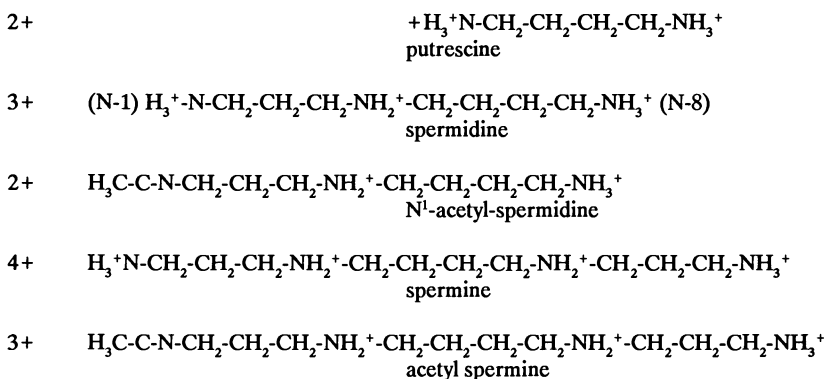
Prostatic Cancer is the leading site for cancer in men over 50 years of age and is the second leading cause of cancer deaths in the US (1). Clinically manifest prostatic cancer is more common in the US than in Japan while the incidence of latent cancer is similar between the two countries. The incidence of prostatic cancer of first generation males of Japanese living in the US is the same as that of US males. The implication is that life style differences are important for the progression of the disease. As prostatic cancer is refractory to most standard chemotherapeutic agents and hormone therapy is only palliative it may be that it will be easier to prevent the progression of prostatic cancer than to cure it. Consideration should be given to 3 omega unsaturated fatty acids, which are common in fish oils, as possible agents in a study of prevention of progression. The 3 omega unsaturated fatty acids have proven beneficial in the prevention of cancer and reduced the growth of prostatic cancer in nude mice (2,3). Other cultural differences in nutrition such as the use of green tea should be investigated to identify other potential anti-tumor progression factors.

Polyamines and Growth

Once prostatic cancer has progressed and metastasized, therapy is aimed at reducing the level of and or blocking the action of androgens. Even total androgen ablation is only palliative and the great majority of patients will relapse. The prostate of normal male rats and humans exhibit a level of polyamine synthesis and tissue content of polyamines that exceeds that of any other tissue (4,5). Normally following castration the synthesis of polyamines and the concentration of polyamines in the prostate drop dramatically. Polyamines are essential for cell growth and function. In hormone dependent tissues such as breast it has become appreciated that with the development of estrogen independent tumors that the independent tumors autonomously produce increased levels of positive growth regulators and decreased levels of negative growth regulators. Growth factors stimulate polyamine synthesis, so whether the result of androgen directly or via growth factor production, stimulation of increased polyamine synthesis is a response to growth stimulation. Indeed the level of ornithine decarboxylase is many fold higher in anaplastic rapidly growing variants of the Dunning prostatic tumors than that found in the normal dorsal-prostate or in the androgen-dependent slow-growing tumors (6).

Polyamines

The polyamines are charged cationic amines at physiological pH, and have the following structures;



spermidine is assymetrical and the beginning and ending carbons are designated N-1 and N-8 respectively. The acetylation of the nitrogen by the enzyme spermidine/spermine N-1 acetyl transferase (SAT) removes one charge. Following acetylation the N-1 acetylated polyamine is a substrate for polyamine oxidase which removes an acetylisopropylamine group and restores the previous polyamine. Thus spermine can be metabolized back to spermidine which can be metabolized back to putrescine by the acetylation and oxidation of the larger polyamine. Inside the cell ornithine is converted to putrescine by the enzyme ornithine decarboxylase (ODC). Putrescine is metabolized to spermidine and spermine by the action of the enzymes spermidine synthase and spermine synthase respectively. The synthases utilize decarboxylated S-adenosyl-methionine as a cofactor to provide the isopropylamine group to the polyamine. Decarboxylated S-adenosyl-methionine is provided by the action of S-adenosylmethionine decarboxylase (SAMDC). Ornithine decarboxylase (ODC), spermidine/spermine acetyltransferase (SAT), and S-adenosyl-methionine decarboxylase (SAMDC) are rate limiting for the various steps involved in polyamine formation and intraconversion.

The polyamines are charged and can be viewed like important physiological cations such as calcium but cations that the cell can metabolically generate, change and inactivate. Like calcium it is probably the level of "free" polyamine that is important for the kinase/phosphatase regulatory activities of the polyamine. Often reported are polyamine concentrations but these are static levels and one cannot appreciate the dynamic flux of synthesis and intraconversion from such measurements. More important are measures of the levels of ODC, SAT, and SAMDC which give an indication of the level of intracellular polyamine flux that is occurring. Each of the enzymes involved in synthesis and intraconversion is able to be inhibited by a selective inhibitor the topic of which has been the subject of recent reviews (7-9).

The first enzyme in the synthetic pathway is ODC which generates putrescine from ornithine. Specific site directed irreversible inhibitors of this enzyme such as alpha-difluoromethylornithine (DFMO) have been valuable in evaluating the role of intracellular polyamine formation. In DFMO treated cells in tissue culture the intracellular levels of polyamines drop with putrescine and spermidine decreasing most rapidly (>90%), and spermine being more resistant to depletion. Indeed spermine can be viewed as an intracellular polyamine storage form. The growth of the cell ceases with polyamine depletion and eventually growth stops. Growth can be reinitiated and intracellular polyamine levels restored by the extracellular addition of the natural polyamines. Even though highly charged, the polyamines are able to cross the cell membrane because of the presence of a polyamine specific transporter. Polyamines are excreted from the cell usually as the acetylated polyamine. Because cells can excrete polyamines as well as take up polyamines a flux of polyamines is possible at the cell membrane. The importance of the intracellular cycle of polyamine formation and intraconversion is becoming clear because of the availability of specific agents which interfere with polyamine intraconversion. the role of the potential intracellular extracellular cycling of polyamines is undefined because of a lack of agents which will selectively block uptake or excretion.

Following intracellular polyamine depletion the rate of polyamine uptake from the extracellular fluid is increased manifold. This has been used to increase the effectiveness of the cytotoxic agent MGBG which utilizes the polyamine transporter for uptake into cells(10-12). MGBG has a number of features which diminish its utility as a chemotherapeutic agent. In terms of its use in concert with polyamine depletion therapy it inhibits polyamine degradation by inhibiting tissue diamine oxidase, and it stabilizes SAMDC,

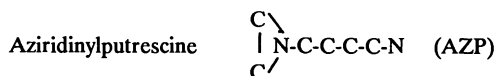
both actions tend to reduce its effectiveness in terms of polyamine depletion, but are more critical *in vivo* than *in vitro*. *In vivo* there are a number of sources that add polyamines to the serum that are not responsive to DFMO induced polyamine depletion. These sources include cellular foods, gut bacteria, and the polyamines released from dying cells that occur with normal cell turnover and tissue remodeling. These serum sources of polyamines make it difficult to control cell growth with agents like DFMO because the cells are able to escape polyamine depletion by way of their transporters enhanced activity. Indeed, it has been observed that DFMO was able to cure mice of L1210 cell leukemia if it was an L1210 mutant lacking the polyamine transporter (13).

We asked the question as to how manipulatable prostatic polyamine transport was *in vivo*, and describe the results of a typical experiment in Table 1. Animals were castrated and seven days later received the androgen dihydrotestosterone propionate (DHTP) 2mg/rat sc daily for three days, or DFMO, an irreversible inhibitor of ODC and polyamine synthesis, 2% in the drinking water for three days, or AMG an inhibitor of diamine oxidase 1hr prior to 14-C putrescine injection, or all three and uptake determined (14).

It appears that we are able to manipulate putrescine uptake into the rodent prostate. We found that we could readily manipulate the uptake of putrescine and cadaverine, both diamines, into the prostate but the tri- and tetra- amines spermidine and spermine and MGBG were not similarly manipulated (15). This implies that there may be multiple transporters which are differentially expressed in different tissues, the prostate being a diamine preferring system and the intestine and kidney preferring the tri-and, tetra- amines or that different tissues differentially modulate polyamine transport.

Targeted Polyamine Toxins

In vivo putrescine is the least toxic polyamine and spermine exhibits the greatest acute toxicity with LD-50 doses of 400mg/Kg for putrescine, 60mg/Kg for spermidine and 10mg/Kg for spermine respectively. Nephrotoxicity is a major site of tissue damage for spermidine and spermine. Because putrescine could be targeted to the prostate and because it was the least toxic of the polyamines we examined whether it could be modified to serve as a vector for a toxic derivative or as an imaging agent (16,17). Aziridine is a simple modification that we felt could be toxic and yet still maintain putrescines ability to use the transporter. So we synthesized aziridinyl putrescine and examined its actions on cell growth.



We found that AZP was a competitive inhibitor of putrescine transport into the cell with a K_i of 0.5-1uM with putrescine having a K_m of 2-5uM. DFMO pretreatment stimulated AZP uptake into the cell, and as seen in Table 2 DFMO pretreatment produced a synergistic response in causing cessation of growth of the human prostatic cancer cell line PC-3.

Table 1: The effect of androgen supplementation, DFMO administration and diamine oxidase inhibition on putrescine uptake in the castrated rat ventral prostate relative to skeletal muscle

Treatment	Uptake Ration (VP:SKEL M)
Castrate Control	3.3+/- 0.4
DHTP	3.5+/- 0.6
DFMO	4.9+/- 0.5
AMG	4.2+/- 0.5
DHTP+DFMO	18.5/- 3.2
DHTP+DFMO+AMG	81.0+/- 12.6

Table 2: Effect of AZP on the growth of PC-3 prostatic carcinoma cells with and without DFMO pretreatment

Treatment	Cell # (x10 ⁴)
Control	414+/- 17
DFMO	340+/- 54
AZP 25uM 1 hr.	123+/- 10
DFMO-AZP	7+/- 2

Thirty thousand PC-3 cells were plated and allowed to attach overnight. The following day the medium was aspirated and replaced with RPMI 1640, 10% FCS, 100uM aminoguanidine to inhibit diamine oxidase (incubation medium), with or without 1mM DFMO. Forty-eight hours later the medium was aspirated the dishes washed once with HBSS, and the cells incubated in the RPMI medium with or without 25uM AZP for one hour. Following the one hour incubation the media was aspirated, the cells rinsed twice with HBSS and the incubation continued for an additional 5 days. The cells were then lifted from the petri dishes with light trypsinization and the cell # and viability determined with the aid of a hemocytometer and by trypan blue exclusion(16).

We are currently examining the activity of AZP *in vivo*. The acute toxicity of AZP is between that of spermine and spermidine being 30mg/Kg or about ten fold more toxic than putrescine.

Dr. Kadmon, Welsh, and colleagues are examining the possibility of developing a putrescine based scan to detect micrometastatic prostatic cancer that has metastasized to the lymph nodes. They are examining positron imaging based on 18 F fluorinated N-propyl putrescine and have achieved substantial prostatic uptake(18). Positron imaging is highly sensitive a can distinguish small relative differences (Ca.25%) in relative distribution of uptake. If the manifold differences in uptake observed in the rodent system continue to be observed in the human it should be feasible to scan for micrometastatic deposits of prostatic cancer in the lymph nodes.

A non alkylating derivative of the polyamines that has interesting properties is N-1,N-14,bis ethyl homospermine.



This compound is one of the hundreds of polyamine analogs that has been synthesized by Dr. R. Bergeron's synthetic group at the University of Florida at Gainesville(19,20). It is a spermine analog but with important differences. Like spermine the nitrogens are fully charged at physiological pH and it does use the polyamine transporter for its uptake into cells. Once inside the cell it turns off polyamine synthesis with both ODC and SAMDC decreasing in activity. It has the advantage that it cannot be intraconverted to other polyamine derivatives because of the ethyl groups. It will not subvert the mitogenic functions of the normal polyamines. Spermine has been observed to prevent the binding of protein kinase C, PKC, to the plasma membrane thus inhibiting its activity (21). Spermine has also been observed to be the most potent of the polyamines in binding to DNA where it alters template activity and B to Z transition states and alteration of intracellular polyamine pools alters the susceptibility of DNA to the action of cancer chemotherapeutic agents(22). Spermine also quenches the uptake of polyamines and so as it enters the cell it is reducing the ability of other polyamines to utilize the transporter for uptake. The polyamine transport system is also energy requiring and it should be noted that many of these polyamine derivatives suppress mitochondria formation so that as the cell divides the number of mitochondria decreases. All of the different derivatives synthesized by Dr. Bergeron differ in the manner in which they affect these processes but the trend is for the bigger polyamines such as diethylspermine and bis ethyl homospermine to exhibit the more useful antitumor growth effects *in vivo* as well as *in vitro*. Of note for this report is that MGBG, like spermidine, is probably handled like a larger polyamine is one of the few cytotoxic agents to demonstrate an objective induction of tumor response in prostatic cancer patients with measurable disease(23). One of these larger non-metabolizable polyamine analogs may prove useful in prostatic cancer as well.

Suramin: A Growth Factor Antagonist

As already stated growth factor stimulation of cells stimulates polyamine synthesis and increases the rate of polyamine transport as well. In the prostate there are a number of potential growth factors that may play a role in the normal and abnormal growth of the prostate. The majority of the growth factor activity in the prostate is associated with the heparin binding growth factors aFGF/HBGF-1 and bFGF/HBGF-2. A number of investigators, many of whom are contributors to this book, have isolated and characterized HBGF found both in the normal and cancerous human and rodent prostate (24-31). As these growth factors have been reported to stimulate the growth of prostatic epithelial, stromal, endothelial and bony osteoblastic cell growth, therapies aimed at blocking the activity of members of the FGF/HBGF family of growth factors may prove useful in controlling the androgen independent growth of prostatic cancer and interfering with its paracrine interaction with these other cell types.

It was noted that the polynaphthylsulfonic acid urea, suramin, inhibited the mitogenic activity and binding of a number of growth factors (32). The growth factor most inhibited was basic FGF followed by TGF β with EGF inhibited but requiring 50 fold higher concentrations for inhibition than bFGF. By inference insulin like growth factor was not inhibited in this study. A major feature of suramin is its ability to inhibit the activity of a large number of a variety of proteins such as polymerases, kinases, phosphatases, proteases and not others of a similar substrate specificity. Thus while suramin is of interest as a growth factor antagonist it has minimal specificity as a growth factor antagonist and has multiple mechanisms of action as well as substantial toxicity, which need to be considered any time suramin is used.

A study examining the role of suramin in the treatment of patients with AIDS occasionally observed regressions in the associated Kaposi's sarcomas. A growth factor isolated from Kaposi's sarcoma is related to the FGF growth factor family. Investigators at the NCI felt prostatic cancer might be an appropriate

Table 3: The dose effect of suramin on the growth of the human androgen-independent PC-3 and DU-145 and Copenhagen rat R-3327 MAT-LYLU prostate derived cell lines *in vitro*

Treatment	Cell Number (x 10 ⁴)
PC-3 Control	16.6 + /- 0.86
50uM Suramin	14.5 + /- 0.79
100 uM	10.2 + /- 0.59
200 uM	8.0 + /- 1.2
DU-145 Control	34.0 + /- 1.9
50uM Suramin	37.2 + /- 1.5
100uM	31.7 + /- 0.8
200uM	27.8 + /- 1.9
	Cell Number (x10 ⁵)
R3327-MAT-LYLU Control	41.8 + /- 0.8
50uM Suramin	41.2 + /- 2.3
100uM	45.3 + /- 1.1
200uM	58.9 + /- 2.7
400uM	70.3 + /- 1.9

PC-3 or DU-145 cells (3x10⁴) or MAT LYLU cells 3x10⁵ were plated and the following day the various concentrations of suramin added to the plate. Five days following plating the incubations were stopped and the number of viable cells determined by cell counts with a hemocytometer and trypan blue exclusion. Two hundred micromolar suramin is considered to be optimal in the human study.

tumor in which to test whether suramin had anti-tumor activity because of the high content of bFGF in the prostate. Initial reports from investigators at the NCI have been encouraging with the majority of patients responding with tumor regressions noted in measurable soft tissue metastases and improvement of serum markers, pain and bone scans in patients with osseous lesions (33,34). One has to be *very* cautious however because these patients are receiving concomitant corticosteroids because suramin tends to be destructive towards the adrenals in humans. It has been observed that hormone refractory patients will occasionally respond to corticosteroid administration. The interplay between adrenal toxicity, antigrowth factor activity, and corticosteroid administration needs to be further defined in appropriate model systems. We have investigated what effect suramin would have on the growth of human and rat prostatic cancer cells *in vitro*.

PC-3 and DU-145 cells were inhibited in growth in a dose response manner by suramin, PC-3 exhibited the greater degree of inhibition with DU-145 exhibiting minimal inhibition at this time point. Studies of the time course of inhibition by suramin demonstrated increasing inhibition with increasing length of exposure. Unexpectedly, R3327-MAT LYL cells were *stimulated* at increasing concentrations of suramin. This suggests that suramin may actually enhance tumor growth of some tumors. In this regard suramin not only inhibits FGF but TGF β and TGF β is an inhibitor of growth for most epithelial tumors. This property may explain some of the growth stimulatory action of suramin with the overall effect being whether it is blocking the cells brake or accelerator for growth. Because suramin effects these two major regulators of cell growth as well as others, and because FGF and TGF β effect so many cell types, and because prostatic tumors grow in a matrix with heparan bound growth factors and interacts in a paracrine manner with different cells, suramin's overall sum of activity *in vivo* will be difficult to evaluate mechanistically. While the report of substantial clinical responses in hormone refractory patients by the investigators at the NCI is encouraging it needs to be further validated at ongoing trials at other centers. If substantiated, then better agents than suramin need to be developed.

Another human prostatic cancer cell line that is considered a useful model of prostatic cancer is the LNCaP line. We have found it to be the most sensitive of the available human lines to the growth inhibitory action of suramin. At this meeting it has been stated that the LNCaP line does not make TGF β and has minimal or no receptors for TGF β , leading one to question whether this is a reason for their enhanced sensitivity to suramin. The LNCaP line is androgen sensitive and androgens stimulate their growth. We were curious as to what action suramin may be enhancing *in vivo* that would result in the reported regression of tumor in patients with prostatic cancer. Because TNF is noted for its cytotoxicity towards many carcinoma cells we investigated whether suramin would enhance or inhibit TNF activity.

Table 4: The effect of suramin, TNF, or both on suppressing the growth of LNCaP human prostatic carcinoma cells *in vitro*.

Treatment	Cell # (x10 ⁴)	
	(-) DHT	(+) DHT
Control	27.6 +/- 1.4	46.5 +/- 2.5
SURAMIN 100uM	11.7 +/- 0.9	31.5 +/- 1.7
TNF 1.0nM	16.0 +/- 3.2	15.5 +/- 3.2
SUR + TNF	4.6 +/- 0.4	5.8 +/- 2.0

LNCaP cells were plated (3x10⁴) and the following day the media removed and fresh media, DME:HAMS F-12,1:1 with ITS supplementation (Collaborative Research) and 5% FCS (2x charcoal extracted) with or without DHT 20nM. The treatments were started with the addition of drug from 100x stock suramin having been dissolved in water and TNF having been diluted in HBSS with 1% BSA and the control receiving an equivalent volume of drug diluent. Eight days following plating the media was aspirated and the cells lifted from the plate with gentle trypsinization and counted with a hemocytometer.

As expected androgen stimulation increased the amount of cell growth. Suramin inhibited the increase in cell number by 60% in the cells not supplemented with DHT, but only 30% in the androgen treated cells. TNF inhibited the growth of the LNCaP cells 40% and the DHT treated LNCaP cells by nearly 70%. Whether the cells were androgen treated or not the combination of suramin and TNF was at least additive in suppressing the growth of the LNCaP cells. This contrasts with other studies in which we found that interferon did not increase the activity of suramin. TNF binding to MEM 180 cells is not reduced by suramin. Thus TNF may be useful for combination with suramin and may be partially responsible for some of the tumor regressions induced *in vivo*.

There are many exciting potential therapies being developed based on our increased understanding of the cellular and molecular biology of the various tumor types. The reports of clinical activity of suramin in the treatment of hormone resistant prostatic cancer raises the hope that growth factor antagonists will be useful in the treatment of this disease. The question is which growth factor or factors will it prove to be? Prostatic cancers may rely on paracrine growth regulation especially in interaction with supporting stroma(35-37). Understanding these interactions with the observation of stromal produced epithelial cell growth factors should allow for the development of more selective and less toxic growth factor antagonists.

ACKNOWLEDGEMENT

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DISCUSSION

Question from the floor:

Was that patient hormone treated?

Dr. Heston:

Yes, that was a hormone resistant patient. He had been through everything.

Question from the floor:

You've drawn that from one patient?

Dr. Heston:

It is not an uncommon backup for patients with prostatic cancer, when they've failed hormone therapy and are in severe bone pain to put them on corticosteroids. Sometimes you see dramatic improvement. That's not the typical case, but that's not an unusual case. That does happen.

Dr. Miller:

During your talk I saw the word coumarin pass by. I would like to know your thoughts on this compound?

Dr. Heston:

We got into coumarin quite by accident. What happens is that the prostate is a good source of a lot of cations, one of which is histamine, so we were looking at the role of H-2 antagonists in prostatic cancer. Dr. Ernie Marshall came up with this business of adding a macrophage activator with an H-2 antagonist to suppress coumarin suppressor cells, to improve therapy. So we tried coumarin. Well, at the same time we were using the H-2 antagonist cimetidine. Cimetidine shrinks the prostate. We examined the animal treated with coumarin and it was smaller. Coumarin also inhibited tumor cell metastasis. Coumarin treatment inhibited the growth of the Dunning tumor models, and now we are looking at *in vitro* human models. Coumarin doesn't bind to the androgen receptor. So, it's not mediated by an androgen receptor mechanism. One of the slides I was going to show is 7-hydroxycoumarin, which is the major metabolite in humans, and which is much more effective than coumarin *in vitro*. The problem is that in humans the minute you form a 7-hydroxycoumarin, it goes like a shot to the glucuronide which is ineffective. So, we are trying to figure out ways of coming up with better compounds that won't be glucuronidated. In all cases that we've looked *in vitro*, we're able to dramatically effect tumor cell growth. The problem is that it is readily reversible. It's not like AZP, where you remove it an hour later and the cells still die. More recent experiments have not reproduced the *in vivo* growth suppression. The coumarins still are active *in vitro* but not *in vivo*. Some have suggested that the early results seen *in vivo* were because of contamination of the coumarin with endotoxin. A possibility that we are investigating.

Dr. Coffey:

There was a lot of excitement from NCI about suramin and it seems to be dampening a bit. Would you sort of summarize what you think of suramin in the Dunning tumor model?

Dr. Heston:

When we've looked at the growth of the Dunning tumor model *in vitro* with the Mat LuLu, we see growth stimulation. Now you've got to remember that suramin is incredibly non-specific. It inhibits not only the basic FGF which may be a mitogen for these cells, but it inhibits TGF beta, which is also going to be an inhibitor of those cells. So, it's going to be possibly related to the relationship between whether you've got your foot more on the accelerator peddle or more on the brake as to what suramin is doing. Suramin does get taken up by cells although very slowly. It's like trypan blue getting into cells. It inhibits a whole series of kinases and phosphatases, so that may be playing a role here too. I think it's going to be a son of suramin we're going after, not suramin itself.

Dr. Coffey:

What about *in vivo* against the Dunning?

Dr. Heston:

We've seen inhibition of the Dunning tumor, but it was at the same time we were losing animals. There was no therapeutic ratio there and it's hard to say whether it was the effect on toxicity in the tumor or whether it was an indirect effect because of a toxic host animal.

Dr. Linehan:

I would add one thing in follow-up to what Dr. Heston has said. Liu, et al. in our group have also evaluated the *in vitro* effect of the combination of suramin and other agents on proliferation of PC-3. *In vitro*, the combination of agents, such as TNF and suramin, appears to have at least an additive effect. A number of times we have also seen that in the early time points where there seems to be a stimulation of proliferation of these cells in culture. Whether suramin is acting to block the early inhibitory effect of a tumor-produced factor such as TGF β or other substance is not known.

Dr. Heston:

That's right. I'd like to say that when we started off, TNF was not my first thought because suramin is a "polyanion", and polyanions have been known to stimulate interferon production by a variety of cell types. We were thinking interferon. We also saw the same thing with interferon. We saw no additive benefit of the suramin being there. Which suggests maybe it's actually a negative interaction.

Dr. Rowley:

The results with glucocorticoid I think were very interesting. I would just like to point out that one of the better known differentiation systems *in vitro* is that of the mesenchymal 3T3 stem cells, where they are proliferative and then quiesce at confluency and differentiate into adipocytes. One of the hormones besides indomethacin that stimulates this is glucocorticoid. Glucocorticoids in that system accelerates the loss of growth associated with the differentiation of these cells. It also has been shown with a number of cell line, that glucocorticoid stimulates the production of unknown growth inhibitory factors.

TRANSFORMING GROWTH FACTORS IN HUMAN PROSTATE CANCER

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INTRODUCTION

Control of epithelial cell growth is poorly understood. Most epithelial cells exist in a tightly controlled state maintained by a balance between the effects of stimulatory and inhibitory factors. Holley proposed that transformed cells escape this control and require less exogenous growth factors in culture than their normal counterparts (1). This observation formed the basis of the autocrine growth control hypothesis whereby transformed cells produce and respond to their own growth factors (2,3).

Transforming growth factors (TGF) which have the ability to support anchorage-independent growth of indicator cell lines include the TGF α and the TGF β family of growth factors. TGF α generally supports growth in a wide variety of cell types (4). TGF β , on the other hand, exhibits both stimulatory and inhibitory properties, depending on the cell type examined. (5). For example, normal rat kidney (NRK) cells require members of both the TGF α and TGF β families to optimally form colonies in soft agar medium whereas mouse embryo AKR-2b cells require only TGF β to grow under anchorage independent conditions. In contrast, the growth of many epithelial tumor cells is inhibited by TGF β . Overall, the effects of TGF β on epithelial cells are multifaceted and are probably influenced by other growth regulatory factors and their receptors that are present in the cell at a given time (6).

Evidence has accumulated in recent years that the role of EGF/TGF α and their receptor in carcinogenesis is complex. Clearly, the addition of EGF to many cell types elicits responses associated with neoplastic transformation, such as proliferation, diminishment of fibronectin production and enhanced secretion of plasminogen activator (7). Secretion of TGF α by a large variety of tumor cells suggests involvement of TGF α in cell transformation and provides evidence supporting an autocrine stimulatory model. More direct evidence was provided by Rosenthal and coworkers who transfected Fischer rat fibroblasts with a human TGF α cDNA construct (8). Synthesis and secretion of TGF α by these cells resulted in anchorage-independent growth and induced tumor formation in nude mice. In addition, anti-human TGF α antibodies prevented the expressing cells from forming colonies in soft agar. To the contrary, Finzi and coworkers were unable to induce a transformed phenotype in NIH 3T3 cells via transfection of the TGF α gene into the NIH 3T3 cells despite high levels of subsequent growth factor production (9).

Using breast cancer as a model of hormone responsive cancer, there is compelling evidence supporting the hypothesis that steroid hormones stimulate a growth response, in part, via locally acting intermediate factors which serve as effectors of steroid hormones. In other words, steroid hormones may

stimulate the production of growth factors which, when secreted, modulate the growth of the cell that secreted them (autocrine growth control) or neighboring cells (paracrine growth control). For example, the rate of proliferation of MCF-7 breast cancer cells is increased by plating the cells at higher densities (10), suggesting conditioning of the media by the cells with factors that support growth. Using anchorage independent growth to assay for activities capable of inducing a malignant transformed phenotype, several growth factor activities have been isolated from steroid responsive cancer cells. Some of these peptide growth factors alter the phenotype of human breast cancer cells *in vitro* and, in some cases, are produced by the breast cancer cells in response to estrogen treatment (11). Similarly, some androgen actions in prostate cancer may be mediated via growth factors (Figure 1). Growth factors may exert their influence via autocrine (Figure 2) or paracrine (Figure 3) mechanisms in prostate cancer. Recent findings have further characterized the effects of some of these factors. In this review, I will focus on the activity of transforming growth factors in prostate cancer.

The hypothesis that constitutive production of autostimulatory growth factors contribute to the transformed phenotype of prostate cancer cells was addressed by examining two androgen-independent human prostate cancer cell lines (PC3 and DU145) (12,13) and one androgen-responsive human prostate cancer cell line (LNCaP) (14) for evidence of autocrine growth control mechanisms (15-21). Autocrine growth loops were defined as present for a particular growth factor when: a) specific high affinity receptors were present on the cell surface; b) growth factor messenger RNA and protein were detectable; and c) a growth response (stimulatory or inhibitory) was elicited by the addition of exogenous growth factor to the media. Two factors which met these criteria in one or more cell lines tested were TGF α and TGF β 1. The details of these experiments are outlined below.

Transforming Growth Factor Alpha

Epidermal growth factor (EGF) is a polypeptide of 6045 molecular weight capable of inducing proliferation and differentiation in diverse populations of cells (22,23). Transforming growth factor alpha (TGF α) is a polypeptide structurally related to EGF (24) which competes with it for binding to the same receptor (25). The EGF receptor is an integral membrane protein which functions as a signal transmitter through an intrinsic, EGF stimulated, tyrosine kinase activity. In addition, autoregulation of the EGF receptor occurs via autophosphorylation of specific regulatory sites on the receptor. This autophosphorylation of the EGF receptor influences receptor degradation and/or intracellular sequestration of the receptor (23). Of interest, the EGF receptor is the proto-oncogene to the *v-erb-B* oncogene of the avian erythroblastosis virus (26). Both EGF and its functional homologue TGF α are considered prototypical autocrine

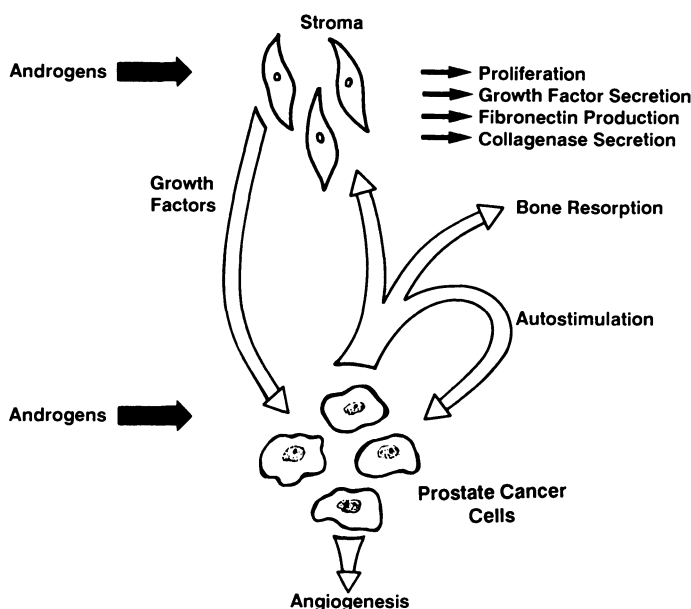


Figure 1: Possible Roles of Androgens in Prostate Cancer

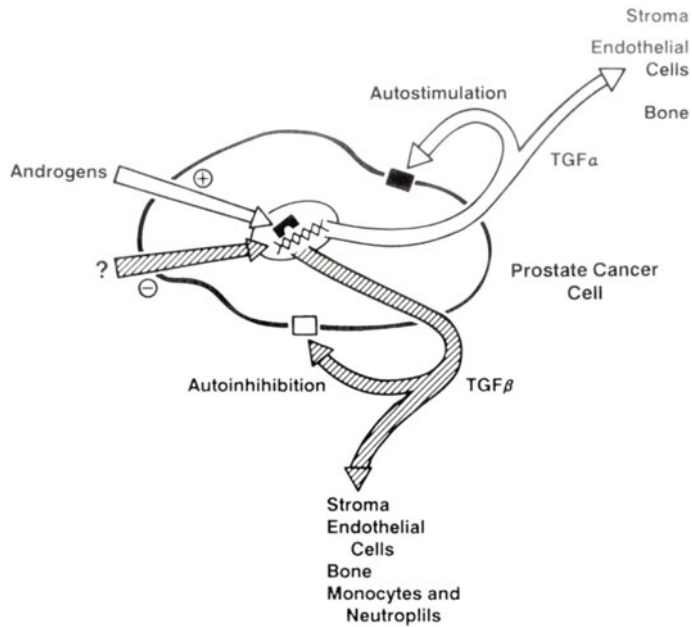


Figure 2: Autocrine Growth Control in Prostate Cancer

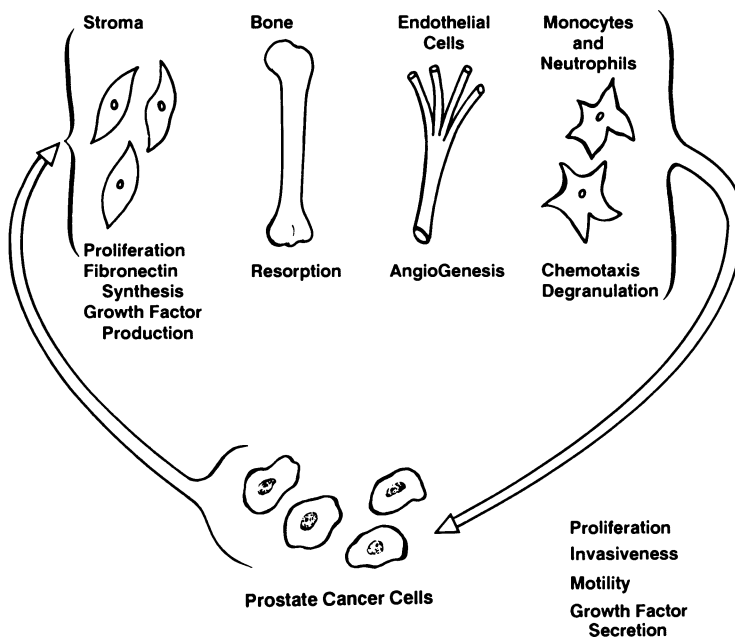


Figure 3: Paracrine Growth Control in Prostate Cancer

control factors. The addition of EGF to noncancerous cells induces specific responses associated with neoplastic transformation. In part, these include proliferation, partial loss of density dependent inhibition of growth decrease in fibronectin production, increase in ornithine decarboxylase activity and enhanced secretion of plasminogen activator (23).

Because other hormone responsive tumors, such as breast cancer, have been shown to produce and/or respond to TGF α , TGF β , insulin-like growth factor I (IGF I), platelet derived growth factor (PDGF), and other growth factors (11,25), we investigated the effects of TGF α on human prostate cancer cells *in vitro*. In addition, we examined the production of TGF α and its receptor, the EGF receptor, by LNCaP human prostatic cancer cells. We demonstrated that LNCaP human prostate cancer cells produce, secrete and respond to TGF α , and we propose that this may represent an important autocrine growth control mechanisms in this disease (15).

Multiple parameters were monitored to assess the response of LNCaP cells to exogenously added TGF α . As a measure of mitogenicity, we determined cell growth in monolayer cultures (Figure 4) and in an anchorage-independent, soft agar assay, which also reflects transforming activity. We measured uptake of ^3H -thymidine and ^3H -uridine as indicators of macromolecular synthesis, though they may also reflect changes in precursor metabolism, transport and pool size changes. Because peptide factors have been shown to increase intracellular signalling via the phosphoinositide second messenger pathway (27), we also examined changes in phosphoinositide metabolism after addition of TGF α to the medium.

TGF α increased the growth of LNCaP cells in monolayer culture in a dose dependent fashion such that after ten days of culture the response to 5, 10 and 20 ng/ml of TGF α was 190,280 and 390% above control cell numbers (Table 1 and Figure 4). No concentration of TGF α was equivalent to dihydrotestosterone (DHT) at 10^{-8}M . Both TGF α and 5- α -dihydrotestosterone (DHT) increased colony formation in soft agar 250% above control levels in LNCaP cells.

TGF α , also, effected macromolecular metabolism as evidenced by increases in ^3H -uridine and ^3H -thymidine uptake. ^3H -thymidine uptake was increased to approximately 3-fold above controls by TGF α (5 ng/ml) while DHT (10^{-8}M) increased ^3H -thymidine uptake to 15-fold above control. Likewise, TGF α or EGF increased ^3H -uridine uptake approximately 2.5-fold above control levels. The uptake of these precursors are dependent on pool size, transport into cells and alterations in DNA and RNA synthesis. In addition, ^3H -thymidine can be incorporated into DNA during reparative, as well as, replicative DNA synthesis. Therefore, these data are reflections of thymidine and uridine metabolism and not just indications of DNA and RNA synthesis.

Finally, as an indication of intracellular signalling, the incorporation of ^3H -myoinositol into phosphatidyl inositol and the subsequent metabolism of phosphatidyl inositol to diacylglycerol and inositol phosphates was examined. In these experiments, labelled inositol phosphates accumulated to 1.9-fold above control levels during treatment with DHT (10^{-8}M). Similarly (Figure 5), TGF α induced a dose-dependent increase in labelled inositol phosphates in LNCaP cells (Figure 5).

Conditioned media was assayed for the presence of transforming activity, EGF receptor binding activity and TGF α immunoreactive activity. Both androgen independent (DU145) prostate cancer cells and the androgen responsive LNCaP cells were found to secrete activity into the media which is transforming, binds EGF receptor and is TGF α immunoreactive. When a TGF α immune specific RIA was used to assay conditioned media from LNCaP cells with and without DHT treatment, TGF α immune specific activity appeared to increase with androgen treatment from 0.15 ± 0.01 to 0.32 ± 0.06 ng of TGF α equivalents per ml of unconcentrated conditioned media (Table 2). Over the span of media collection cell numbers were equivalent in the control and DHT treated flasks. This suggests TGF α secretion into the media by LNCaP cells might be modulated by androgens.

Expression of the TGF α messenger RNA was documented by Northern analysis (Figure 6). All three prostate cancer cell lines tested, the androgen-independent PC3 and DU145 cells and the androgen responsive LNCaP cells, showed expression of the appropriately sized TGF α message. Steady state TGF α mRNA levels in LNCaP cells after treatment with estradiol or DHT did not change compared to controls.

Using a polyclonal antibody to the EGF receptor, a specifically immune precipitated protein at approximately 180 Kd was observed in all prostate cancer lines examined (Figure 7). The breast cancer line MDA 468 was used as a positive control and is known to have an amplified EGF receptor gene. Scatchard

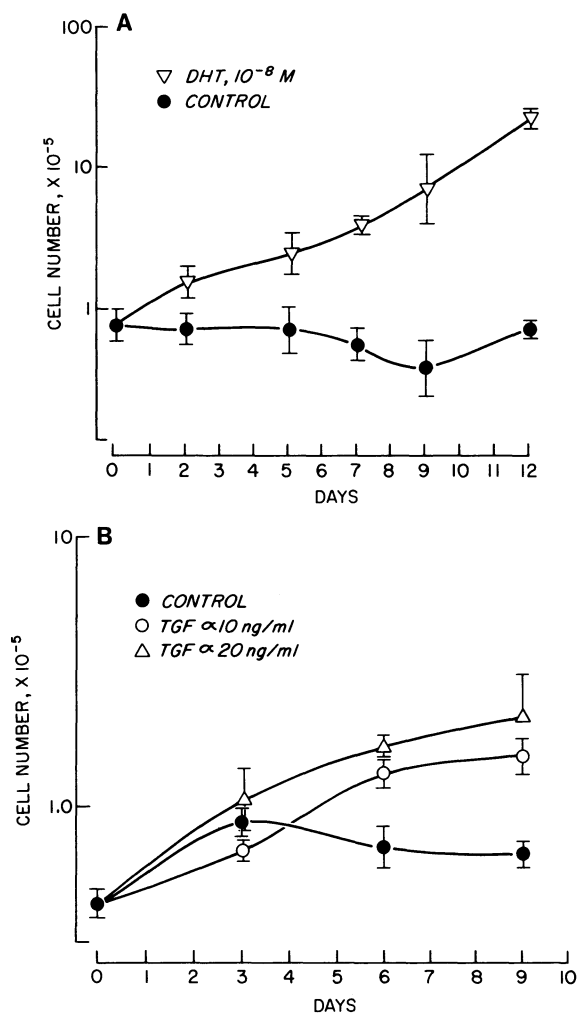


Figure 4: Growth in monolayer of LNCaP cells treated with A. DHT (10^{-8} M) (Δ) or B. TGF α 10 ng/ml ($\circ$) or 20 ng/ml (Δ). Control curves ($\bullet$) are shown in each part. Cells were grown in IMEM plus 5% CCS with or without TGF α or DHT (15).

analysis of LNCaP cells, using a whole cell assay for EGF receptors, showed an increase in the number of EGF receptor binding sites from approximately 40,000 sites per cell ($K_d = 0.5 \times 10^{-9}$ M) to more than 80,000 sites per cell ($K_d = 1.3 \times 10^{-9}$ M) in the presence of DHT. DU145 cells had 140,000 EGF receptor binding sites per cell ($K_d = 2.0 \times 10^{-9}$ M).

The above data indicate that TGF α might play an important role in modulating the proliferative states of prostate cancer cells. Could TGF α play the role of an autocrine growth factor in human prostate cancer? Derynck and coworkers demonstrated the expression of TGF α and EGF receptor messenger RNA in androgen independent PC3 cells (28). In addition, Traish and Wotiz showed the presence of EGF receptors in rat prostatic tissue and demonstrated that the level of EGF binding decreased with castration (29). The presence of EGF receptors on LNCaP cells was demonstrated by Schuurmans and coworkers, who also showed modulation of the EGF receptor sites to higher levels with androgens (30). Fernandez-Pol et al had previously shown that DU145 cells were stimulated in an anchorage-independent assay by TGF α (31), and Connelly and Rose confirmed this observation by demonstrating EGF binding and mitogenic activity in DU145 cells (32).

Table 1: LNCaP Cell Growth: Monolayer

Treatment	Percent control (day 0)	
	6 days	10 days
Control	129	123
EGF (5 ng/ml)	130	187
TGF α (5 ng/ml)	167	193
TGF α (10 Ng/ml)	158	282
TGF α (20 ng/ml)	233	387
DHT (10 ⁻⁸ M)	300	650

*Percent control (day 0) = data expressed as the mean cell number at days 6 and 10 divided by the mean cell number at day zero. Each data point was determined in triplicate. The standard deviation from the mean for each point was $\leq 10\%$.

The data presented from our laboratory demonstrate an autocrine growth stimulatory loop in human androgen responsive prostate cancer cells (15-17). The LNCaP cells in our experiments responded to exogenous TGF α by showing increased anchorage-dependent growth in monolayer, as well as, increased anchorage-independent colony formation in soft agar. In addition, we demonstrated increased thymidine and uridine metabolism, which may be reflective of TGF α induced changes in pool sizes, transport, RNA synthesis and DNA reparative and/or replicative synthesis. Finally, TGF α increases intracellular signalling via the phosphatidyl inositol pathway (15-17,21). These data substantiate the ability of TGF α to influence human prostate cancer cells.

With regards to the presence of EGF receptors on prostate cancer cells, the binding of EGF and TGF α occurred with the appropriate affinity on LNCaP cells and DU145 cells (15). In fact, similar to Schuurmans and coworkers (30), we observed an increase in EGF receptor numbers after treatment with androgens. The presence of EGF receptor on human prostate cancer cell lines was also confirmed by immunoprecipitation.

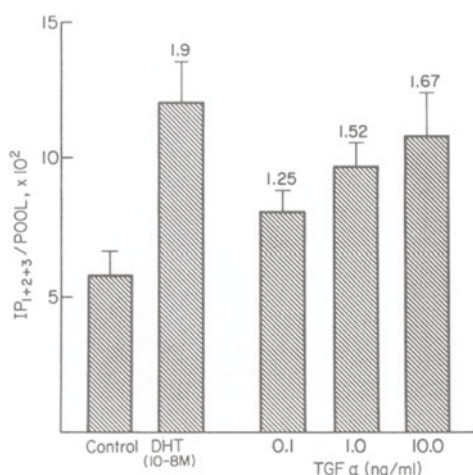


Figure 5: Accumulation of ³H-inositol phosphates in LNCaP cells treated with DHT or TGF α for 48 hr. All cells were incubated in media containing ³H-myoinositol for 24 hr to achieve isotopic equilibrium (15).

Table 2: Evaluation of Conditioned Media for Transforming Activity

Cells ^a	NRK ^b colony forming assay	TGF α ^c radio-immuno assay
LNCaP (control)	0.51 $\pm$ 0.04	0.15 $\pm$ 0.01
LNCaP (DHT)	0.63 $\pm$ 0.17	0.32 $\pm$ 0.06
DU145	0.73 $\pm$ 0.18	0.27 $\pm$ 0.02
MDA-MB-231	0.53 $\pm$ 0.04	0.22 $\pm$ 0.01

^a DHT = 10^{-8} M; MDA-MB-231 = breast cancer cells used as positive control.

^b Nanogram EGF equivalents per milliliter of unconcentrated media (cell numbers were equivalent).

^c Nanogram TGF2 equivalents per milliliter of unconcentrated media (cell numbers were equivalent).

Although it is not known how androgens increase EGF receptors, one can hypothesize that androgens increase EGF receptors by inducing the production of EGF or TGF α . Clark et al (33) have shown that EGF can modulate its own receptor in KB cells, a human epidermoid cancer cell line, and they demonstrated that EGF increased EGF receptors in these cells. Kudlow (34) showed a similar induction of EGF receptor synthesis by EGF in MDA 468 human breast cancer cells.

The production and secretion of transforming and EGF receptor binding activity by the LNCaP cells and DU145 cells was also demonstrated. This activity was shown to be TGF α by an immune-specific radioimmunoassay and our data suggest that TGF α secretion by the androgen-responsive LNCaP cells is increased by DHT (15-17). We conclude, therefore, that LNCaP cells produce and respond to TGF α in an autostimulatory fashion (Figure 8). In addition, androgens might modulate this autocrine loop by increasing the secretion of TGF α and by increasing EGF receptor levels. Better model systems for human prostate cancer and additional studies are needed to confirm this finding and to clarify the role of a TGF α autostimulatory loop in human prostate cancer.

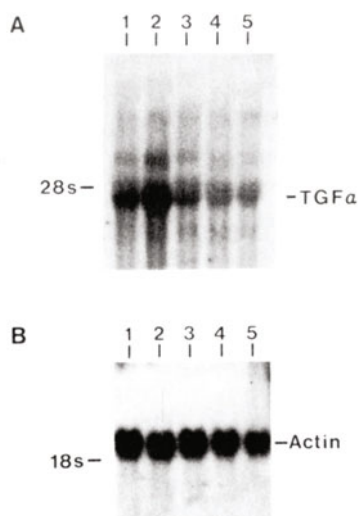


Figure 6: Northern blot hybridization of total RNA from PC3 (lane 1), DU145 (lane 2), and LNCaP cells treated under control conditions (lane 3) or with 17β estradiol (10^{-8} M) (lane 4) or DHT (10^{-8} M) (lane 5). A. Hybridization to a TGF α probe. B. Hybridization to an actin probe (15).

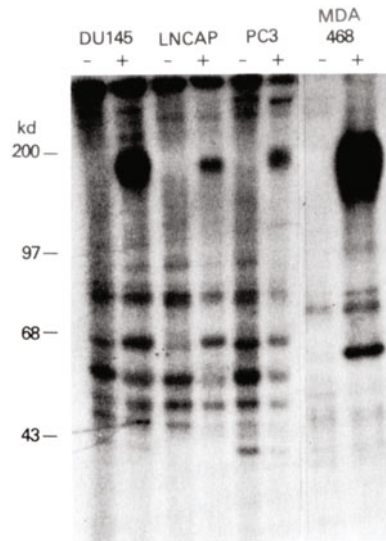


Figure 7: EGF receptor immunoprecipitation. +, immune specific lanes; -, immune-nonspecific lanes. MDA 468 breast cancer cells were used as positive controls. Cells were metabolically labeled with ^{35}S -methionine. Exposure was for 6 hr. (15).

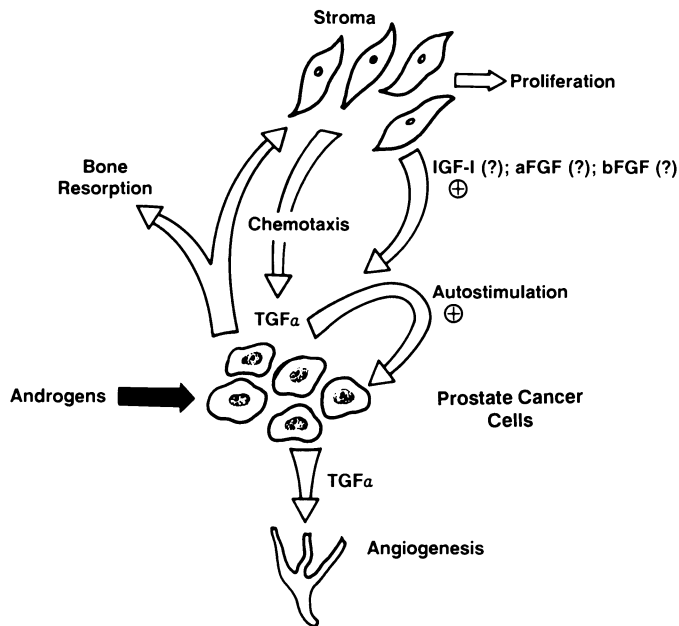


Figure 8: Possible Roles of TGF α in Prostate Cancer.

Transforming Growth Factor Beta

Transforming growth factor beta1 (TGF β 1) is a disulfide-linked homodimer of 25 kd that is unrelated to EGF structurally and functionally and it binds to its own specific receptor (35,36). TGF β 1 is synthesized by many cell types, and it is capable of regulating the action of many peptide growth factors. While TGF β 1 has mitogenic effects on various fibroblast systems, it usually exerts negative growth modulatory influences in epithelial cell systems (37,38).

Several human prostate cancer cell lines have been examined for response to TGF β 1. Wilding et al. (17,18,20) has shown that the human, androgen receptor (AR) negative, prostate cancer cell lines DU145 and PC3 transcribe and secrete TGF β 1. In addition, these cells have receptors for TGF β 1 and are inhibited in anchorage dependent and independent assays by TGF β 1 (17,18,20). The AR positive, prostate cancer cell line LNCaP, however, did not express TGF β 1 mRNA, have TGF β 1 receptors nor did not respond to TGF β 1. TGF β 1 thus might serve as an autocrine inhibitory factor in prostate cancer, or, because of its effects on a wide range of cell types, it may contribute an important paracrine function in the development of tumor stromal tissue and metastases. We describe below our data demonstrating the inhibition of human prostate cancer cells by TGF β 1.

Growth curves performed using DU145 and PC3 cells showed initial inhibition of growth by TGF β 1 in both cell lines in a dose responsive fashion (Figure 9). DU145 cells were maximally inhibited to 23% control values at 6 days of treatment with TGF β 1 (5 ng/ml) whereas PC3 cells were inhibited to 40% control values under the same conditions. As the experiments progressed, the rate of growth of the PC3 and

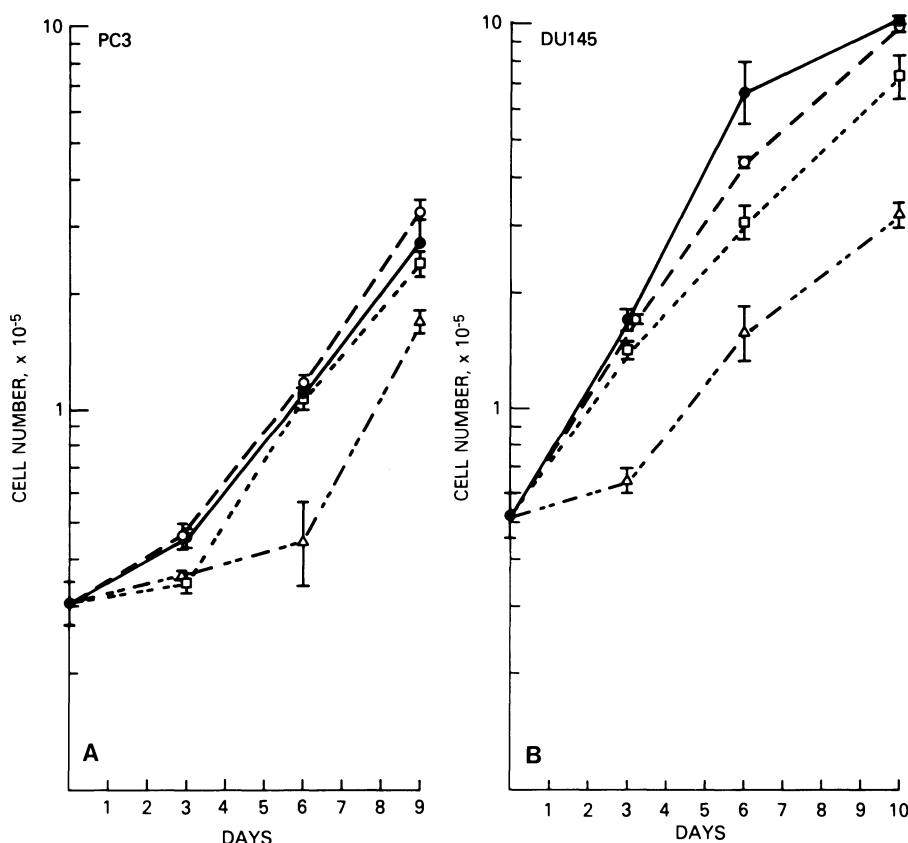


Figure 9: Growth response to TGF β in monolayer. Cells were plated at a density of approximately 5×10^4 cells per well in 6 well dishes. Medium was changed and treatment added every 3 days. A: PC3 cells and B: DU145 cells. (●) control; (○) TGF β 1 = 4 pM; (□) TGF β 1 = 40 pM and (△) TGF β 1 = 200 pM (20).

DU145 cells treated with TGF β 1 returned to control levels despite the addition of fresh media containing TGF β 1. However, the number of cells in treated wells plateaued at densities lower than control levels. LNCaP cells grown in the presence of DHT (10^{-8} M) were not inhibited by TGF β 1. It is not known if the resumption of control rate of growth by the PC3 and DU145 cells was due to the emergence of TGF β 1 resistant clones or to the production of stimulatory factors capable of overcoming the inhibitory effects of the TGF β 1. Tritiated thymidine uptake was also inhibited by TGF β 1 in DU145 cells. In contrast, tritiated thymidine uptake was minimally affected by TGF β 1 treatment in PC3 and LNCaP cells (Table 3).

Cultures of DU145 cells in serum free media in the presence of TGF β 1 (10 ng/ml), TGF α (10ng/ml) or both for 5 days yielded cell numbers of 60% control with TGF β 1 alone and 55% control with both TGF β 1 and TGF α present in the media. Cell numbers were 78% of control in the presence of TGF α alone. Thus, TGF α was unable to reverse the inhibitory effects of TGF β 1.

DU145 and PC3 cells were plated in a soft agar bilayer assay in the presence of 10% fetal calf serum. After 10 days colonies > 60 μ m in size were counted and showed a decrease in colony formation in the presence of TGF β 1 (5 ng/ml) to 16% control levels for DU145 cells and 55% control levels for PC3 cells. LNCaP cloning was unaffected by TGF β 1. The LNCaP cells were grown in the presence of 10% FCS which contained DHT. Addition of exogenous DHT did not increase the cloning efficiency. This is in contrast to cells grown in the presence of charcoal-stripped calf serum where the addition of DHT doubled the colony formation but did not increase cloning efficiency to levels achieved with 10% FCS.

Immunoprecipitation of ^{35} S cysteine-labeled media from all three cell lines with a polyclonal antibody to TGF β 1 revealed a polypeptide that comigrated with ^{125} I-labeled human platelet TGF β 1 on SDS-PAGE and was present only in media from the hormone-independent cell lines, PC3 and DU145 (Figure 10). This was confirmed by radioreceptor assay using A549 cells and conditioned media from the 3 cell lines. In these experiments, serum free media from DU145 and PC3 cells, collected over 48 hours with confluent monolayers of cells, showed the presence of TGF β 1 at concentrations of 100 pg/ml and 75 pg/ml, respectively. LNCaP cells did not appear to secrete TGF β 1 under control or DHT treated conditions.

Using a 1Kb TGF β 1 probe, Northern analysis of RNA from all three lines revealed a 2.5 Kb transcript in the hormone-independent PC3 and DU145 cells but not in LNCaP cells (Figure 11).

Scatchard analyses were performed on whole cells to determine the existence of high affinity TGF β 1 receptors (Figure 12). DU145 cells had 1500 binding sites/cell with a $K_d = 6.9 \times 10^{-12}$ M ($r = -0.93$) and PC3 cell had 2900 binding sites/cell with a $K_d = 1.2 \times 10^{-11}$ M ($r = -0.99$). High affinity binding sites could not be determined on LNCaP cells despite performance of the assay on cells in monolayer and in suspension. However, low levels of binding sites could not be ruled out.

Table 3: ^{3} H-Thymidine Uptake

Cells	TGF β (pM)	DHT (10^{-8} M)	^{3} H-Thymidine uptake (dpm $\times 10^{-3}$)
DU145	--	--	414 $\pm$ 56 (12) ^a
	40	--	333 $\pm$ 77 (5)
	200	--	212 $\pm$ 55 (5)
PC3	--	--	217 $\pm$ 12 (12)
	40	--	213 $\pm$ 22 (6)
	200	--	189 $\pm$ 19 (6)
LNCaP	--	+	104 $\pm$ 9 (12)
	40	+	94 $\pm$ 4 (6)
	00	+	82 $\pm$ 3 (6)
	400	+	84 $\pm$ 3 (6)

^a Number of determinations in brackets.

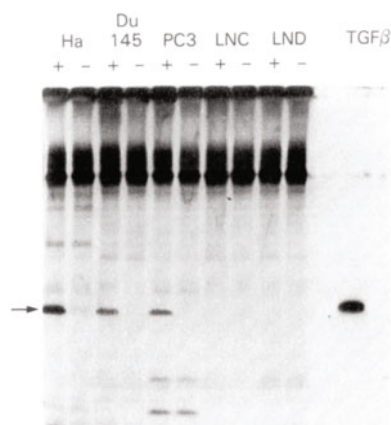


Figure 10: Immunoprecipitation of conditioned medium with polyclonal anti-TGF β 1 antibody, (+) = labeled medium and antibody, (-) = labeled medium, antibody and cold TGF β 1. Ha = H-ras transfected 3T3 cells. LNC = LNCaP cells labeled in the absence of DHT. LND = LNCaP cells labeled in the presence of DHT 10^{-8} M. TGF β 1 = purified TGF β 1 immunoprecipitated with anti-TGF β 1 antibody. Arrow indicates specific immunoprecipitated TGF β 1 (20).

The data described above demonstrate that the androgen-independent PC3 and DU145 human prostate cancer cells produce, secrete and are inhibited by TGF β 1 while hormone responsive LNCaP cells neither produce nor respond to TGF β 1. These observations, though limited to a few cell lines, raise interesting questions as to how TGF β 1 might control cell growth in human prostate cancer in an autoinhibitory fashion (Figure 13). The observation that density-arrested BSC-1 cells secrete a growth inhibitor identical to or closely related to TGF β 1 initially raised the possibility that autocrine inhibitory factors may be contributory factors in growth control (39). Similarly, the androgen-independent DU145 and PC3 cells have high affinity TGF β 1 binding sites and are growth inhibited by exogenous TGF β 1. In addition, both cell lines also transcribe, produce and secrete significant quantities of TGF β 1. These findings suggest that growth control is a balance between inhibitory and stimulatory factors. Although most cells secrete primarily an inactive form of TGF β 1 (40), our evidence suggests that some of the endogenously produced TGF β 1 is active since it has the appropriate molecular weight on polyacrylamide gel electrophoresis, it is immunoprecipitable and it competes effectively with exogenous TGF β 1 for binding to AKR-2B cells. Therefore, at least some of the TGF β 1 is present in its activated form in the conditioned media of PC3 and DU145 cells.

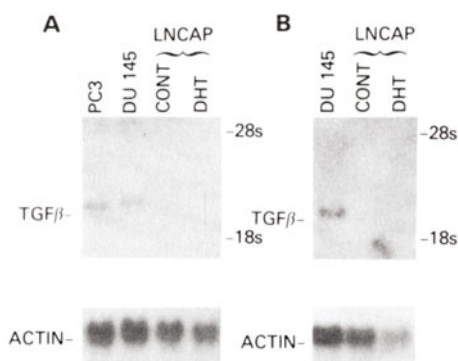


Figure 11: Northern analysis of TGF β 1 messenger RNA in human prostate cancer cells. A. Total RNA (20 μ g/lane) and B. poly (A)⁺ RNA (10 μ g/lane). Actin hybridization confirms the presence of RNA in each lane (20).

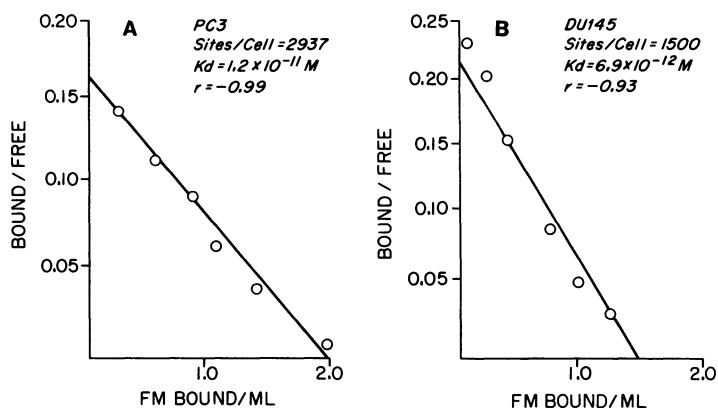


Figure 12: Scatchard analysis of TGF β 1. Specific binding of ^{125}I -TGF β 1 was determined in the presence and absence of a 100-fold excess of TGF β 1. Each point was performed in duplicate (20).

TGF β 1 receptors appeared to be intact as evidenced by their ability to bind exogenous TGF β 1 at a $K_d = 10^{-12}$ to $10^{-11}M$. Second, Massague and Kelly (41) have demonstrated that, in BALB/c 3T3 mouse fibroblasts, the level of TGF β 1 receptors at the cell surface is not depleted by sustained exposure to the ligand. This is due to rapid recycling of the receptor after ligand-induced internalization, or rapid replenishment of surface receptors from a large intracellular pool. Wakefield et al. (42) confirmed that TGF β 1 causes only minimal down-regulation of its receptor in a wide variety of cell types. In addition, they showed the degree of TGF β 1 binding in any given cell type was unaffected by agents that affect the biologic action of TGF β 1 in that cell type, such as other growth factors, retinoid acid, phorbol esters and epinephrine. They suggested that modulation of binding may not be an important control mechanism in the action of this growth factor, but instead that cellular response may be modulated at subsequent steps.

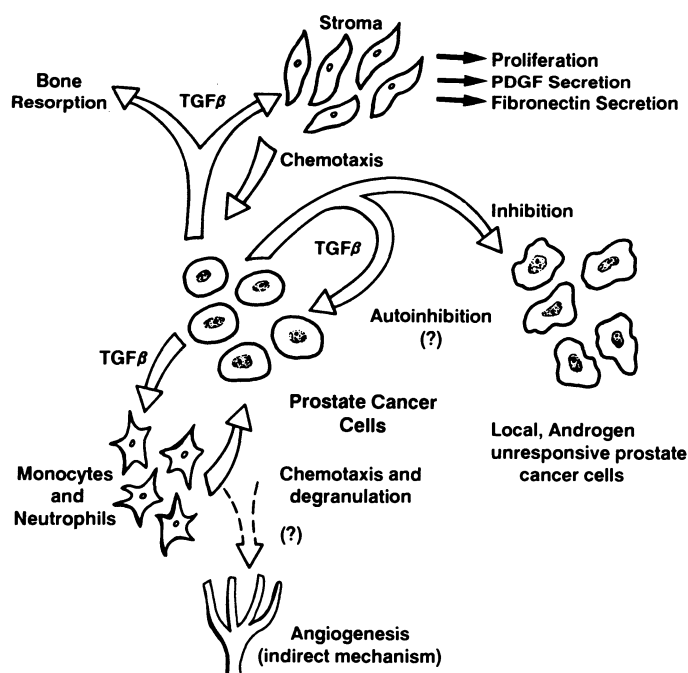


Figure 13: Possible roles of TGF β 1 in Prostate Cancer.

The failure of the LNCaP cells to produce detectable levels of TGF β 1 while PC3 and DU145 cells produce significant quantities may reflect the important role TGF β 1 could play in the more aggressive malignant phenotype displayed *in vitro* and *in vivo* by these androgen-independent prostate cancer cells.

The role of TGF β 1 in prostate cancer biology may not be limited to negative growth regulation. Its paracrine effects on stromal cells may be more important (Figures 3 and 13). One of the primary targets for TGF β 1 are fibroblasts. TGF β 1 has been shown to enhance the proliferative effect of epidermal growth factor on fibroblasts (43), as well as, enhance the production of collagen (43,44) and fibronectin by fibroblasts. Increased production of these proteins by fibroblasts exposed to TGF β 1 suggests TGF β 1 may play an important regulatory role in the synthesis and deposition of extracellular matrices during wound healing and tumor formation (46). In addition, TGF β 1 appears to modulate the action of other growth factors such as basic fibroblast growth factor, as well as, the expression of collagenase and proteinase genes (47). Immune surveillance may also be impaired by TGF β 1, since it inhibits the activation of B lymphocytes (48), T lymphocytes (49) and natural killer cells (50). Of interest, TGF β 1 induces monocyte chemotaxis and production of growth factors such as IL-1 (51,52). In contrast, endothelial cell proliferation and motility are inhibited by TGF β 1 (53,54) while osteoblasts are mitogenically stimulated by TGF β 1 (55,56), an indication that TGF β 1 may be important in the regulation of bone remodeling. All of these actions may constitute important steps in the establishment of a prostate tumor and the development of metastases. The limited number of human cell lines available, prevent us from making conclusions in regards to hormone responsive and hormone unresponsive characteristics. However, because of the capability of androgen-independent prostate cancer cells to bind, respond to, and produce TGF β 1, TGF β 1 may serve as an autocrine inhibitory factor in some prostate cancers. The vast array of effects that TGF β 1 exerts on a variety of other cell types suggests this growth factor may also play an important role as a paracrine factor in the establishment of primary tumors and their metastases. For example, prostate cancer commonly metastasizes to bone and forms blastic lesions. As noted above, osteoblasts are stimulated by TGF β 1 (55,56). One could hypothesize that the blastic characteristics of prostate cancer bone metastases are the result of TGF β 1 production by the prostate cancer cells.

As we noted above, there are similarities between breast and prostate cancer with regards to transforming growth factors. Human breast cancer cells secrete TGF β 1. Using the MCF-7 cell line, Knabbe et al demonstrated that, while TGF β 1 secretion is inhibited by the addition of estrogen and insulin, growth inhibitory antiestrogens and glucocorticoids strongly stimulated its production in a clone of MCF-7 breast cancer cells (57). TGF β 1 from antiestrogen treated MCF-7 cells inhibited the growth of an estrogen receptor negative cell line, MDA-MB-231. This growth inhibition was reversed by the addition of a polyclonal antibody directed against native TGF β 1 (57). To the contrary, Arteaga and coworkers demonstrated that other clones of MCF-7 cells do not respond to antiestrogens in this fashion and that most estrogen receptor negative breast cancer cell lines are inhibited by TGF β 1 (58). Zugmaier et al have recently shown TGF β 2 to be equipotent to TGF β 1 in its inhibition of human breast cancer cells, while Mullerian inhibitory substance had no effect (59). The effects of other members of the TGF β family on prostate cancer cells are unknown.

SUMMARY

In summary, I have described some of the effects the transforming growth factors, TGF α and TGF β 1, exert on human prostate cancer cells *in vitro*. In particular, I have emphasized the evidence supporting the potential roles of TGF α and TGF β 1 in possible autocrine growth control mechanisms of prostate cancer cell growth. The data presented describe possible autocrine growth loops for both TGF α and TGF β 1 in human prostate cancer. It is evident that a number of growth factors, besides the TGFs, play a mediating or modulatory role in prostate cancer cell proliferation. For example, the heparin binding growth factors are covered elsewhere in this volume.

Current efforts to control hormone responsive tumors, centered around inhibition of steroidogenesis or interference with hormone/receptor binding, universally fail when hormone independent clones emerge. As evident from the *in vitro* studies presented in this chapter, one mechanism by which tumors may become hormone unresponsive is by constitutively activating autocrine growth pathways normally under hormonal control. The elucidation of autocrine growth pathways, as described above, might create a wide array of new therapeutic approaches aimed at disrupting the hormonal cellular response. With more complete knowledge of the mechanisms of regulating prostate cancer cell growth and malignant progression, modes of experimental prostate cancer therapy could be designed to interrupt growth stimulation by TGF α , enhance the growth inhibition by TGF β 1 or to block the paracrine effects of these factors on surrounding endothelial cells, stroma, bone, and the immune system. A major obstacle in utilizing such antigrowth factor

strategies in experimental therapy is a lack of knowledge of the role of these growth factors in the proliferation and function of normal tissues. Such knowledge would also provide insights into the carcinogenesis and prevention of prostate cancer.

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DISCUSSION

Dr. Story:

Have you done any of this at all with fresh human prostate cancer?

Dr. Wilding:

Just in terms of response to EGF and TGF beta. We haven't looked at expression or secretion yet, but, in terms of response we have been able to see inhibition of primary cultures of epithelial cells from prostate cancer by TGF beta.

Dr. Story:

Would you know offhand whether it has been demonstrated in the past that tissues which did not produce these factors or express these messages in the normal state acquire these as sort of an *in vitro* artifact? Has that been reported?

Dr. Wilding:

No, except in the breast cancer system where obviously a lot more work has been done, especially by Lippman's group. When they looked at primary epithelial breast cells, they were able to show that those cells produce TGF alpha. Initially, it was felt that TGF alpha was being produced in estrogen independent breast cancer cell lines much more than in the estrogen response breast cancer lines. The EGF receptors are higher in the estrogen independent cell lines, and that maybe both of these contributed to hormone independence. All of that was turned around when they looked at primary epithelial breast cells and found that the primary epithelial breast cells made more TGF alpha and had even more receptors than the hormone independent cancer cells under certain conditions.

Dr. Coffey:

In relation to factors that affect angiogenesis, we have the TGF alpha, the TGF beta, and the beta FGF. Which is the most angiogenic of those groups as far as the growth factor occurs on the endothelial cells?

Dr. Wilding:

I don't know.

Dr. Coffey:

There's a growing literature now that there's something besides the autocrine effect. And that is, it doesn't have to go outside the cell, but actually works inside the cell. Now that's a different type of autocrine from the autocrine where you have to have receptors that you can measure by your definition. What does your lab call that type of growth factor?

Dr. Wilding:

I think now that you would still have to consider it an autocrine growth loop. I think in the case of the LNCaP cells and TGF beta, we didn't even see TGF beta expressed. So, I don't really feel that there was something happening within the LNCaP cells with TGF beta. I think that is a question in regards to TGF alpha. How much TGF alpha is within the cells? Are there other growth factors? Someone had mentioned earlier that the fibroblast growth factors do not have signal peptides, but whether there is something internal going on in the LNCaP cells is unknown.

Dr. Coffey:

It's going to make a world of difference as to whether you're going to find it in the media, and what you're going to do therapeutically if it's an intracrine as opposed to an autocrine process.

Dr. Barrack:

Ikeda et al published a paper in *Biochemistry* 26:2406, 1987, that PC3 cells synthesized TGF beta 2, but were not inhibited by TGF beta. Do you recall that paper.

Dr. Wilding:

There was a report, I thought it was 2 or 3 years ago, where a group took PC3 cells and they treated them with very high doses of tamoxifen and hydroxy-tamoxifen in approximately micromolar doses. They showed that PC3 cells produce TGF beta 2. I haven't put TGF beta 2 on PC3 cells to see if they are inhibited. Other studies done with Gernard Zugemaier in Mark Lippman's group on breast cancer cells didn't show any difference between TGF beta 1 or TGF beta 2 effects on breast cancer cells.

Dr. Barrack:

But you are seeing an inhibitory effect of TGF beta 1 on PC3 cells, whereas in this other report TGF beta 2 did not inhibit PC3 cells. Is that correct?

Dr. Wilding:

No, Ikeda's report examined the effects of TGF beta 2 purified from PC3 cells on mink lung cells and found inhibition.

Dr. Chung:

I was wondering about DHT effects on EGFR. DHT increases EGFR in LNCaP cells. Is that because the effect was androgen receptor mediated? How about DHT effect on PC3 and DU145 EGFR?

Dr. Wilding:

We observed some increase in the cell number when we treated the cells with DHT, and Schuurmans has published in *The Prostate*, previously, that he had seen an increase in EGF receptors with DHT treatment of those cells. When we looked at EGF receptor expression with Northern blots and then with RNAase protection it was variable. Sometimes we would see an increase in a message, and sometimes we wouldn't. PC3 and DU145 cells do not have androgen receptors, therefore, we did not examine the effect of DHT on EGF receptors in these cells.

Dr. Chung:

Yes, because in the normal rat, androgen is supposed to be inversely regulating EGFR (Traish and Wotiz, *Endocrinol.* 121:1461, 1987) which is the opposite to what you have described in cell culture. I wonder whether that's a species difference or because it's a cancer cell line, as opposed to normal tissue?

Dr. Wilding:

I don't know. In Ira Paston's group Clark had a paper a number of years ago examining EGF receptors. Though the study was not in breast or prostate, they showed that EGF stimulated an increase in EGF receptors. I guess one could postulate, though I have absolutely no proof in prostate, that if by some account you were increasing the production of TGF alpha or EGF that you might get an increase in receptors if that mechanism can be extrapolated to prostate and breast.

GENE PRODUCTS AS THE MOTIVATING FORCE IN THE PROSTATE CELL'S RESPONSE TO ANDROGENS

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Considering that the prostate gland is simply an accessory organ of the male reproductive tract and that its function is still poorly understood, it, none the less, provides a significant focus for modern biomedical research. The prostate is a model organ to study the mechanism of action of steroid hormones. It is unique in its exquisite dependence on androgenic steroids for development, growth and function. It has also provided an important model system to study the interaction between stromal and epithelial cells in organ function. Beyond this, the preponderance of human pathological conditions which are the result of benign and malignant prostate disease provides strong impetus to our need to understand this gland and the mechanism of its hormonal dependence.

The complexity of this mechanism is evidenced by the multitude of diverse cellular functions that depend upon testosterone. Any manipulation of the circulating testosterone levels can lead to changes in the size and shape of the gland while drastically altering the differentiation state of the prostatic glandular cells. Prostate cells require androgenic steroids to synthesize and secrete proteins. In addition, testosterone governs both the proliferation rate of prostate cells and their ultimate lifespan. In a reductionist view, all of these parameters can be related to the progress of three cellular processes, differentiation, proliferation and programmed death. As we now understand from molecular studies of other cell systems, each of these different levels of regulation require the activation and expression of a distinct set of cellular gene products. If we can identify the various gene products that are required for prostate cell growth, differentiation or death, in response to testosterone, we will gain a great number of molecular targets to aid in the fight against benign and malignant prostate disease.

It is with this goal that we have pursued studies to determine how manipulations of testosterone levels might acutely alter the gene expression patterns of prostate cells *in vivo*. Our approach is based on the understanding that cells respond to environmental or hormonal stimuli by altering the activity (expression) of certain genes. This can occur in a positive fashion (gene products increase) and will be referred to as an induction response. In general, gene products that participate in the onset or progression of a cellular process are induced, even if only for a transient period. Alternatively, certain gene products can decrease in concentration in response to hormonal stimuli (or lack of) and this process will be referred to as a repression response. A gene product repression might occur if a cell is changing states (as in differentiation) and no longer requires that product. However, it is also important to consider that a loss of a gene product (such as a specific transcription factor) might be an important determinant in driving the cell to an altered state. In our studies, neither of these terms (induction or repression) should directly imply a mechanism for the change in gene expression; they refer to a condition of increased or decreased expression of any particular gene product by prostate cells in response to testosterone stimulation. Simple cellular responses may require the altered expression of only one or a few gene products. More complex cellular responses, as we are interested in, may require a change in the expression of a large number of gene products at different times as the process progresses.

In our initial approach to understanding the link between androgenic steroids, gene action and prostate growth and function, we have chosen to study a well-characterized animal model system, the rodent ventral prostate gland. This model is extremely suitable for molecular analysis because of the rapidity and intensity with which the gland responds to hormonal stimulation. Within days after castration of a mature rat, the highly differentiated secretory epithelial cells of the prostate gland undergo a programmed death response (1-3). By this mechanism, up to 85% of the cells of the ventral prostate gland will be deleted from the long-term castrated rat. Prostate cells that survive the period of regression, while no longer dependent on testosterone, retain the potential to respond to androgens. By returning testosterone to a long-term castrated rat, the cells of the vestigial prostate gland can be rapidly stimulated to regrow and differentiate so as to completely restore the gland to the pre-castrate state (4-6).

We were also aided in these studies by the incredible growth in our knowledge concerning the role of various individual gene products in the processes of cell proliferation and differentiation. The expanded utilization of molecular biological cloning and characterization techniques have provided a large number of probes for genes with potential relevance to androgen-regulated response processes. The genetic probes that we utilized for our studies fall into two different classes. The first class includes a variety of probes capable of detecting genes associated with cell proliferation or growth in other systems. Often, these genes are classified as proto-oncogenes because of their relationship to viral genes (oncogenes) that act dominantly to stimulate uncontrolled proliferation. The normal cellular homologues of these genes may be expressed transiently during proliferation and their products seem to be required for regulated proliferation. This class includes probes for *c-fos*, *c-myc*, *cHa-ras*, *cKi-ras*, *c-myb*, *c-sis*, heat shock 70K (*hsp-70*) and basic fibroblast growth factor (bFGF). The second class includes two genes required for cellular homeostasis, actin and tubulin. These genes are generally considered to be "housekeeping" genes because they encode cellular structural proteins that are required at all stages of the cell cycle. However, since the requirement for these structural proteins often increases during mitogenesis and cytokinesis, their activity also changes during the cell cycle. With the use of these cDNA probes to analyze mRNA expression patterns and certain antibody probes to measure the synthesis of related proteins, we can monitor changes in activity of a large number of genes during the acute period of testosterone stimulation of the rat ventral prostate gland or during its regression following castration.

We have found the use of Northern Blot analysis procedures to be of most value for this type of analysis. In this technique, we extract poly-adenylated mRNA from pooled groups of rat ventral prostate tissues at various time intervals after castration or after androgen restimulation of a castrate rat. RNAs from control tissues and from tissues at sequential periods following the stimulation are co-electrophoresed in a denaturing agarose gel and then transferred to a nitrocellulose filter paper by Northern blotting procedures. The Northern blot is then hybridized to radiolabeled probes for individual gene products, as shown for the C3 gene probe in Figure 1. Exposure of the hybridized blot to X-ray film gives a visible picture of the intensity of hybridization across the time period being analyzed. The X-ray film intensity for a transcript band at any given time can be quantitated by the means of densitometry. These numbers can be corrected for potential RNA loading inequities by comparing the transcript band density to the relative density of hybridization of the 18S ribosomal RNA probe in the same lane. By this means, we can assign a number to the expression of the appropriate transcript for any given gene and compare these numbers over time, as shown in Figure 2. In summary, our analysis of the expression of the C3 gene, a subunit of the major prostatic secretory protein of the rat ventral prostate, shows a steady decline in activity during the acute period following castration, and a continuous increase in activity when testosterone is returned to a one week castrated rat, consistent with the known stringent requirement of testosterone for C3 expression (7,8).

Gene Expression During Androgen-Stimulated Proliferation of Rat Ventral Prostate Cells

The vestigial ventral prostate gland in a long-term castrated male rat provided our model for studies of the gene activity associated with androgen-stimulated proliferation. Testosterone supplementation of a sexually immature rat will stimulate the development of the mature prostate gland. In a similar manner, the vestigial gland in a castrated rat will respond to testosterone replenishment with the rapid induction of DNA synthesis (4-6). Seven day-castrated mature male Sprague Dawley rats were treated at 12 hour intervals with 2 mg testosterone propionate in sesame oil. Flow cytometric quantitation of the number of proliferating ventral prostate cells was performed by counting the number of propidium iodide- stained nuclei in the S or G₂M phase as a function of time following testosterone replenishment. An approximate time course of the onset of DNA synthesis in the vestigial ventral prostate subsequent to testosterone replenishment is shown in the graph of Figure 3. In this experiment, we found that the fraction of prostate nuclei in S or G₂M of testosterone-treated rats begins to show significant differences from control, non-treated castrate rats after 12 hours. The time of onset of DNA synthesis in our model is early when compared to other reported studies of this parameter (4-6).

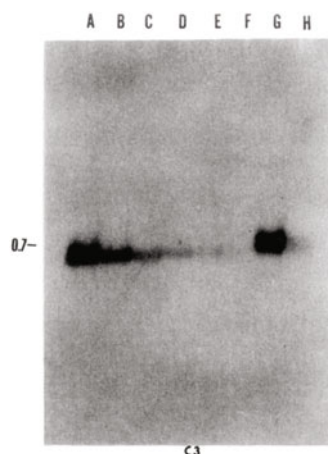


Figure 1: Northern blot analysis for the expression of the transcript encoding the C3 gene in the ventral prostate gland following castration. Polyadenylated mRNA was extracted from the ventral prostate glands of mature Sprague Dawley rats (A) or from ventral prostate glands at 24 (B), 48 (C), 72 (D), 96 (E), or 120 (F) hours after castration of the rat. These RNAs were co-electrophoresed, blotted onto a Nylon filter paper and probed for C3 transcripts with a radiolabeled cDNA probe for C3 provided by Dr. Malcolm Parker (Imperial Cancer Research Fund, London). X-ray film autoradiography (shown above) provides a picture showing the relative intensity of the transcript band in any given RNA sample compared to the control levels. The continuously declining expression of this transcript after castration can be compared to its reinduction following the 24-hr return of a bolus dose of testosterone (2 mg) to a 96-hr castrated rat (G).

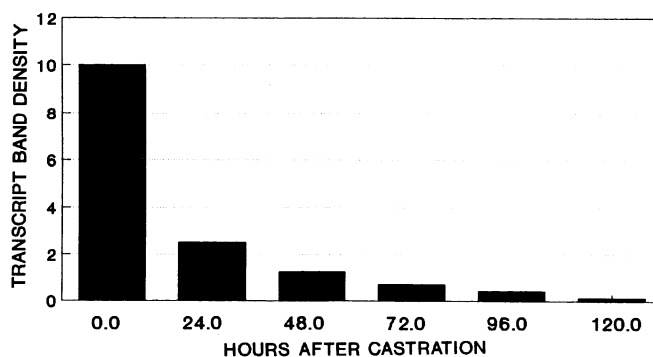


Figure 2: Prostatic Regression C3 Activity. Quantitation of activity of the C3 gene in the rat ventral prostate following castration. Densitometry was performed on the Northern blot shown in Figure 1. The numbers corresponding to the density of the film exposure for the C3 transcript at each day following castration was corrected for any fluctuations in the 18S ribosomal RNA levels and are presented quantitatively by the relative transcript band density in the graph shown above.

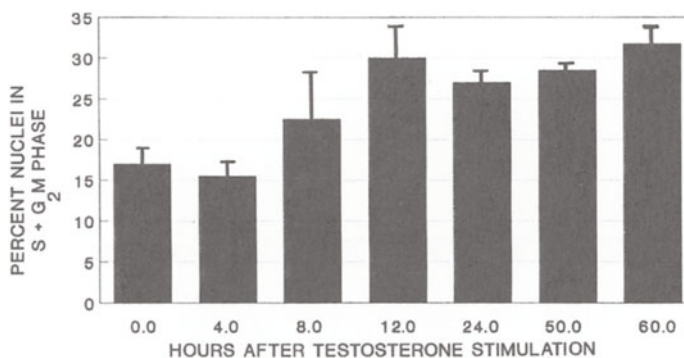


Figure 3: Prostatic Growth Proliferative Index. Graphical presentation of the fraction of cells in the vestigial ventral prostate gland that are undergoing mitosis in response to testosterone restimulation. Seven-day castrated rats were replenished for testosterone by 12-hour bolus doses of 2 mg testosterone propionate. At the indicated times, individual glands were removed, mechanically minced into single cell suspensions and stained with propidium iodide and RNAase A. Cell suspensions were run through a Coulter flow cytometer set to calculate the percentage of cells in the G₀/G₁-, S- or G₂M-phase of the cell cycle. The mitotic index is determined by the percentage of cells in S + G₂M.

By this time, the stimulated vestigial prostate glands in our model system have already exhibited some major fluctuations in gene activity (9). mRNA extracted from ventral prostates of rats treated 1 hour earlier with testosterone demonstrated a 6 to 8 fold induction of transcripts encoding the *c-fos* product compared to RNA from control, untreated rats. This induction is transient; levels of *c-fos* mRNA fall to untreated levels by two hours after treatment. The early, abrupt and transitory induction of *c-fos* mRNA is a landmark seen in other circumstances where cells are reacting to an environmental or hormonal stimulus (10,11). In our study, by far the most active of the growth-related genes found during the early phase of prostate regrowth was the gene for *cHa-ras*. Induced transcript levels for *Ha-ras* were evident by 2 hours after testosterone-stimulation and this transcript continued to increase in concentration during the period of the experiment so that by 24 hours after testosterone replacement, it demonstrated a 20-fold induction in expression. Transcripts encoding the *cKi-ras* gene were also induced during this period, but not to the extent of the *cHa-ras* gene. Each of these three gene products, *c-fos*, *cHa-ras* and *cKi-ras*, then fall into a class of early-response genes (Figure 4).

Later (6 to 8 hours after testosterone stimulation) a series of transcripts encoding the *c-myc*, *c-myb*, *hsp-70*, *actin* and *tubulin* gene products show a virtually coordinate induction. These gene products fall into an intermediate class of response most notable in that it closely precedes the onset of DNA synthesis in testosterone-stimulated prostate cells (Figure 5). At least some of these genes, most prominently *c-myc*, *actin* and *tubulin*, have functions that might directly relate them to the onset of the mitotic phase of prostate growth. *Tubulin* proteins drive the segregation of replicated chromosomes during late mitosis while *actins* are required for events during late cytokinesis when the cellular structures are reforming. Antibodies to the *c-myc* protein, when injected into a mitotic cell, can block any further DNA synthesis (12). In addition, the temporal pattern of a brief and early induction of *c-fos* followed by a later induction of *c-myc* marks many types of cells entering the mitotic cycle (10,11,13). Thus, while the function of the protein encoded by *c-myc* is still not completely understood, it is clearly relevant for successful DNA synthesis. In summary, we can say that the early molecular events associated with androgen-stimulated replication of prostate cells closely mimic the molecular response of a wide variety of cells to a multiplicity of growth-inducing factors.

This type of analytical system might also be of value in evaluating the role of various growth factors in prostate growth. If testosterone is acting on the prostate gland by stimulating the production of a growth factor responsible for the mitogenesis of prostate cells, we should expect to detect an increase of gene products for that growth factor, at least preceeding the onset of DNA synthesis in the regrowing gland. Hybridization studies using the *c-sis* probe showed little or no change in the minor expression of the corresponding transcripts for this gene during the 24 hours after testosterone. Since *c-sis* is homologous to a

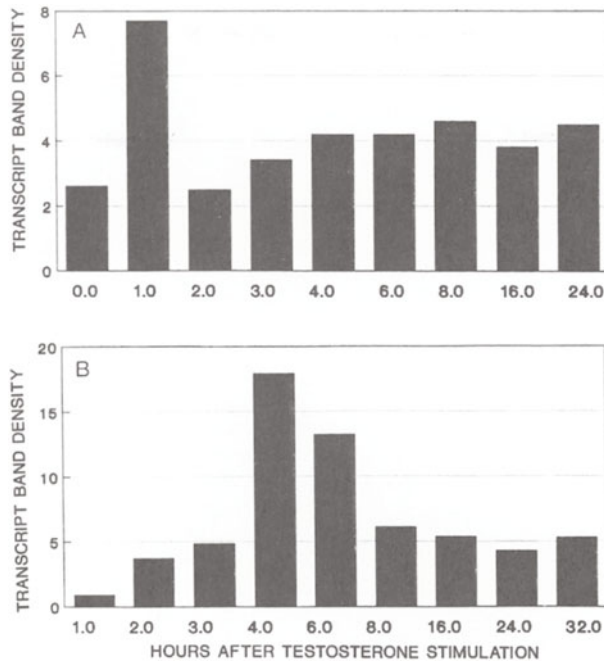


Figure 4: Prostatic Growth and *c-fos* (A) and *cHa-ras*(B) Activity. Graphical representation of the relative transcript band density for the *c-fos*, *cHa-ras*, or *cKi-ras* genes in RNA extracted from vestigial ventral prostate glands during the time interval following replenishment of testosterone to 7-day castrated rats.

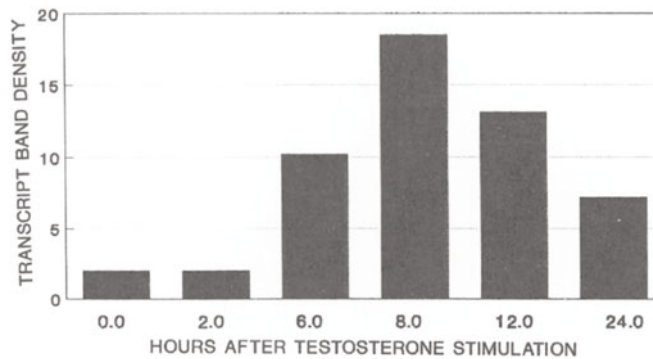


Figure 5: Prostatic Growth and *c-myc* Activity. Graphical representation of the relative transcript band density for the *c-myc*, *actin*, and *hsp-70* genes in RNA extracted from vestigial ventral prostate glands during the time interval following replenishment of testosterone to 7-day castrated rats.

subunit of the platelet derived growth factor, we think it is unlikely that this factor plays an important role in ventral prostate regrowth stimulated by testosterone. There is, however, a sharp induction of basic fibroblast growth factor (*bFGF*) mRNA expression during the acute period of prostate regrowth (Figure 6). It is interesting to note, that the apparent induction of *bFGF* mRNA in our model is relatively late (after 12 hrs testosterone stimulation), occurring subsequent to the induction of intermediate response gene products, and almost coordinate, if not later than, the onset of DNA synthesis. This would suggest that *bFGF* might play a secondary role in acute prostate regrowth, perhaps related to its known angiogenic activity (14).

Gene Expression During The Androgen-Programmed Death of a Rat Ventral Prostate Cells

Any procedure that blocks the access of dihydrotestosterone to the mature rat ventral prostate will induce massive cell death in the gland (1-3). Under histological examination, the dying prostate cells are viewed as undergoing "apoptosis" (15), an expression referring to a set of distinct physical changes that include cytoplasmic shrinkage, margination and condensation of nuclear chromatin combined with fragmentation of the nucleus (16). Biochemically, androgen-deprived prostate cells show evidence of regularly degraded nuclear DNA (17). All of these physical manifestations are characteristic of a unique cellular process referred to as "programmed death" (18,19). The molecular basis of this programmed death response is poorly understood. It is clear, however, that androgen-programmed death is not a passive response. RNA or protein synthesis inhibitors can block the onset of programmed death in ventral prostate cells following castration (2,20). These early experiments strongly imply that some gene product(s) synthesized *after* castration is required for prostate cells to undergo programmed death. In this sense, programmed death can be thought of as a cellular suicide.

Our preliminary analysis of gene activity in early regressing rat ventral prostate glands is consistent with the concept of androgen-programmed death as a genetic response to androgen deprivation (21). Following castration of a mature male rat, specific shifts in gene activity mark the onset of prostate regression far earlier than the histological evidence of cell death (accumulation of apoptotic bodies) can be detected. The dying prostate cell will exhibit a cascade-like series of molecular events, including an early (36 hrs after castration) induction of transcripts encoding *c-fos*, a later induction (after 48 hr) of *c-myc* transcripts and even later (after 72 hrs), an induction of transcripts encoding heat shock 70k-related gene products, as summarized in Figure 7. Since a similar genetic response is demonstrated by androgen-dependent rodent tumor cells in the acute time period following castration of the host (22), we would suggest that this cascade pattern of gene activity (*c-fos* → *c-myc* → hsp-70) would be characteristic of a cell undergoing androgen-programmed death.

Paradoxically, as we have described in our study of gene activity during ventral prostate regrowth, this genetic response to androgen-deprivation resembles the appropriate genetic response of cells to a proliferative stimuli (10,11,14). The coincidental occurrence of such similar gene activity during the early period of two distinctly different cellular processes would argue that the onset of both cell proliferation and cell death might share a common early signal pathway. Clearly this pathway deviates before the onset of DNA synthesis and we are currently evaluating other molecular markers of proliferation onset such as protein kinase C activation and RB protein phosphorylation in regressing prostate cells to determine how far these pathways coincide. It is of interest that other systems of inducible cell death such as chemotherapeutic toxicity or programmed death mediated by surface antibody binding require the cell to be in a proliferative state for effective death-stimulation. While we tend to think of the cells of a mature prostate gland as being in a non-proliferative state, perhaps the first step to their imminent death is a proliferative stimulation that is aborted in the absence of androgens.

This kind of descriptive study of the gene activity associated with the onset of androgen-programmed death in prostate cells has also led to the discovery of a gene product with a remarkable degree of specificity for this process, TRPM-2. Originally cloned from a cDNA library made from RNA of regressing rat ventral prostate tissue by Dr. Martin Tenniswood of the University of Ottawa (23), mRNA transcripts homologous to the TRPM-2 gene have now been shown to be intensely induced during the onset of programmed death in a wide variety of mammalian cell types (24). The systems so far analyzed include rat renal cells dying in response to renotoxic agents (gentamycin) (25), ischemia/reperfusion injury (26), or dying as a result of pressure following a ureteral obstructive procedure (27). Rodent embryonic cells dying during the tissue remodeling phase of ontogeny express high levels of RNA for TRPM-2 (24) as do mouse bladder tumor cells following effective chemotherapy (24). Even more recent independent studies have found that RNA gene products related to the TRPM-2 gene mark Alzheimer's diseased brain regions or brain tissue suffering degeneration subsequent to Scrapies Virus infection (28). Each of these degenerative conditions has one common element; cells undergoing a programmed death response. *In situ* hybridization

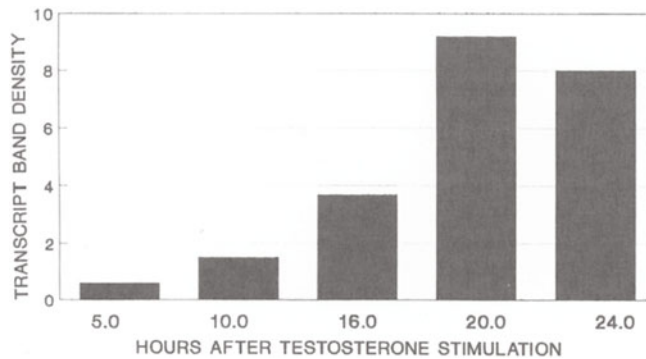


Figure 6: Prostatic Growth and basic FGF Activity. Graphical representation of the relative transcript band density for the basic fibroblast growth factor gene in RNA extracted from vestigial ventral prostate glands during the time interval following replenishment of testosterone to 7-day castrated rats.

analysis for TRPM-2 gene products in this various array of degenerating tissues proves that the cells dying as a result of the condition are the source of the TRPM-2 products (24). In addition, procedures that reduce the level of cell death in a tissue coincidentally reduce the expression of the TRPM-2 gene (29). We can thus convincingly link the expression of this gene to the progression of a programmed death response in mammalian cells.

As a first step towards understanding the possible function of TRPM-2 in a programmed death response, we utilized procedures to characterize potential protein product(s) encoded by this gene. Sequencing studies of the transcript for TRPM-2 gave the first clue as to its identity (24,30). Based on the partial sequence of a cDNA clone homologous to the TRPM-2 probe isolated from regressing rat kidneys, we were able search available gene banks and confirm a strong, but not complete, homology between the TRPM-2 cDNA and the reported sequence of a gene expressed constitutively by mammalian Sertoli cells, sulfated glycoprotein-2 (SGP-2) (31). This identity explained our ability to detect constitutive expression of a TRPM-2-homologous transcript in RNA from rodent testis. More important was the realization that antibodies made against the SGP-2 protein might be capable of detecting proteins encoded by TRPM-2 in dying cells. Using antibodies provided by Dr. C. Yan Cheng of Rockefeller University made against purified rat SGP-2 (clusterin), we were able to confirm the intense synthesis of homologous proteins, both in regressing rat ventral prostate tissue and in ureterally obstructed rat kidneys (24,30).

At this time, we can only speculate as the function of the TRPM-2 gene products in androgen-programmed cell death. While we initially hoped that TRPM-2 might encode a lethal gene product, as has long been suspected to play a role in inducing programmed death, the constitutive synthesis of a very homologous protein by both Sertoli cells and epididymal epithelial cells would argue against this possibility. Recently, evidence indicates that the SGP-2 protein can bind to a terminal complex of complement proteins, inactivating their ability to penetrate the membrane of a target cell (32). If the main function TRPM-2/SGP-2 is a complement inhibitor, one might ascribe a protective function to this protein. Its relatively late time of synthesis by the dying cell, however, would indicate that it is not effective in saving the dying cell. Rather, we might speculate, that the intense expression of the TRPM-2/SGP-2 protein might have more of a protective effect on surrounding tissue, protecting it from non-specific activation of complement when basement membranes are exposed during regressive changes. We hope to be able to answer this question soon, but more important, we believe that our acceptance of this gene product as a late marker of programmed cell death provides us with a starting point to decipher the genetic regulatory system controlling this process.

Genes Products as the Motivating Force in the Prostate Cell's Response to Androgens

In the past few years, much of the attention of molecular biological research on the prostate has focused on the role of an individual gene product in the prostate cell's response to androgens. This might be related to whether a particular growth factor has relevance to prostate gland biology or if a singular proto-oncogene controls benign or malignant prostate growth. It is important to remember the complexity of the

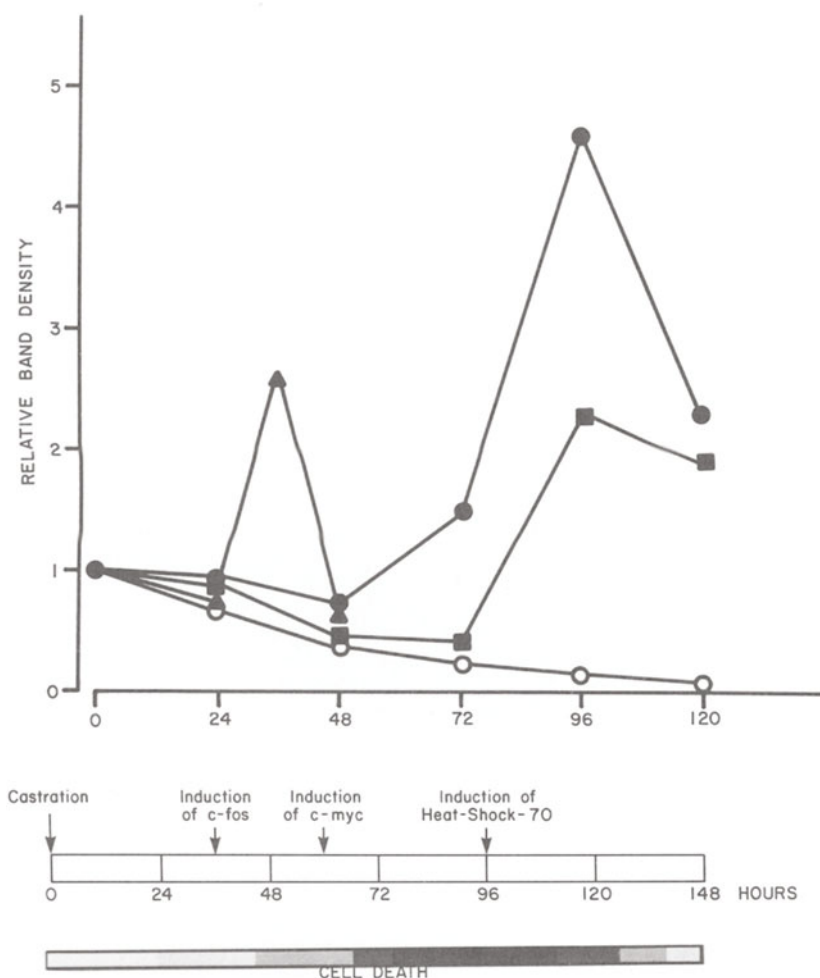


Figure 7: Graphical representation of the relative transcript band density for the *C3*, *c-fos*, *c-myc*, and heat shock 70K genes in RNA extracted from ventral prostate glands during the acute time period following castration of the rat. Numerical values were derived following hybridization of a Northern blot with a probe for the *C3* gene (o), *c-fos* gene (▲), *c-myc* gene (●) or *hsp-70* gene (■). Shown below is a temporal map designed to relate the wave of programmed cell death in the ventral prostate gland to the time after castration and the relative gene activity in the gland.

cellular apparatus in that any individual gene product only acts in conjunction with hundreds of other physically dissimilar gene products to produce a given cellular action. As has been found from biochemical studies of numerous cell systems, primary stimuli are transmitted when changes in one cellular protein initiates a cascade reaction, changing the physical characteristics of other proteins down the chain, and inducing the appearance of new proteins in a regulated order. Each individual gene product down the chain of reaction thus has an important role in converting the initial signal into the final cellular reaction. Integrated studies of this coordinate action of gene products should have more emphasis in future research into the molecular biology of the prostate gland.

It is intrinsically apparent that a key genetic component required for the initiation of any of these cellular processes in response to testosterone, must be the androgen receptor protein. This receptor is the primary signal transducer of testosterone action and it has been demonstrated, mainly from studies on androgen-dependent tumors, that tumor cells with low or absent androgen receptor content show little growth regulation by testosterone (33). Similar to the other members of the steroid receptor gene family, the activated androgen receptor complex is believed to act as a transcriptional modulator, controlling the production of mRNA from genes that contain a cis-acting androgen regulatory element (ARE) in proximity to the DNA coding sequence. The recent success in cloning the androgen receptor transcript and gene should lead to rapid progress in deciphering its role in prostate cell biology. Distinct AREs on genes inducible by androgens have proven difficult to define. However, it has been found that regulatory elements for other steroid receptors, most notably the glucocorticoid receptor, can function as an androgen receptor binding site and enhancer element *in vitro*, and *in vivo* (34). Considering that glucosteroids are proving to have some influence on the biology of the rat prostate gland (35), this aspect deserves further investigation to determine its relevance to the development of human prostatic diseases.

Understanding the black box beyond the androgen receptor lies in determining the primary gene products induced by direct androgen receptor action. Of the many gene products we have examined in our study, it is not likely that many (or any) of them are expressed because of androgen-receptor mediated inducible transcription. Rather, many of these genes are characteristically induced as part of a response cascade in any cell subject to a proliferative stimuli or a signal for programmed death. Therefore, for the future, we can hope that by working back to the initial gene products activated by androgen action, we can gain understanding to the complex cellular mechanisms that generate prostate cell proliferation and prostate cell death.

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DISCUSSION

Dr. Coffey:

In the aschemic kidney that looks necrotic, are TRPM-2 only seen in programmed cell death? Dr. Isaacs had a case where the calcium increases wouldn't go on up. Are those events ever separated?

Dr. Buttyan:

Cells undergoing a "programmed death" response are probably best identified through histologic means by the presence of apoptotic bodies in tissues. Apoptotic bodies are dense, shrunken cells with condensed and fragmented nuclei. These types of bodies can be easily distinguished from cells undergoing a "necrotic cell death" that appear with swollen, low density cytoplasmic regions, broken outer membranes and intracytoplasmic material spewed out into the extracellular spaces. Prolonged renal ischemic injury is more associated with the necrotic type cell death while the renal ischemia/reperfusion type of injury that my colleagues and I have been working with is more associated with the appearance of large numbers of apoptotic bodies in the post-reperfusion kidney, consistent with an exaggerated programmed death response.

Dr. Coffey:

If you don't see the reperfusion, aschemia won't do it?

Dr. Buttyan:

The transcript for the TRPM-2 gene is induced during chronic rat renal ischemic injury. However, its expression is very much depressed, both in time and intensity, when compared to its profound induction during injury caused by a brief period of renal ischemia followed by renal reperfusion. I would also like to comment on Dr. Isaacs' statement this morning that cell death following calcium ionophore treatment was not accompanied by the induction of the so called TRPM-2 gene. This gene product, like many others, is transiently expressed during programmed cell death. We have found in certain cases, for example neomycin treatment of cultured cells, that this gene induced to a maximum within 6 hours after treatment and return to undetectable control levels by 12 hours after treatment. So before one decides that this gene is completely inactive during any form of cellular injury response, one would have to check for its expression at multiple, perhaps narrower time intervals during the cell death process.

Dr. John Isaacs:

Agents like water and ouabain which kill cells in less than an hour are agents that are known to produce necrotic death and in those systems TRPM-2 isn't elevated. So treatment with agents known to produce necrotic death usually results in lyses of lysosomes and mitochondrial membranes before they have time to activate this cell death program. The point that Dr. Coffey is bringing up, and the one you've also alluded to, is that if you can elevate calcium and induce DNA fragmentation, apoptosis can be induced without TRPM-2 induction. TRPM-2 doesn't need to come up in order for the cell to die. So I agree, I don't think TRPM is an absolutely essential requirement.

Dr. Buttyan:

The protein encoded by this gene is highly related, if not identical to, a protein that has recently been shown to function as a complement inhibitor *in vitro*. Apparently it has a close structural relationship to a family of proteins that include the C9 complement protein. It has been suggested that the TRPM-2/SGP-2 protein binds to the complement complex, replacing C9 and effectively inactivating the ability of the complex to penetrate a target cell membrane. So in a sense, one might ascribe a more protective function to this protein during cell death. Even though it is synthesized too late to protect the dying cell, perhaps it is synthesized and secreted to protect surrounding tissues from the nonspecific activation of complement, as might occur when basement membranes are exposed during tissue remodeling phases.

Dr. Matusik:

I don't quite understand why there seems to be hesitancy in saying if TRPM-2 is identical to the Sertoli factor or not.

Dr. Buttyan:

First, we have detected some minor sequence differences between the cDNA clone isolated from a hydronephrotic kidney and the cDNA clone for SGP-2 isolated from rat testis. Second, the molecular weight of the protein synthesized and secreted from regressing prostate and hydronephrotic kidney is slightly higher than the corresponding SGP-2 protein synthesized by Sertoli and epididymal epithelial cells. Both of these differences might be accounted for by strain polymorphisms or protein processing differences in dying versus normal cells. Since there are several large DNA fragments homologous to the cDNA clone as detectable by Southern blotting of rat DNA, we will not know how many genetic units encode these products until we have mapped, sequenced and analyzed the corresponding genomic DNA.

PANEL DISCUSSION

Growth Factors-2

Panelists: William Catalona, Aaron Katz, John Petros, and Edward Sherwood:

Dr. Katz:

Dr. Rowley, urogenital sinus tissues are exclusively sensitive to androgens, and I was wondering if you had performed any experiments which documented the fact that this inhibitor factor may be somewhat androgen regulated. The second part of my question is whether you had performed any tritiated thymidine uptake labelling studies to see if the inhibitor factor somewhat interfered with the cell cycle or possibly delayed the onset of DNA synthesis?

Dr. Rowley:

Well, with regards to your second question, we haven't done anything with cell cycle. We really don't know where it's happening in the cell cycle. We have studies like that planned. As far as androgen responsiveness, that's one of the first things that we wanted to address. The problem that we faced is that it's been very difficult to get this into completely defined medium. We can cycle the cultures through just DMEM for a few days to condition the DMEM and use that for purification. But to get any kind of a medium that maintains the production of the factor, we can put it into defined medium and maintain viability and growth of the cells, but invariable when we do that, we cease production of factor. We can even use charcoal stripped serum to try to get rid of steroids and we still see loss of factor secretion when we do that. So there seems to be some low molecular weight component in the serum that is regulating this protein. We just don't know what it is yet. So it's been very difficult to address those questions.

Dr. Petros:

We have heard quite a bit about TGF beta and everyone seems to agree that it's generally inhibitory to the epithelial cells. I'm just wondering first of all if some of the panelists who talked about TGF beta would address the effects of tamoxifen in the various systems they've worked with, and if any of the clinicians would know about the results in clinical trials, and has that been effective? Are there other things that have been known to induce the production TGF beta. Tamoxifen has been studied in breast cancer and prostatic cell lines.

Dr. Wilding:

There originally was a paper by Canobi in Cell that showed a clone of MCF7 cells expressed and produced more TGF beta in response to tamoxifen and that TGF beta expression and secretion went down in response to estrogen. Subsequently, Kent Osbourn looking at other clones of MCF7 that do not show any decrease in TGF beta with estrogen or increase with tamoxifen. Therefore, it would be difficult to say that TGF beta is the way that tamoxifen works. I don't think that anyone believes that that is the case. In terms of prostate cancer and antiandrogens, we were specifically looking at human cell lines and the only human prostate cancer cell line was the LNCaP. We were a little surprised at the fact that when we added hydroxyflutamide or RU23908, which is Anandron, or cyproterone acetate that we stimulated the cells to grow. Subsequently, Carlos Soninshine at Tufts has shown that the LNCaP cells are stimulated to grow with antiandrogens and I believe one of the European groups has similar data. So if you want to look at human prostate cell lines and you look at LNCaP, it is going to be difficult to look at beta since we didn't see expression in beta and we stimulated the cells when we did treat them with anti-thormones. In terms of clinical trials with TGF beta, treating animals, Gerhardt Zugmeier, Bob Dickson, and I treated nude mice injected with MDA 231 cells, which is an estrogen independent breast cancer cell line, and we also injected mice with DU145 androgen independent prostate cancer cells. We put mini

pumps on their backs and infused them with TGF beta. The experiment took close to 2 milligrams of purified TGF beta. Mice over a two-week period were receiving in the range of 5-10 micrograms per day. We didn't see any change in the tumors. The tumors were injected in the abdomen of the mice. Those tumors all grew as expected, and as with the sham controls, we also injected tumor cells in the nape of the neck right at the head of the mini pump. Though in the first week those tumors lagged behind the tumors on the abdomen, by 2 weeks they had caught up. When we started injecting TGF beta intraperitoneally into the mice, basically the mice started dying of things other than the cancer that we injected. We found the TGF beta to be very toxic. We did look at some of the pharmacokinetics of the injected TGF beta, by spiking it with labelled TGF beta, and could find it in the bloodstream and in most organs, except the brain by looking for TCA precipitable counts. That would mean that it would be TGF beta, unless the 125I came off and was connected to another protein.

Dr. John Isaacs:

In a follow up, besides malignant cells, we have attempted to drip beta TGF onto the normal prostate of rats. We castrated an animal, let the prostate involute, you place an Alzet mini pump with a catheter to drip beta TGF right into the prostate. You allow the TGF beta to drip into the involuted for several days before giving androgen, and then you watch the restoration kinetics versus animals that are dripped with just the carrier. We could see absolutely no change in the restoration kinetics, suggesting that the TGF beta as we gave it, in a relatively high dose also, was unable to block the restoration of the rat prostate induced by the androgen.

Dr. Sherwood:

Dr. Rowley, concerning the urogenital sinus inhibitory factor, have you looked at the other proteins in the TGF beta family such as inhibin or Mullerian inhibitory substance, and do you think that these agents may play a role in urogenital sinus inhibitory activity?

Dr. Rowley:

We haven't specifically looked at any of those. It's hard to speculate just what role they might have. But I certainly wouldn't just rule that out.

Dr. Ichikawa:

Fibroblast growth factor gene doesn't have signal sequences which are characteristics for secretory proteins. Can genetic changes make cells begin secreting FGF into the culture medium?

Dr. Catalona:

So the question is can the genetic changes in the cells cause them to begin secreting FGF? Can anyone address this?

Dr. McKeehan:

Well, the FGF family consists of seven different gene products that have varying degrees of homology. We seem to cross react so far with the same receptors so several of these proteins all have signal sequences, and they are all different genes compared to acidic and basic FGF which have no signal sequences. Several were isolated by oncogenic technologies, for example 3T3 soft agar growth assays, HST Kaposi sarcoma genes. So obviously these are either overexpressed or activated abnormally because they don't seem to be present in the normal tissue. Along this line, I would like to mention we have screened most of the FGF gene products in the models that I've showed, the simple one, the normal prostate, regenerating prostate, slow and fast tumors, and we don't find a large impressive amount of any of the oncoprotein gene products compared to acidic FGF. The one that has not been looked at in detail, because it's the newest one, is the one cloned by Stew Aronson. It is a good candidate. It has a secretory signal that should be looked at closely because he's claiming that it is solely of mesenchymal origin and also specific for epithelial cells. That is, it's not a fibroblast growth factor, and does not act on 3T3 cells and other fibroblasts. So this is one we are now looking at in the prostate system.

Dr. Coffey:

Dr. Lelund Chung isolated some of these growth factors for doing motility, and Dr. Isaacs said something about discussion of motility.

Dr. Isaacs:

The issue that I was trying to bring up was in terms of metastasis. We've talked about growth factors, and we've talked about what makes cells grow. But really, clinically one of the most important issues is what makes the cells metastasize. One can argue what to call the histological lesions that patholo

gists are presently calling prostatic cancer in autopsy series. I think it's fair to say that whatever they are, they stay in the prostate. When they leave the prostate, they now become a clinical entity. Motility characteristics are something that we hope are going to separate cells that are going to stay in the prostate, versus the ones that go out. But would you change the response of a cell that you knew was motile, for example, a cell that could move around and responded to certain growth factors, and, if you changed its motility, in other words stopped it from being motile, would you change its growth factor requirements? Maybe cells respond differently to growth factors depending on where they are migrating, whether they are arrested, whether they are basically in the prostate sitting there quiescently or actively involved in motility. We haven't heard about one of the most interesting factors which is the autocrine motility factor, which seems to stimulate cell motility. It would be very interesting to see if you took a cell that had been characterized on its response to standard growth factor, gave it autocrine motility factor, making it more motile, and see if that changed the cell's response to the other growth factors. That is, when a cell changes its shape, it may actually respond totally differently to different growth factors.

Dr. Coffey:

In two issues ago in *Nature*, they reviewed a lot of the motility and spreading factors and there's a whole series of these coming out now. A summary was published in *Current Concepts of Cell Biology* a few months ago about what we do know about cell motility at the moment, in a generalized scheme. Based on these reviews, I do not think that cell motility guarantees metastasis, but I will be surprised if anything metastasizes that doesn't have cell motility.

Dr. Freeman:

Dr. Buttyan, is it possible or is there any data that TRPM-2 is in fact a death gene, a suicide protein, but it requires a co-factor that's not present in Sertoli cells? The second question is do you or anybody here have any data on how potentially oncogenic membrane proteins, such as the EGFR or *c-neu*, respond to androgen replacement and withdrawal?

Dr. Buttyan:

At present, the data linking the TRPM-2 gene product to the cell death process is its near ubiquitous expression in many, many different types of cells and tissues undergoing a programmed death response, regardless of the death stimulus. We do not yet understand whether this gene product might be lethal protein, as so often hypothesized to explain the programmed death response, or whether in fact it might serve as a cofactor with some other product to have a lethal effect. It may even be a protective factor for cells near the area of the injury, as I have mentioned. To answer the second part of your question, we have not yet analyzed for the role of those particular gene products in cell death. I would like to emphasize again that many gene products probably participate in the cell death response. We have only analyzed a few of hundreds of potential factors involved in cell death. By sorting out how the various gene products change during specific cellular processes, such as programmed death or proliferation, we hope to gain knowledge of the integrated molecular mechanism that drives prostate cells to grow abnormally or to understand why some human prostate cancer cells fail to die during androgen deprivation therapy.

MOLECULAR BIOLOGY OF PROSTATE-SPECIFIC ANTIGEN

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INTRODUCTION

PSA has been categorized as a member of human glandular kallikrein gene family (1,2). The fully-glycosylated protein has a molecular weight of 34,000 and contains approximately 7% carbohydrate (2,3). The PSA polypeptide consists of a single chain of 240 amino acid residues (3,4). The amino acid sequence homology of the PSA with human glandular kallikrein is 78%; and with pancreatic kallikrein, 57% (4-6). The cDNA sequence homology with the glandular kallikrein is 85%; with the pancreatic kallikrein, is about 65% (5,6). PSA is synthesized exclusively in prostate epithelial cells and secreted into the prostatic tubules to become a component of the seminal fluid (7). Although the precise physiological function of PSA has not been determined, a physiological substrate (a human seminal protein) has been identified recently (8). This suggests that PSA plays a fibrinolytic role in semen (9).

Expression of PSA protein in prostatic tissue has been correlated with fluctuating androgen levels during development in man (10). Other studies (11) also have shown that the level of PSA protein may be reduced when prostatic cells either lose their androgen response or increase their anaplasticity. Therefore, PSA should be a useful marker for studying androgen action in human normal or abnormal prostatic cells. The advantage of the PSA-LNCaP model for studying androgenic regulation of human gene expression is that these cells respond to androgens *in vitro* by increased growth and production of PSA. Moreover, antibodies to human PSA are available commercially and have been used to isolate a full-length PSA cDNA. These probes can be used to examine the tissue- and steroid-specificity of PSA gene expression and to determine whether this effect occurs at the transcriptional level. PSA specific nucleotide probes can also be used to isolate human PSA genomic clones, whose DNA structure will be analyzed to identify specific *cis* regulatory elements which are important for control of PSA gene expression.

Cloning of cDNA and Genomic DNA for the PSA Gene

The full-length PSA cDNA ($\approx$ 1.5 kb) was cloned from a normal human prostate lambda gtlI cDNA library in this laboratory using anti-PSA antibody. The PSA cDNA was subcloned into a pGEM4 vector. The deduced amino acid sequence matched the published reports of the amino acid sequence of the entire protein (6). A radioactive labeled 418 bp Sty I fragment, corresponding to the 3' untranslated region, was used to screen a PSA genomic DNA library. Presently, two of 10 positive genomic clones appear to contain the 5' flanking region of the PSA gene. Since these two clones were recognized by a PSA-specific

oligonucleotide but not by a kallikrein-specific oligonucleotide they appear to be PSA-specific. These two clones are now being subcloned and sequenced and will be used for identifying *cis*-acting elements for the PSA gene.

Androgenic Regulation of PSA Expression in LNCaP Cell Line

We have utilized the PSA cDNA probe to determine whether androgens affect PSA mRNA levels in LNCaP cells (a human prostate metastatic cell line). Whereas, low levels of transcripts were evident in androgen withdrawn cells, PSA mRNA (≈ 2.9 kb) was significantly increased within one hour after addition of androgen. Control cells of either human genital skin fibroblasts or HeLa cells were negative for PSA mRNA. Transcript levels remained high at the following androgen addition for the cells. Very minor PSA reactive transcripts (9 kb and 1.2 kb) were also detected after androgen stimulation.

PSA protein secretion from LNCaP cells was also examined. In the absence of androgen, there was very little change in PSA protein levels. However, in the presence of androgens there was a marked increase in PSA secretion beginning at 24 h after androgen addition. PSA secretion continued to increase for 96 h following androgen addition.

In Situ Hybridization of PSA mRNA in Human Prostate Tissues

We next determined if PSA mRNA could be detected in epithelial cells of human prostate tissue. For these studies we utilized the technique of *in situ* hybridization with the sty 1 fragment of PSA cDNA as our probe. We found specific transcript exclusively in epithelium cells of the prostate, but not in kidney or pancreas. The specific hybridization on prostate tissue section could be abolished by RNase treatment, indicating that the specific hybridization was due to the mRNA within the cells. A non-specific DNA used as a probe showed only background activity.

REMARKS

Although the phenotypic expression of PSA protein is prostate-specific, the regulatory factors and mechanisms which restrict and/or promote PSA expression remain to be characterized fully. Since PSA levels in prostatic cells can be induced by androgens, it will be of interest to determine the molecular mechanism of this induction. The classical model of steroid hormone action holds that the steroid-occupied receptor protein can modulate transcription of a target gene by binding to specific *cis*-acting regulatory sequence(s), termed steroid responsive elements (SRE's), in or near the transcription unit of the target gene (12-14). Sequences of glucocorticoid and progesterone response elements are extremely similar (15), whereas estrogen response elements have a distinct consensus sequence (16). Progress in the identification and characterization of androgen response elements (ARE's) has lagged behind that of the other steroid receptors because of: 1) a lack of highly purified androgen receptor for *in vitro* binding studies; and 2) the need to establish a stable cell line that contains androgen receptors and an androgen-inducible gene, for which antibody and molecular probes are available.

Molecular studies of the mechanism of androgen action in the prostate will undoubtedly be facilitated by the recent development of antibody and cDNA probes for the androgen receptor, as well as by the availability of antibodies, cDNA and genomic DNA for PSA, whose expression in prostate cells is androgen regulated.

Our initial data demonstrated that LNCaP cells contain high affinity androgen receptors (> 800 mol/mg protein) and can respond to androgens with increased cell growth and expression of PSA. The recent progress in cloning of the human androgen receptor cDNA in this lab will permit sufficient *in vitro* production of recombinant receptor to localize and characterize androgen regulatory elements (ARE's) of the PSA gene.

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DISCUSSION

Dr. Thompson:

In that last experiment, were you co-transfecting the receptor expression vector and the reporter gene?

Dr. Tindall:

No, these were in LNCaP cells, which already contained high concentrations of androgen receptor. We were using the endogenous receptor to induce the CAT activity.

Dr. Matusik:

The obvious question you alluded to at the end of your talk. Have you transfected your first vector for the DNA binding domain into a cell line to see if you get constitutive expression of a GRE - CAT gene?

Dr. Tindall:

No, we have not done that. That's a very good suggestion. In fact, we're planning to do that experiment in the near future.

Dr. Barrick:

It appears from the molecular weight (150KD) of your protein that you have extra sequence. Which part of the sequence is it? Is it larger than what it should be?

Dr. Tindall:

Right. That's larger than what you'd expect for the androgen receptor, because it contains both part of the receptor and beta-galactosidase. So the combination of those two proteins gives you a larger

molecular weight. We can in fact cleave that to a smaller molecular weight forms using collagenase, since a collagenase recognition site was inserted between the two proteins.

Dr. Barrick:

Does the binding affinity exhibit more than the one reported before? Do you think those sequence affect binding affinity?

Dr. Tindall:

Recently, we haven't compared the binding affinities of the fusion protein with the endogenous receptor. The two affinities were very similar (approx. 1nM).

STRUCTURE AND EXPRESSION OF THE ANDROGEN RECEPTOR IN NORMAL TISSUES AND IN PROSTATE CARCINOMA CELL LINES

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The mechanism of androgen action is believed to be similar to that of the other members of the steroid receptor family. Three facets of androgen action are distinctive, however. The first is its profound effects on phenotypic sexual maturation and development. The second is that the action of the principal circulating androgen, testosterone, is modulated by an intracellular metabolite, that is, 5 α -dihydrotestosterone. The third pertains to the growth promoting effects of androgens on normal and cancerous cells of the prostate and other androgen target tissues. Despite the central importance of the androgen receptor, the details of androgen receptor physiology have been inferred principally from studies of the distribution and behavior of the receptor labelled with tritiated steroids. This is because until the cDNAs encoding the androgen receptor were cloned, the tools were not available to study directly the structure of the androgen receptor protein or to examine the expression of the androgen receptor gene. For these reasons, we have undertaken the development of tools to allow us to examine the expression of the androgen receptor in tissues and in cell culture. The following discussion summarizes two aspects of the work that is ongoing in our laboratory: the use of antibodies that recognize the androgen receptor and examination of androgen receptor gene expression in human prostate carcinoma cell lines.

Production and Use of Antipeptide Antibodies that Recognize the Human and Rat Androgen Receptors

To produce antibodies specific for the androgen receptor, we have employed several approaches. First, the cDNA for the native human androgen receptor was expressed in eukaryotic cells. This has been possible by using an expression vector driven by the CMV promoter in tissue culture cell lines. Second, a fusion protein has been produced in *E. coli* that contains the entire coding segment of the human androgen receptor except for the 37 amino terminal amino acids present in the native receptor protein. Third, antibodies were made using synthetic peptides as immunogens. The first of these peptides is derived from the predicted amino terminus of the human and rat androgen receptors (anti-N-terminal). The second is derived from the 20 amino acids that comprise the predicted carboxy terminus of the human and rat androgen receptor proteins (anti-C-terminal). The work described below employed both the anti-C-terminal and the anti-N-terminal antibodies.

When the purified anti-C-terminal antibodies are used in Western analysis to examine extracts prepared from COS cells transfected with the human androgen receptor cDNA, a major band is detected at approximately 106 kD that is believed to correspond to the native receptor. The reactivity of the anti-N-terminal antibodies in Western blots is similar to that seen with the anti-C-terminal antibody.

We have examined the genesis of this differential expression by analyzing these cell lines using Western and Northern analysis. When immunoblots were prepared using equal amounts of cell extract prepared from the LNCaP and DU145 cell lines, no immunoreactive proteins were detected in the DU145

cell samples, while a large amount of the 106 kD androgen receptor was visualized in the LNCaP cell extracts. Notably, this experiment was performed using an antipeptide antibody that recognizes an internal segment of the amino terminal portion of the human androgen receptor. This analysis has been further extended to the examination of androgen receptor mRNA. When samples of total RNA prepared from the LNCaP, DU145 and PC3 cell lines were examined by Northern blotting, it was evident that while the LNCaP cell line expresses a large quantity of androgen receptor mRNA, the PC3 and DU145 cell lines express no detectable mRNA.

As the PC3 and DU145 cell lines appear to express little or no androgen receptor mRNA, the question arises as to whether these cell lines have undergone some alteration of the androgen receptor gene that has removed all or part of the gene. This question has been examined by analyzing the androgen receptor gene structure by genomic Southern blotting. This analysis indicates that all eight coding exons of the androgen receptor gene are intact in the DU145 and PC3 prostate carcinoma cell lines. Taken together, these results suggest that the androgen gene may be transcriptionally inactive in these receptor-negative prostate carcinoma cell lines.

ACKNOWLEDGEMENTS

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DISCUSSION

Question from the floor:

Your exon probes for the Southern blot probably wouldn't pick up a mutation that's 5' upstream from it, is that correct?

Dr. McPhaul:

No, we've actually done further experiments, although the data we have presented addresses only the 8' coding exons. In other experiments, we have defined the transcription start site and the 5' to the coding region of the gene. In these experiments we don't see any abnormalities in the DNA specimens that we've examined. But again, as you're well aware, such analyses are very insensitive. There could be a point mutation in the region 5' to the coding segment that might affect the expression of the androgen receptor. Don't forget that we have not even shown that this is transcriptional. We show that there is no RNA and we infer that it may be transcriptional. Theoretically, this result could be due to some alteration of the region 3' to the coding segment that effects the stability of the RNA, and the androgen receptor genes in these cell lines may be actually transcriptionally active.

Dr. Tindall:

Your immunohistochemistry data suggests that the C-terminus antibody recognizes the receptor differently in the presence and absence of steroid. That's one possibility, although there are other possibilities. Have you used sucrose gradient analysis to see if there's a difference in recognition in the presence and absence of androgen?

Dr. McPhaul:

We have not been able to get convincing shifts on sucrose gradients using these antibodies. I am not sure if it's because these are very specific regions of the receptor. I am not sure if it's because those regions are not exposed in the native receptor when it's in a form that we can recognize in sucrose gradient, or if in fact it is something methodological, like our titers are not high enough. I don't know, but at this point we have not been able to produce convincing shifts in our gradients. I wish to point out that even though I presented my single slides here, these peptides have been injected into multiple rabbits. So we're not looking at just one rabbit's response to these peptides. We have a consistent finding as far as the antibodies that are produced in response to these different epitopes.

Dr. Matusik:

I was interested in the first vector system where you showed that you are making the AR receptor and a smaller protein around 64K. Have you done any analysis to see how the smaller protein is being formed?

Dr. McPhaul:

We don't see such bands consistently in cells that express the androgen receptor from the endogenous gene. In cells in which we transfect the receptor cDNA we see it. I didn't go into the data, but we have antibody probes to a number of different portions of the molecule. We think it is due to mRNA splicing. The expression vector that a lot of people have used for the androgen receptor is the CMV vectors. Those don't contain a splice donor, or artificial splice acceptor sites. Paul Berg and that school of thought concluded that such structural motifs were necessary for efficient expression. The people who developed the CMV vector were able to get very high levels of expression of a number of things without those structural entities being present. But I would be willing to bet that when we finally analyze where those are coming from, that they in fact represent an internal splicing within the androgen receptor cDNA using cryptic splice acceptor and splice donor sites. The reason I say this is because when we've analyzed those same extracts with antibody probes directed at different epitopes, they don't simply correspond to amino terminal or C terminal truncations, they appear for example to be missing large loops of internal domain which would imply that part of the middle has been removed. So I don't think they're physiological, I think they're an artifact of the transfection system that we're using.

Dr. Matusik:

I was raising this question because of O'Malley's work on the A & B form of the progesterone receptor, and wondering if you may have seen similar phenomenon here.

Dr. McPhaul:

In that respect though, with the A & B forms, when they looked at their seating, it appeared to be a clear cut explanation as far as this methionine being used or that methionine being used. In this case, if I use amino terminal or C terminal antibodies, you come to the conclusion that these bands are internally deleted. And, as I said, we have never seen it in any of our native expressing.

Dr. Matusik:

I am curious to see that the androgen receptor is so clearly nuclear from your results, and likewise I think more and more that we're accepting the fact that most receptors are nuclear, yet no one seems to be addressing the question of how the steroid gets into the nucleus. Do you have any ideas?

Dr. McPhaul:

No I don't, but given they're so hydrophobic, I would think that an active transport mechanism would not necessarily be required. But I don't have any evidence for any data in that respect.

Dr. Catalona:

On your initial schematic, you showed a testosterone receptor and a dihydrotestosterone receptor and you referred to these sort of generically as the androgen receptor protein. Are they one in the same?

Dr. McPhaul:

Our laboratory, particularly Jean Wilson, has been very interested in this question. There are papers in press (Endocrinology) that examine in detail the binding of the ligand to the receptor. They've come to the conclusion that it may be possible to account for the observed difference by simply a differential affinity of these ligands to the same receptor protein. I think that at this point at least our institution believes that the most important modulators of classic androgen action of those two ligands is mediated through the same receptors.

Question from the floor:

So these two antibodies would be essentially the same receptor and the differential affect of DHT in testosterone is just due to the affinity?

Dr. McPhaul:

Yes. It depends on which system you use, but it appears that the off rate is faster for T than it is for DHT.

Dr. Young:

Can your immunostaining using the anti-androgen receptor antibody raised from androgen receptor-specific peptide be blocked by the peptide?

Dr. McPhaul:

Yes, we've done that and it specifically competes, it obliterates the immunostaining entirely. I just didn't have time to show all of the control slides.

Dr. McPhaul:

I'd like to know whether or not you looked at the ability of other compounds that are not metabolized, like mibolerone or R1881, to induce the TR2 or TR3 gene products in your LNCaP cells?

Dr. Chang:

No. All of our experiment so far are only done with DHT.

Dr. Tindall:

You called TR2 a hormone receptor, but it's still an orphan gene, right, looking for a ligand?

Dr. Chang:

Right.

Dr. Tindall:

Have you tested any anti-androgens or other anti-steroids? There has been much speculation that such receptors exist.

Dr. Chang:

Yes, but the homology (in the hormone-binding domain) between TR2/TR3 receptors and other known steroid receptors (including androgen receptor) is very low. So I suspect that TR2/TR3 may be a new ligand. All major known steroids tested so far did not bind to these two new receptors. Another possibility is these two receptors may have no ligand to bind.

Dr. Tindall:

Also, people have been looking for the cloned dioxin receptor. They find the protein but they haven't got the gene. Have you tested your protein for binding to dioxin?

Dr. Chang:

No. But we don't think that its dioxin receptor because dioxin is very very similar to a glucocorticoid receptor (M.W. 94K) and TR2 receptor protein is only about 67K.

Dr. Chung:

I am curious. Are you suggesting that there is a receptor in the embryonic development in the epithelial compartment that may be lacking steroid binding domain during early development as measured by antibody to detect specifically that receptor?

Dr. Chang:

All I can say at this moment is that our study showed that the fetal epithelium, which had shown no ligand binding activity by autoradiographic analysis, expressed the same amount of the androgen receptor protein (by immunostaining) and its own RNA (by *in situ* hybridization) as the fetal mesenchyme. The results may suggest that androgen receptors are expressed in the fetal epithelium, but it may not be able to bind to androgen until postnatal development.

Dr. Chung:

Have you done *in situ* hybridization?

Dr. Chang:

Yes, *in situ* also. So that's an indicator of mRNA androgen receptor expressed in the epithelium at this stage. But again, we can not show it can bind to DHT.

Dr. Smith:

I'm interested in the function of the TR3 protein. Since the androgen receptors are transcription factor and possibly TR3 is also a transcription factor, have you taken a cell type which contains neither of those and done co-transfection studies with an androgen response element, MMTV-CAT for example, to see whether or not you can get synergy between the two forms? I imagine you could do the same thing with other receptors to see if you could amplify the effect of the receptor with expression of TR3. Have you tried any of those experiment?

Dr. Chang:

No, but this is a very good suggestion. We are about to try to find out a function of TR3 by just injecting anti-sense or antibody-neutralizer.

Dr. Barrack:

In what species did you do the immunochemical staining in the embryonic study?

Dr. Chang:

The mouse in the embryonic study; rat, human, and mouse in the androgen receptor immunochemical staining.

Dr. Barrack:

Why have you given this orphan receptor the name TR. Given the question that Dr. Catalona asked about multiple androgen receptors, i.e., is there a DHT receptor and a T receptor, it could be confusing calling these orphan receptors TR.

Dr. Chang:

In the earlier screening of the androgen receptor cDNA from human testis library, we found several types of receptors and simply named these receptors from testis as TR1 (which turn out to be androgen receptor), TR2 receptor, TR3 receptor.

Dr. McPhaul:

In your embryologic studies, you used your monoclonals. Which epitopes were those directed against? Was that your C-terminal?

Dr. Chang:

Forty percent of the N-terminal domain and 25% of DNA binding domain.

STRUCTURAL ANALYSIS AND GENE EXPRESSION OF TR2 RECEPTOR AND TR3 RECEPTOR

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SUMMARY

We describe the isolation, cloning, and structural characterization of cDNAs encoding TR2 receptor and TR3 receptor and the expression of the receptor proteins. The natural ligands for these nuclear receptor-like proteins are unknown. TR2 receptor exists in four variant forms which differ in the putative ligand-binding domain. The expression of TR2 receptor in rat ventral prostate and TR2 receptor and TR3 receptor in the human prostatic carcinoma cell line LNCaP is discussed.

INTRODUCTION

Nuclear receptors are grouped in a superfamily of gene regulators which function in the control of specific gene activity to regulate growth, development and maintenance of homeostasis (1,2). Receptor proteins are characterized by the linkage of three main structural regions: a variable N-terminal domain, a short, highly conserved cysteine-rich domain and a less well conserved C-terminal domain. While the function of the N-terminal region is not well understood, the central cysteine-rich domain is thought to fold into the so-called "zinc fingers" which participate in the specific binding of the receptor to target DNA hormone response elements. The C-terminal domain functions in the binding of the hormone ligand, and of the 90 k heat shock protein (3) and in receptor dimerization. Because of these common structural features, in particular the highly conserved amino acid sequence of the DNA-binding domain, it is possible to isolate cDNAs encoding proteins which possess these features and assign them to the nuclear receptor superfamily knowing neither the hormone ligand nor the target genes which the "receptor-ligand" complex regulates.

In the process of screening human cDNA libraries for androgen receptor cDNA, we isolated two such cDNAs which encode nuclear receptor-like proteins we have termed "TR2 receptor" and "TR3 receptor" (4-7). TR2 receptor expression was shown to be negatively regulated by androgen in rat ventral prostate (5) and both TR2 receptor and TR3 receptor expression appear to be sensitive to androgen in the prostatic carcinoma cell line LNCaP. These findings suggest that TR2 and TR3 receptors may be important gene regulators in the normal and diseased prostate. Here we summarize the work our laboratory has carried out to characterize TR2 receptor and TR3 receptor structure and expression.

Abbreviations used: cDNA, complementary DNA; AR, GR, PR, ER, MR, receptors for androgen, glucocorticoid, progesterin, estrogen, and mineralcorticoid, respectively; DHT, 5 α -dihydrotestosterone.

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MATERIALS AND METHODS

Materials. Tritiated steroids, ^{32}P and ^{35}S -labeled compounds were purchased from New England Nuclear and Amersham. *In vitro* translation and transcription kits were from Promega. Restriction and other enzymes were from Boehringer Mannheim, New England Biolabs and BRL. The human testis lambda gt10 cDNA library was obtained from Clontech.

cDNA screening and sequencing. cDNA library screening was performed using procedures described previously (4). TR2 and TR3 receptor clones were digested with restriction nucleases and fragments were inserted into M13 vectors for dideoxy sequencing (8) and into pGEM-3Z vectors for *in vitro* transcription and translation.

***In vitro* transcription and translation.** pGEM-3Z plasmids containing TR2 receptor and TR3 receptor cDNAs were linearized and transcribed as described previously (4). Translation was carried out using procedures supplied by the supplier.

Other methods. DNA-cellulose affinity chromatography was carried out as described before (5). Steroid binding assays were performed using the hydroxylapatite filter method (9). RNA isolation and Northern blot analysis was performed as described previously (10) and also using the procedure of Mahmoudi and Lin (11).

RESULTS AND DISCUSSION

cDNA isolation. A human testis lambda gt10 cDNA library was screened with a 5' ^{32}P end-labeled 41-base oligonucleotide probe homologous to a highly conserved portion of the DNA-binding domain common to steroid hormone receptors (4,5,12). The probe was designed for the general selection of clones encoding steroid receptor proteins, some of which we hoped would encode the androgen receptor (AR). cDNAs encoding glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR) were eliminated by screening the collection of clones positive for the 41-base probe with end-labeled 24-base probes specific for each of these receptors. The bulk of the clones which were negative for all of the known steroid receptor probes fell into two classes: AR-type and TR2-type. One clone, which contained a partial sequence of TR3 receptor, comprised a third unknown class. AR-type cDNAs were later confirmed, from steroid binding and immunoprecipitation experiments and from X-chromosome localization, to encode AR. TR2 receptor clones isolated from the human testis library fell into three subclasses: TR2-5, TR2-7 and TR2-9 receptor (5-7). A fourth TR2 subclass, TR2-11 receptor, was isolated from a human prostate lambda gt10 cDNA library (7). The TR3 receptor sequence was used to screen the same human prostate library to obtain full-length clones.

TR2 receptor and TR3 receptor structural characterization. The four TR2 receptor variants have identical N-terminal regions but differ in the C-terminal putative ligand-binding domain. Figure 1 shows a schematic comparing the variant forms of TR2 receptor. The cDNA for TR2-5 was 2029 bp in length with an open reading frame capable of encoding a protein of 483 amino acids with a calculated molecular weight

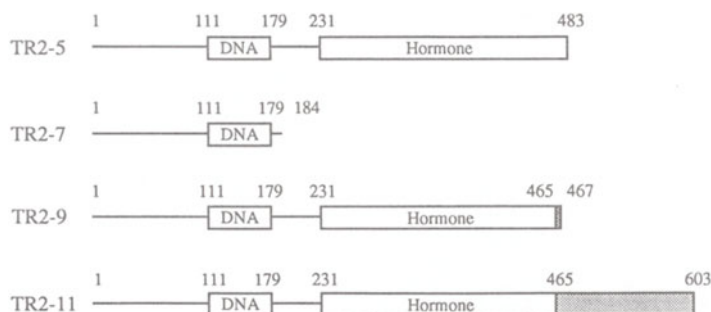


Figure 1: TR2 receptor variants. Numbers indicate amino acid position. The putative DNA- and androgen-binding domains are boxed.

of 53 k. The cDNA for TR2-7 contained a 429 bp insertion between nucleotides 669 and 670, which shortened the open reading frame to 184 amino acids because of the presence of a termination codon near the beginning of the insert. The TR2-7 receptor gene product has a calculated molecular weight of 21 k. The cDNA for TR2-9 was 1785 bp in length and had an open reading frame encoding a protein of 467 amino acids with a calculated molecular weight of 51 k. The three C-terminal amino acids of TR2-9 receptor are different from the corresponding positions in TR2-5 receptor and the TR2-9 sequence has 16 fewer amino acids than the TR2-5 sequence, because a 244 bp deletion in the TR2-5 receptor sequence occurs between nucleotides 1518 and 1763. (Or, conversely, a 244 bp insertion occurs in the TR2-9 receptor clone to give rise to TR2-5 receptor.) A termination codon is present three positions after the deletion/insertion site. The cDNA for TR2-11 was 2221 bp in length and had an open reading frame encoding a protein of 603 amino acids with a calculated molecular weight of 67 k. TR2-11 receptor, therefore, has 120 more amino acids in the putative ligand-binding domain than does TR2-5 receptor, and amino acid residues are different from amino acid position 465 on, the same position in the TR2-5 receptor sequence that the deletion occurs to give the TR2-9 sequence.

The cDNA for TR3 receptor was 2473 bp in length and had an open reading frame capable of encoding a protein with 598 amino acids with a calculated molecular weight of 64 k. The typical eukaryotic polyadenylation signal AATAAA was located upstream of the poly(A) tail on the TR3 receptor sequence and all four TR2 receptor variant forms.

The deduced amino acid sequences of the DNA-binding domains of TR2 and TR3 receptor show a high degree of homology with the DNA-binding domains of other known members of the steroid receptor

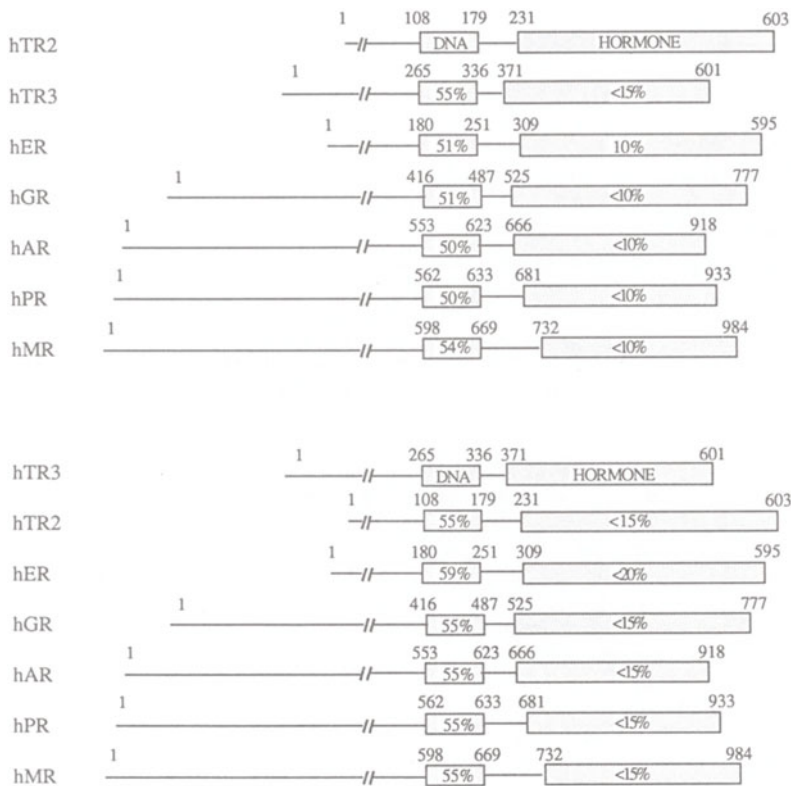


Figure 2: Amino acid homology between TR2 receptor, TR3 receptor and steroid receptors in the putative DNA- and androgen-binding domains (boxed). Numbers indicate amino acid position.

super-family. The positions of 26 amino acids (9 Cys, 3 Lys, 3 Arg, 2 Try, 2 Gly, 2 Phe, 1 Met, 1 Asp, 1 His, 1 Ala, 1 Ser) are completely conserved among TR2 and TR3 receptors and all other known steroid receptors. The homology in DNA-binding domain amino acid sequence between TR2 and TR3 receptor and other steroid receptors ranges from about 50 to 60% (Fig 2). The amino acid sequences of the putative ligand-binding domains of TR2 and TR3 receptor, the roughly 200-250 amino acids at the C-terminal (1,2), show low homology (< 20%) with each other or with the hormone-binding domains of other known nuclear receptors. TR3 receptor, however, shares considerable homology with NUR/77, a cDNA derived from mRNA of serum-stimulated mouse BALB/c 3T3 cells (13). Disregarding three extra serines in NUR/77, TR3 receptor and NUR/77 show 86% nucleotide and 91% amino acid homology, suggesting that TR3 receptor may represent the human homologue of the mouse protein. A cDNA for a similar protein, called NGFI-B, has been isolated from a rat pheochromocytoma PC12 cell cDNA library (14). Expression of NGFI-B mRNA is induced by nerve growth factor.

A common feature of the hormone-binding domains of nuclear receptors is high methionine content. Interestingly, TR2 receptor shows a high methionine content and TR3 receptor does not. TR2-5 receptor has 11 methionine residues in the putative ligand-binding domain, while TR2-11 receptor, with its extended ligand-binding domain, has 17 methionine residues in this region. TR3 receptors has only 1 methionine residue in its entire ligand-binding domain.

***In vitro* expression, DNA- and ligand-binding.** mRNAs produced from *in vitro* transcribed TR2 and TR3 receptor cDNAs were translated in a rabbit reticulocyte lysate reaction. Translation products labeled with [³⁵S] methionine had molecular weights, after SDS-polyacrylamide electrophoresis, that matched the weights deduced from the nucleotide sequences.

Translation products labeled with [³⁵S] methionine were passed over calf thymus DNA-cellulose columns to determine if TR2 and TR3 receptors could bind heterologous DNA. Both TR2 receptor (TR2-5, -7, and -11 receptor; TR2-9 receptor was not tested) and TR3 receptor bound to DNA in low salt (TE) and were eluted with high salt (0.5 M KCl). Neither bound to cellulose not conjugated with DNA.

Unlabeled TR2 and TR3 receptor translation products were incubated with tritiated steroids in the androgen, estrogen, progesterin and glucocorticoid steroid classes. No specific binding was detected. The low homology of the putative ligand-binding domains of TR2 receptor and TR3 receptor with other known receptors may indicate that the unknown TR2 receptor and TR3 receptor ligands are very different in structure from the steroid and non-steroid ligands of the known nuclear receptors.

***In vivo* expression.** Northern blot and dot hybridization analysis of TR2 receptor tissue distribution in the rat revealed that TR2 receptor mRNA was most abundant in the androgen-sensitive male reproductive organs (5). With ventral prostate as 100%, and using TR2-5 receptor DNA as probe, the following relative amounts of TR2 receptor message were found: seminal vesical, 92%; testis, 42%; submaxillary gland, 18%; liver, 13%; kidney, <1%; uterus, <1%. TR2 receptor mRNA levels in rat ventral prostate were found to be negatively regulated by androgen. TR2 receptor mRNA increased about two fold over normal two days after castration; the increased level could be reduced to normal by 5 α -dihydrotestosterone (DHT) injection. In addition, injection of the antiandrogen flutamide into intact rats increased TR2 receptor mRNA levels up to about two and one half-fold over normal. It might be added here that rat TR2 receptor cDNAs have been isolated in our laboratory from a rat ventral prostate library and show great homology to the human cDNAs.

Using TR2 receptor and TR3 receptor cDNA as probes, TR2 receptor and TR3 receptor mRNAs were detected in total RNA isolated from LNCaP cell cultures. LNCaP is an androgen-dependent human prostatic carcinoma cell line originally isolated from a lymph node metastatic lesion (15,16). Using the 1.6 Kb Eco RI restriction fragment of TR2-11 receptor as a probe, two 2.5-3.0 Kb bands were visualized. To determine if the two bands represented two variant forms of TR2 receptor which are coexpressed in LNCaP cells, RNA blots were probed with a TR2-5 (TR2-7 and 9 receptor also) -specific 0.4 Kb Sac I-Eco RI fragment from TR2-5 receptor cDNA and with a TR2-11-specific 0.6 Kb Eco RI-Eco RI fragment from TR2-11 receptor cDNA. Both bands were positive for the TR2-11-specific probe and negative for the TR2-5-specific probe, indicating that TR2-11 receptor is the sole variant TR2 receptor form expressed in LNCaP cells (Fig 3). This observation and the fact that TR2-5, 7 and 9 receptor cDNAs were isolated from a testis library and TR2-11 receptor cDNAs were isolated from a prostate library suggest that variant TR2 receptor expression may exhibit a certain degree of tissue specificity. Assuming that the variant TR2-5, 7 and 9 receptor forms are all, in fact, expressed forms and not just cloning or splicing artifacts, the coexpression of the three forms in the testis brings up the possibility of competition between the receptor forms for ligand

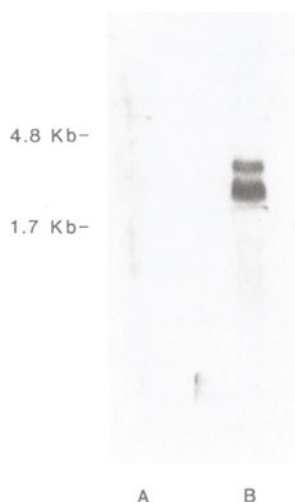


Figure 3: Northern blot analysis of total RNA from LNCaP cells. 25 ug of total RNA was separated on a formaldehyde agarose gel. After blotting, filters were hybridized with ³²P-labeled TR2-5 receptorspecific 0.4 Kb Sac I-Eco RI fragment (lane A) or ³²P-labeled TR211 receptor-specific 0.6 Kb Eco RI-Eco RI fragment (lane B).

binding. The different receptor forms may bind different natural ligands or may bind the same ligand with different affinity; the activation of target genes could be therefore regulated by control of the expression ratio of the variant receptor forms.

Preliminary data from our laboratory show that DHT treatment of LNCaP cells repressed the level of TR2 receptor mRNA, consistent with the effect of androgen on TR2 receptor expression in rat ventral prostate (5). Androgen, however, had an opposite effect with TR3 receptor and appeared to elevate TR3 receptor expression in LNCaP cells.

Serum growth factors similarly induced TR3 receptor expression in LNCaP cells. Cells were incubated in low serum (0.5%) for three days prior to the addition of medium with high serum (10%). Cells were then incubated in the high serum medium for increasing lengths of time. TR3 receptor expression is induced to a maximal level 30 minutes after the addition of high serum medium. A similar rapid increase in NUR/77 transcription was observed after the addition of fetal calf serum, platelet derived growth factor or fibroblast growth factor to quiescent mouse cells (17). NUR/77 cDNA was isolated along with several other cDNAs by virtue of induction by serum growth factors (18).

TR2 receptor and TR3 receptor are nuclear receptor-like proteins which may function with androgen and autocrine growth factors in the regulation of gene activity in the prostate and perhaps other male reproductive organs. TR2 receptor and TR3 receptor expression is high in the prostate and is sensitive to factors which are known to regulate prostate growth and cell division. Although the natural ligands of TR2 receptor and TR3 receptor are not yet known, the two proteins are nevertheless potentially important. Work is currently underway in our laboratory to identify the natural ligands through the use of chimeric constructions of the androgen receptor DNA-binding domain with TR2 receptor and TR3 receptor ligand-binding domains. Such chimeric receptors, in the presence of the unknown ligand, should be able to activate reporter gene transcription under the control of the androgen-sensitive mouse mammary tumor virus hormone responsive element (19). A similar approach was taken in the identification of retinoic acid as the ligand for the retinoic acid receptor. (20,21). In addition, monoclonal antibodies against TR2 receptor and TR3 receptor are being prepared. Such antibodies should be useful in the subcellular localization of the receptor proteins, in the determination of tissue distribution, in the isolation of the ligand molecules through affinity purification of receptor-ligand complexes, and in the analysis of receptor expression during development. An understanding of how TR2 receptor and TR3 receptor function in the development and maintenance of the prostate may lead to a better understanding of the conditions which lead to prostatic hypertrophy and malignancy.

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cDNA CLONING, ANTIBODY PRODUCTION AND IMMUNOHISTOCHEMICAL LOCALIZATION OF THE ANDROGEN RECEPTOR

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SUMMARY

We describe the isolation and cloning of cDNAs encoding human and rat androgen receptors (ARs) and characterization of AR proteins expressed from the cDNAs. In addition we describe the production of *tpE*-AR fusion proteins and their use as antigens in the production of polyclonal and monoclonal anti-AR antibodies. Immunohistochemical staining of mouse, rat and human tissues revealed widespread tissue distribution of AR and subcellular localization of AR in the cell nucleus.

INTRODUCTION

Androgens are essential for normal male sexual differentiation, development and function. Androgen effects are mediated through the androgen receptor (AR), a protein which belongs to a superfamily of *trans*-acting nuclear receptors which modulate gene transcription through high affinity and specific binding to hormones and DNA (1-3). Mutations in the AR gene are thought to cause the abnormalities associated with various forms of the androgen insensitivity syndrome (4,5). Until the recent isolation of cDNAs encoding androgen receptors (6-9), it was difficult to obtain sufficient quantities of the protein for structural and functional characterization, or to provide antigen for the production of anti-AR antibodies. With the availability of *in vitro* synthesized AR, bacterial AR fusion proteins, and anti-AR antibodies, much has been learned about AR function and expression. Here we summarize our recent work in AR cDNA cloning, AR characterization, antibody production, and immunohistochemical staining.

MATERIALS AND METHODS

Materials

Tritiated steroids,³²P and ³⁵S-labeled compounds were purchased from New England Nuclear and Amersham. *In vitro* translation and transcription kits were obtained from Promega. Restriction and other enzymes were from Boehringer Mannheim, New England Biolabs and BRL. The human testis lambda gt11 cDNA library was obtained from Clontech. Crl:CD-1 (ICR) mice were purchased from Charles River, Inc., and Sprague-Dawley rats were from Taconic. pATH vectors were provided by A. Tzagaloff. pSVL and pMSG-CAT vectors were from Pharmacia.

cDNA Screening and Sequencing

cDNA library screening was performed using procedures described previously (6). hAR and rAR clones were digested with restriction nucleases and fragments were inserted into M13 vectors for dideoxy sequencing (10) and into pGEM-3Z vectors for *in vitro* transcription and translation.

In Vitro Transcription and Translation

pGEM-3Z plasmids containing hAR and rAR cDNAs were linearized and transcribed as described previously (6). Translation was carried out using procedures supplied by the supplier.

AR Fusion Protein Production

E. coli *trpE*-AR gene fusions in the expression vector pATH were constructed for the purpose of making *trpE*-AR fusion protein antigens in large quantity (11). The fusion proteins were also insoluble and therefore could be easily isolated. Three fusion proteins, designated A, B, and C, were made using three overlapping segments of hAR and rAR cDNAs (Fig. 1). For the production of fusion protein A, the 726 bp *Sac* I fragment of hAR was ligated to *Sac* I-digested pATH 10. For the production of fusion protein B, the 835 bp *Sau* 3A fragment of rAR was ligated to *Bam* HI-digested pATH 1. For the production of fusion protein C, the 435 bp *Eco* RI-Pst I fragment of rAR was ligated to *Eco* RI/Pst I-digested pATH 11.

Vectors containing the AR sequences were transfected into *E. coli* RRI, and transformed clones were screened for the desired constructs by restriction mapping. Transformed *E. coli* clones were amplified in supplemented M9 medium and *trpE* transcription was induced with 3 β -indoleacrylic acid (12). Cells were lysed and insoluble fusion protein was collected by centrifugation, solubilized by heating in SDS and purified by SDS-PAGE.

Antibody Production

Polyacrylamide gel pieces containing the fusion proteins or the electroeluted proteins were emulsified with Freund's complete adjuvant and were injected intradermally in male Lewis rats and female New Zealand white rabbits. Animals were given biweekly intradermal boosts using the incomplete adjuvant. For the final boost, 100 μ g of fusion protein was electroeluted from excised gel pieces and injected intravenously in saline. Rat serum was tested for the presence of antibody by peroxidase-linked immunoassay using fusion protein as antigen. Positive serum was tested by a double antibody precipitation method using [³H]DMNTreceptor complexes from rat ventral prostate (13). For cloning monoclonal antibody-producing hybridomas, spleen cells from serum-positive rats were fused with SP2/0 mouse myeloma cells in the presence of 50% polyethylene glycol in DMEM. Hybridomas were selected in medium containing hypoxanthine, aminopterin and thymidine. Cells from positive wells were cloned by limiting dilution and media from culture wells were screened for the presence of anti-AR antibodies. Monoclonal antibodies were precipitated from medium from large scale cultures with ammonium sulfate and were purified by affinity chromatography with agaroselinked anti-rat IgG antibodies. Purified IgG fractions showed only two bands representing heavy and light chain IgG peptides after SDS-PAGE. Polyclonal antibodies were similarly purified by passage over agarose-linked anti-rabbit IgG.

Staining Procedure

Rat tissues were stained with polyclonal anti-AR antibodies raised in rabbits and mouse and human tissues were stained with monoclonal anti-AR antibodies (clone ANI-15) For polyclonal staining, frozen

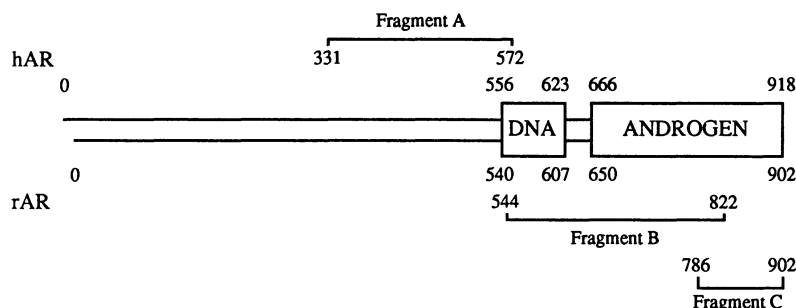


Figure 1: Location of hAR and rAR fragments present in *trpE* fusion proteins A, B and C. The numbers indicate hAR or rAR amino acid positions. The putative DNA- and androgen-binding domains are boxed.

embedded tissues were cut at 5 μ m thickness, mounted on slides and fixed with paraformaldehyde and picric acid. The sections were then stained using the peroxidase-streptavidin-biotin method (14) using polyclonal anti-AR as first antibody and biotinylated goat anti-rabbit IgG as second antibody. For monoclonal staining, frozen sections were fixed and stained with monoclonal anti-AR antibody as first antibody and biotinylated rabbit anti-rat IgG as second antibody. Sections were then incubated with peroxidase-streptavidin. As negative controls, tissue sections were incubated with preimmune serum and AR fusion protein-absorbed immune serum.

Other Methods

Steroid binding assays were performed using the hydroxylapatite filter method (15). Sucrose gradient centrifugation was carried out as described previously (11). Chloramphenicol acetyl transferase assays were performed according to Gorman (16).

RESULTS AND DISCUSSION

cDNA Isolation

Human testis and rat ventral prostate lambda gtl1 cDNA libraries were screened with a 5' ^{32} P end-labeled 41-base oligonucleotide (6) homologous to nucleotide sequences in the highly conserved DNA-binding domain of the glucocorticoid receptor (GR) (17,18), progesterone receptor (PR) (19-21), and estrogen receptor (ER) (22,23). Clones encoding known steroid receptors such as GR, PR, and mineralocorticoid receptor (MR) were eliminated by screening clones positive for the 41-base probe with 24 base probes specific for each of these receptor sequences. The remaining clones fell into two major classes which were designated TR2-type and AR-type. TR2-type clones were about 2.1 kb in length and were considered too short to code for full-length AR. The AR-type clones were partial sequences which overlapped to produce initially a 2.7 kb hAR cDNA (6) and, after further screening, a 3.7 kb cDNA sequence encoding the full-length protein (7). The rAR clones overlapped to produce a 3.2 kb cDNA sequence encoding the full-length rAR. The hAR and rAR cDNA open reading frames were capable of encoding proteins of 918 and 902 amino acids, respectively, with a calculated molecular weight of about 98 k. Translation starting at the second ATG produced a protein with a calculated molecular weight of 79 k. (The molecular weights were initially reported as 94 and 76 k due to an error in the computer software used to calculate peptide molecular weights from deduced amino acid sequences.)

Receptor Characterization

After insertion of hAR and rAR sequences into the pGEM-3Z expression vector, mRNA was transcribed using T7 RNA polymerase and the mRNA was translated in a rabbit reticulocyte lysate reaction. The molecular weights of [^{35} S] methionine-labeled translation products separated by SDS-PAGE closely matched the weights calculated from the deduced amino acid sequences. In addition to the major 98 k bands, 79 k and smaller molecular weight bands were present, due either from translation initiation at internal ATG codons or from proteolytic degradation (7).

In vitro translated proteins were used in a steroid binding competition assay to ascertain binding affinity and specificity (15). After incubation of translation products with [^{3} H]R1881, a potent synthetic androgen, unlabeled competing R1881 and other androgens and non-androgenic steroids were added. The results showed that the *in vitro* translated proteins bound androgens with high affinity and specificity. From Scatchard analysis (24), the apparent R1881 dissociation constant was 0.3 nM and 0.5 nM for hAR and rAR, respectively, in agreement with the affinity constant obtained by others (8). The identification of the cDNAs as coding for androgen receptor was also confirmed by localization to the X chromosome, by the specific precipitation of *in vitro*-translated ARs and by anti-AR autoimmune antibodies (13). Incubation with autoimmune serum also shifted the sedimentation coefficient of labeled ARs in a sucrose gradient from 4S to 9S.

The 3.4 Kb EcoRI-EcoRI fragment of hAR was inserted into the eukaryotic expression vector pSVL for hAR expression in cultured mouse L cells. hAR-pSVL was co-transfected with pMSG-CAT, a plasmid having the chloramphenicol acetyl transferase (CAT) gene under the control of the mouse mammary tumor virus (MMTV) long terminal repeat. The MMTV sequence contains a hormone response element to which androgen-AR, as well as hormone-GR, -PR and -MR complexes, bind and thus activate CAT transcription (25-28). Treating cells transfected with hAR-pSVL and pMSG-CAT with as low as 1 x

10⁻⁹ M 5 α -dihydrotestosterone (DHT) induced CAT activity (Fig. 2), indicating that the cloned hAR gene expresses a receptor capable of transcriptional activation.

AR Fusion Proteins and Anti-AR Antibodies

AR-*tpE* fusion protein A (62 k) contained a 242 amino acid sequence in the hAR N-terminal domain extending 16 amino acid residues into the DNA-binding domain (Fig. 1). Fusion protein B (70 k) contained a 279 amino acid sequence spanning the bulk of the rAR DNA-binding and hormone-binding domains. Fusion protein C (47 k) contained the 117 C-terminal amino acids of rAR. The parent *tpE* peptide (35 k) consisted of the 323 N-terminal *tpE* amino acids followed by a short multicloning site linker sequence. Of the three fusion proteins produced, fusion protein A, containing mainly N-terminal domain amino acids, was the most antigenic. Three cell lines (H-ANI-6, H-ANI-7, and H-ANI-15) producing monoclonal antibodies against fusion protein A were obtained (11). One cell line producing monoclonal antibodies against fusion protein B was obtained (H-ANI-21). Sera from rats and rabbits immunized against fusion protein C contained no measurable anti-AR antibody activity.

The four monoclonal anti-AR antibodies were specific for androgen receptor. Double antibody immunoprecipitation assays using as antigens other tritiated hormone-receptor complexes, including glucocorticoid receptor from rat liver, estrogen receptor from human breast tumor MCF-7 cells, and progesterone receptor from human breast tumor T-47D cells, showed that the monoclonal anti-AR antibodies precipitated only [³H]DMNT-AR complexes (11). Monoclonal anti-AR antibody also shifted the sedimentation coefficient of [³H]DMNT-AR complexes during gradient centrifugation.

AR Subcellular Localization and Tissue Expression

A wide range of normal rat and mouse tissues and organs as well as tissue samples of human prostatic carcinoma and benign prostatic hyperplasia were examined for AR staining (14). Polyclonal anti-AR antibodies gave the same pattern of staining in rat tissues as the monoclonal anti-AR antibodies did in mouse and human tissues. In all the tissues examined, positive staining was noted exclusively in cell nuclei and not in cytoplasm, in agreement with other published findings (29,30) and consistent with estrogen and progesterone receptor subcellular localization (31). Monoclonal antibodies prepared against fusion protein A (ANI-15) containing the 5' segment of hAR (Fig. 1), which shares 77% homology with the analogous rAR segment (11), cross-reacted with mouse and rat AR present in the tissue sections.

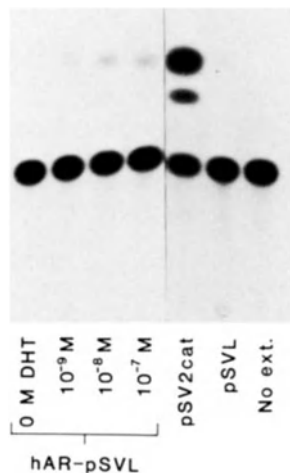


Figure 2: CAT activity induced by increasing concentrations of DHT. 24 hours after transfection of vectors, the appropriate amounts of DHT were added and cells were incubated a further 24 hours. Cells were harvested and 60 μ g of cytosolic protein were assayed for CAT activity (16). pSV2cat was transfected alone and represents a positive control. Parent vector pSVL-transfected cells received 1 x 10⁻⁷M DHT. No ext. = no protein extract added to assay tube.

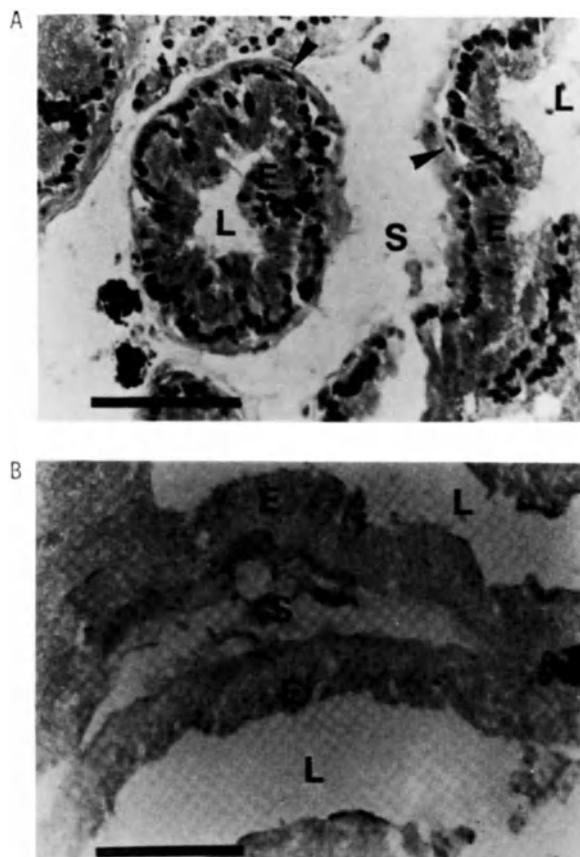


Figure 3: A. Immunohistochemical localization of AR in rat prostate stained with the polyclonal anti-AR antibody. AR is present in most of the epithelial cells as indicated by the diaminobenzidine reaction product within cell nuclei. A few stromal cells surrounding the epithelium also show positive staining (arrows). B. Rat prostate stained with the control serum. The polyclonal antibody absorbed with antigen (AR fusion protein) was used as the control serum. No nuclear staining is observed. L, lumen; E, epithelium; S, stroma. Bar = 100 μ m

Cells in the male reproductive organs, particularly secretory epithelial cells, were in general stained very strongly. In the prostate, the nuclei of epithelial cells lining the lumen showed intense positive reaction, although a few stromal cell nuclei were also stained (Fig. 3). In the seminal vesicle, epithelial cells exhibited nuclear staining and again a few stromal cells showed a positive reaction. The smooth muscle layer surrounding the seminal vesicle also showed a strong reaction. Glandular epithelial cells and stromal cells of the coagulating gland displayed less intense staining. The epithelium of distal and proximal ends of the epididymis showed strong AR-staining, while the central region showed weaker staining. All cell types of the testis, with the exception of spermatogenic cells, stained positively for AR, but the reaction was not as strong as in the other male reproductive tissues.

Female reproductive organs showed weak but significant AR-staining. AR-positive cells were found in the epithelium and stroma of the vagina, the myometrium and stroma of the uterus and granulosa luteal cells of the ovary.

Of all the non-reproductive organs examined, only spleen showed no detectable AR-staining. Liver hepatocytes showed weak positive staining which was weaker in female tissue than in male. While the adrenal medulla was negative, the adrenal cortex had some positively staining cells. Cells in the zona fasciculata and zona reticularis exhibited AR-staining while cells in the zona glomerulosa were stained poorly or not at all. In the kidney, epithelial cells of the convoluted tubules and parietal cells of Bowman's capsule had a positive reaction for AR. The nuclei of nearly every skeletal muscle cell examined stained positively for AR, while most nuclei in cardiac muscle were positive. Female muscle tissue was stained only slightly less than male tissue. Cells of the anterior pituitary gland showed variable staining, and cells of the intermediate part, called the pars intermedia, were weakly stained. Some pituicytes in the posterior pituitary gland showed a positive reaction for AR. No differences were detected between male and female pituitary glands. AR-positive cells were found in the Purkinje cell and granule cell layers of rat cerebellum.

Monoclonal anti-AR antibody was used to stain cells from benign prostatic hyperplasia and prostatic carcinoma. Epithelial cell nuclei stained positively for AR while stromal cells exhibited little or no nuclear staining. The function of AR in prostatic carcinoma cells is of clinical interest because tumor growth which is initially androgen-dependent usually becomes androgen-independent over time (32).

CONCLUSION

Human and rat AR cDNAs were isolated by screening for the highly conserved DNA-binding domain common to members of the nuclear receptor superfamily. The cloning and characterization of human and rat AR cDNAs made feasible the purification of large quantities of AR fusion proteins for use as antigens in the production of polyclonal and monoclonal anti-AR antibodies. These antibodies have proven useful in our initial studies of AR subcellular localization and distribution in mouse, rat and human tissues. Presently a more extensive immunohistochemical survey of human prostatic carcinomas and benign prostatic hypertrophies is being carried out in our laboratory. The use of monoclonal antiAR antibodies will undoubtedly facilitate the investigation of such areas as AR expression and function during early sexual differentiation and involvement of AR in male-specific disorders.

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NEW APPROACHES TO STUDIES ON THE ANDROGEN RECEPTOR

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INTRODUCTION

Recent advances in studies on the androgen receptor (AR) have led to the application of new technology to the question of receptor status in prostate disease. Critical to these advances has been the cloning of AR complementary DNA (cDNA) and genomic DNA, the determination of AR gene intron/exon boundary DNA sequences and the development of antibodies that recognize AR. Receptor analysis in prostate disease previously relied on biochemical assays of androgen binding activity and autoradiographic localization (1). With specific antibodies, AR expression can be determined at the cellular level independent of hormonal status. At the DNA level, the polymerase chain reaction (PCR), a powerful DNA amplification technique, can be used to identify mutations associated with androgen resistance in prostate disease. PCR technology is being applied to the study of androgen insensitivity (2,3) and has made possible the identification of natural mutations which can serve as guides in determining structure/function relationships of the AR.

Brief review of AR cDNA structure

DNA sequence analysis reveals that the AR is a member of the nuclear receptor family and shares sequence homology with the progesterone, glucocorticoid and mineralocorticoid receptors (Fig. 1). It was this high degree of homology within the DNA binding domain that made possible the initial cloning of AR, both in our laboratory (4-6) and that of others (7-11). AR sequence shows little homology with the estrogen or vitamin D receptors (Fig. 1). The most divergent region of all steroid receptors is the N-terminal sequence which is thought to be involved in transcriptional regulation. Comparison of rat and human AR amino acid sequence reveals an identical steroid and DNA binding domain (5). There are repeats of single amino acids within the N terminal region which are striking, differ between the species, and vary in the human AR sequence (5,8,10).

Immunochemical analysis of AR

Purification of AR sufficient to raise antibodies has remained a difficult task. However, this problem is somewhat circumvented by deriving primary AR amino acid sequence from the cDNA. Synthetic peptides with sequence corresponding to AR are effective immunogens in raising antibodies that selectively recognize

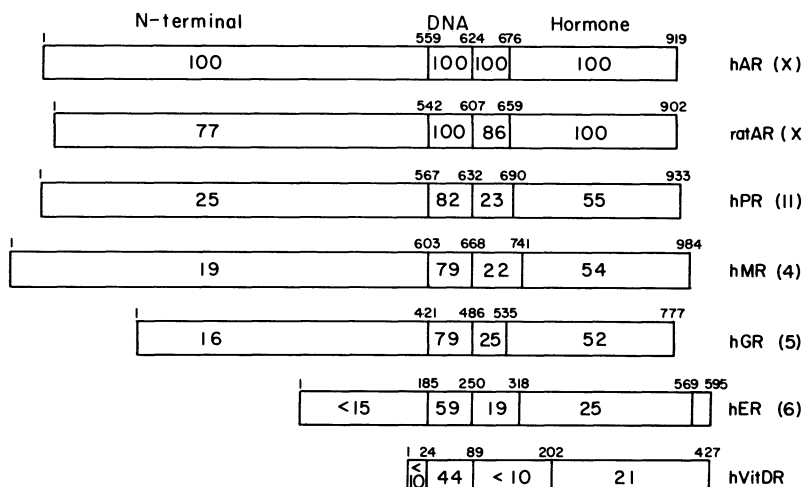


Figure 1: Comparison of human AR deduced amino acid sequence with that of other members of the nuclear receptor family. The percent sequence similarities are shown within the boxes for human receptors for progesterone, mineralocorticoid, glucocorticoid, estrogen, and vitamin D (5). Amino acid residue numbers are indicated above and chromosome location in parentheses on the right.

both denatured and native AR (6). A peptide with sequence just 5' of the DNA binding domain, shown in Fig. 2, has 15 amino acids of AR sequence followed by a three amino acid spacer to the coupling protein, Keyhole limpet hemocyanin. High titer antisera were obtained from rabbits immunized with the peptide. As shown in Fig. 3, the antibody recognizes native AR such that increasing amounts of immune IgG causes receptor sedimentation to increase from 4.5S to approximately 7 and 10S, depending on the antibody concentration. We have shown previously that this antibody recognizes AR immunocytochemically and localizes receptor to nuclei of rat prostate (6) and other reproductive tissues. The technique of western blot analysis has revealed an AR molecular weight of about 114,000 for the native receptor (12).

The peptide sequence used in antibody production (Fig. 2) is identical in rat and human AR. The antibody is effective in immunocytochemical localization of AR in human prostate as shown in Fig. 4. Sections of human prostate tissue obtained by radical prostatectomy contained regions of benign prostatic hyperplasia (Fig. 4A) and adenocarcinoma (Fig. 4B). Strong nuclear immunostaining was detected in acinar epithelial cells of glandular benign prostatic hyperplastic tissue, with weaker immunostaining in nuclei of stromal cells (Fig. 4A). The arrangement of the acinar gland was characteristic of normal prostate histology. In contrast, a region of human prostatic cancer showed a breakdown of the acinar structure with heterogeneous immunostaining of epithelial cell nuclei (Fig. 4B). The larger neoplastic epithelial cells showed weak or no immunostaining, whereas the more normal shaped epithelial cells have intense staining like that seen in benign prostatic hyperplasia. Immunocytochemical analysis of AR should prove useful in the therapeutic evaluation of prostate cancer and in the design of strategies for management.

More recently, additional AR synthetic peptides of the N-terminal and carboxyl-terminal regions have been prepared for antibody production. In western blot analysis, they show reactivity with a common 114,000 molecular weight band. The availability of additional antibodies makes it possible to distinguish true AR species from cross-reacting proteins which share a common epitope. A battery of antibodies to short synthetic peptides from different regions of AR is valuable in identifying truncated AR proteins that might result from naturally occurring mutant genes or from alternate gene splicing events. Multiple AR antibodies will allow the development of a "sandwich" ELISA technique useful as a quantitative companion to immunohistochemical analysis.

Northern blot hybridization of AR messenger RNA

The cloning of AR cDNA makes possible the detection of AR mRNA by northern blot hybridization analysis. AR mRNA is approximately 10 and 7 kb in human prostate (5), while only the 10 kb species is detected in tissues of the rat (6). In human prostate specimens which display low to undetectable levels of

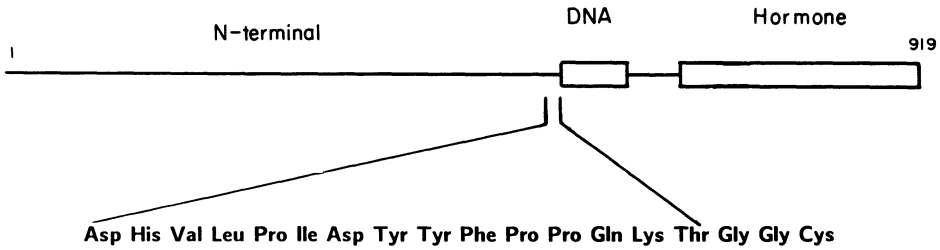


Figure 2: A synthetic peptide used in production of antibodies that react with AR. An 18 amino acid immunogen was prepared with sequence derived from the deduced AR amino acid sequence just 5' of the DNA binding domain was coupled to Keyhole limpet hemocyanin through a three amino acid spacer as previously described (6). Computer analysis in the MicroGene data base indicated that this sequence was unique to AR. Two New Zealand White adult female rabbits were immunized by intradermal injection of a suspension containing the coupled peptide, the free peptide and complete Freund's adjuvant. ELISA titers of polyclonal antisera produced in rabbits were high after the first month of immunization. The position of the synthetic peptide sequence is shown relative to the three functional domains of the AR. The human AR is 919 amino acids in length.

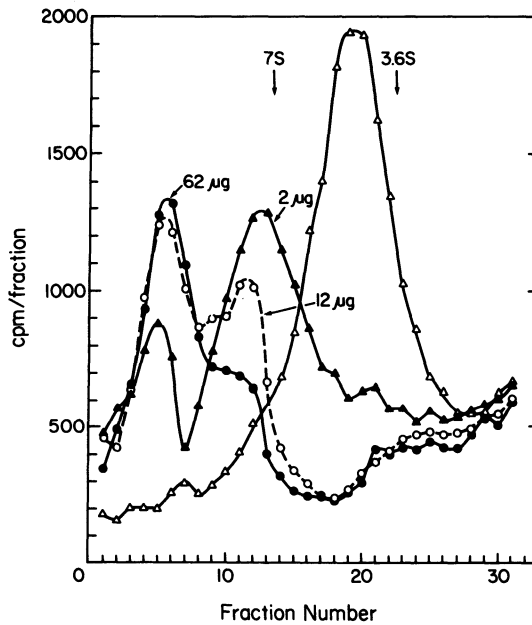


Figure 3: Sucrose gradient analysis of AR following incubation with polyclonal antibody raised to a synthetic peptide. A Dunning tumor (R3327G) AR fraction prepared by $(\text{NH}_4)_2\text{SO}_4$ fractionation was labeled with $[^3\text{H}]$ dihydrotestosterone (15 nM) and incubated for 3 hr at 0°C without () or with increasing amounts of immune IgG: 2 μg (), 12 μg (), 62 μg (). The IgG fraction was obtained by chromatography on protein A-agarose (6). Sucrose gradients were linear 2-20% sucrose in 10% glycerol, 5 mM mercaptoethanol, 0.15 M KCl and 50 mM Tris, pH 7.2. Arrows indicate the sedimentation migration of ovalbumin (3.6S) and bovine gamma globulin (7S).

AR protein, northern blot analysis may reveal defects in AR gene expression that could arise from altered transcription or mRNA translation. Certain receptor gene mutations might result in a change in size or concentration of the mRNA, perhaps due to deletion of part of the gene, an altered rate of transcription, or a change in RNA processing. A low level of mRNA might result from a mutation that increases the mRNA degradation rate. Estrogen resistance was recently reported in a human breast cancer cell line that expresses a mutant estrogen receptor (13). Variant estrogen receptor mRNAs are also reported in human breast tumors (14-16).

Studies in the rat have shown that AR mRNA is down regulated by androgen (17). Within 24 hr of androgen withdrawal by castration, a 2-5 fold increase in the level of AR mRNA is observed. Treatment of the castrated rat with testosterone propionate suppresses the level of AR mRNA to that below the untreated control rat. A metastatic human prostate cancer cell line, LNCaP, shows a similar response; removal of serum from the medium causes a 10 fold increase in AR mRNA in the LNCaP cell line, while androgen supplementation suppresses AR mRNA (17). Interestingly, cyproterone acetate, an antiandrogen, had an agonist-like effect in these cells by also down regulating AR mRNA (17). Thus, both in the rat and human prostate, androgen acts to suppress the level of AR mRNA. Analysis of autologous down regulation in prostate cancer cells in vitro may reflect its state of hormone responsiveness.

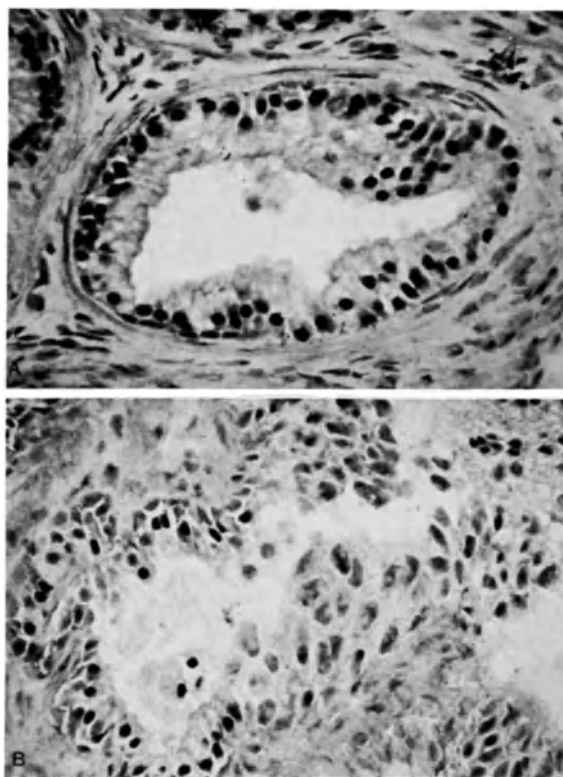


Figure 4: Immunocytochemical localization of AR in human benign prostatic hyperplasia and prostate cancer. Tissue was obtained by radical prostatectomy from a patient diagnosed as having prostate adenocarcinoma, Gleason's score 2+3. The tumor of about 2 cm in diameter was contained to the prostate gland but there was evidence of lymph node metastases. Frozen sections (10 μ m) of the tumor were fixed with 4% paraformaldehyde, pH 7.4, incubated with the IgG fraction of androgen receptor antibody (10 μ g/ml), and stained by the avidin-biotin-peroxidase method (33). Rabbit polyclonal antibody, AR1-52, was raised against a conjugate of the synthetic peptide shown in Fig. 2 and the IgG fraction was purified (6). Shown are sections of benign prostatic hyperplasia (A) and adenocarcinoma (B). Tissue sections were taken from different regions of the same prostate gland. Magnification, X460.

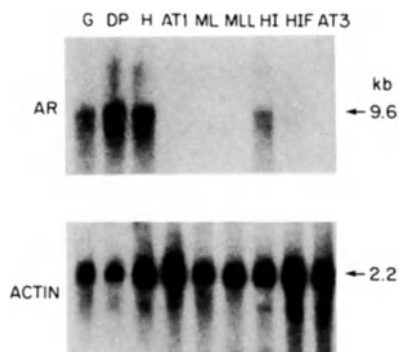


Figure 5: Northern blot analysis of poly(A) RNA in dorsal prostate and Dunning tumors. Poly(A) RNA (15 μ g) was analyzed by northern blot hybridization using a human AR (top panel) or actin (lower panel) ^{32} P-labeled cDNA as probe. Shown are RNA samples for the G tumor (G), normal dorsal prostate (DP), H, AT-1, MAT-Lu (ML), MAT-Ly-Lu (MLL), HI, HIF and AT3 tumors. Molecular weight markers used for calibration were HindIII digested lambda DNA. Actin hybridization was a control for RNA loading and showed some variation between the tumors.

Dunning prostate adenocarcinoma

The Dunning prostate tumor sublines were developed as models for human prostate tumor progression (18). Some of the techniques described above were recently applied to a study of this group of tumors (19). All Dunning tumor sublines express a 10 kb AR mRNA, but each line shows a different level of expression (Fig. 5). Highest amounts of AR mRNA were observed in the H and G lines, which correlates with their relatively high concentration of AR protein as determined in androgen binding studies. On the other hand, prolonged x-ray film exposure times of northern blots were required to observe AR mRNA in the AT1, AT3, MAT-Lu and MAT-Ly-Lu tumor lines, indicating a very low level of AR mRNA in these androgen resistant sublines. The HI and HI-F sublines contain intermediate levels of AR mRNA. There were no detectable aberrations in AR mRNA size. In general, the level of AR mRNA in the Dunning tumor sublines paralleled the level of AR protein as determined by androgen binding (19).

Immunocytochemistry, using the antibody described above, revealed AR predominantly in acinar epithelial cells and in locally invasive adenocarcinoma cells within the stroma of the H tumor (19). The G subline showed strong immunostaining while the androgen resistant AT and MAT-Lu sublines lacked detectable immunostaining for AR, in agreement with their low levels of AR mRNA. Thus, loss of androgen responsiveness in the AT and MAT-Lu Dunning tumor sublines appears to reflect a low level of expression of the AR gene. Southern blot analysis indicated that no major AR gene rearrangements were associated with loss of androgen sensitivity in the Dunning tumor lines.

Rapid analysis of AR gene mutations

Androgen and its receptor are essential factors in the normal regulation of prostate growth and development. An alteration in receptor gene structure and consequently receptor function, might be associated with androgen resistance and uncontrolled growth that characterizes some prostate cancers. The absence of AR binding in recurrent, androgen resistant prostate cancers, may result from a number of factors affecting transcription of the AR gene, in which case AR mRNA would be undetectable. On the other hand, the presence of AR mRNA in tumors without AR binding activity would indicate the AR gene is transcribed and suggest that loss of binding activity resulted from a mutation requiring more sophisticated techniques for detection.

To evaluate possible alterations in the AR gene coding sequence from genomic DNA derived from prostate cancer tissue, it is necessary to know intron/exon boundary sequences of the AR gene. These regions were recently determined (2) to allow the preparation of synthetic oligonucleotides for use in primed exon amplification using the polymerase chain reaction (PCR). A diagram of the human AR gene and how it is amplified using PCR is shown in Fig. 6. In determining intron boundary sequences, six 15 kb genomic

fragments were isolated from a human X chromosome library using human AR cDNA and oligonucleotides as probes. The AR gene consists of eight exons, A through H (2): the first 1700 bp of the N-terminal coding region comprises the first exon; exons B and C code for the two zinc finger motifs of the DNA binding domain. Exon D forms the "hinge" region containing nuclear translocation signals. The 3' end of exon D together with exons E-H comprise the steroid binding domain based on structural homology with other steroid receptors and our earlier expression studies (4). Since DNA sequence is known only for the coding exons and short flanking regions of the introns, the exact size of the AR gene has not been determined, but probably exceeds 60 kb. Splice site locations are similar to those of the progesterone and estrogen receptor, supporting a common evolutionary pathway for the steroid receptor genes (2).

Methods for rapid detection of single base mutations associated with androgen resistance can now be applied to the analysis of amplified AR DNA derived from prostate cancer. Exons are amplified by PCR from normal human genomic DNA and DNA extracted from prostate cancer tissue. Amplified exon DNA is analyzed by gel electrophoresis. The presence of equal sized DNA bands from normal and cancer tissue eliminates the possibility of a major deletion in the coding region of the AR gene. Further analysis to detect point mutations in the coding region is based on the formation of hybrid DNA molecules between normal and mutated sequences. DNA/DNA hybrids with nucleotide mismatches are susceptible to chemical cleavage. The necessity of sequencing entire coding regions in the early stages of identifying gene mutations can thereby be avoided. Chemical cleavage was developed for the purpose of selectively cleaving hybrid molecules which contain nucleotide mismatches (20-22). Mismatched thymines are cleaved with hydroxylamine and mismatched cytosines with osmium tetroxide. A particular advantage of chemical cleavage is that it identifies all possible mismatches in heteroduplexes formed between wild type and variant DNAs. This method was recently used to detect a single base mutation associated with the androgen insensitivity syndrome (3).

The GC-clamp technique is another method for rapid detection of DNA fragments amplified by the polymerase chain reaction which contain a base mismatch relative to wild type sequence (23). This method was used recently by Brown and Migeon in studies on AR mutations associated with androgen insensitivity (24). It takes advantage of the differential electrophoretic mobility of native and mutant DNA/DNA heteroduplexes in a gradient denaturing gel based on a change in melting temperature. GC tails added to the oligonucleotide primers improve the resolution of normal and mutated gene fragments in the gel. A limitation of the GC-clamp method is that all possible mutations may not be identified.

Examples of mutations causing androgen resistance in the syndrome of androgen insensitivity include deletions of part of the steroid and DNA binding domains, and single base mutations within the steroid binding domain (25). An interesting possibility that remains to be investigated is that an AR mutation in prostate cancer could abolish androgen binding activity, or perhaps change the binding characteristics so that the receptor recognizes another ligand, leaving intact its transactivation function. The function of such a receptor might be analogous to that of an oncogene product (26). Transactivation functions of steroid receptors are believed to reside in all three domains: N-terminal, DNA binding and steroid binding. The steroid binding domain of the receptor suppresses transactivation prior to ligand binding and transformation. However, deletion of the steroid binding domain converts the glucocorticoid receptor to a constitutively

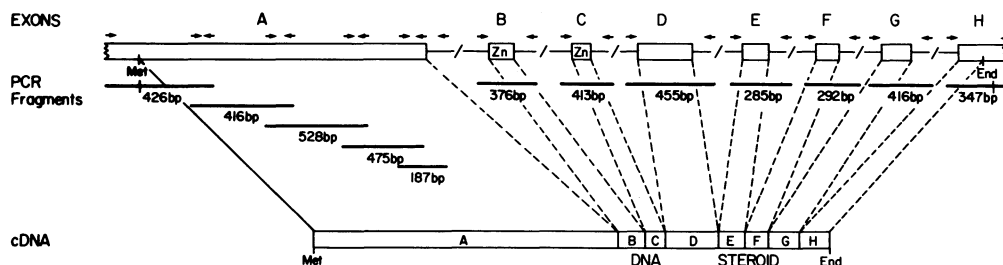


Figure 6: Diagram of the human AR gene showing exons, intron/exon junctions, and the location of oligonucleotide primers used for the polymerase chain reaction. Amplified fragments and the location of exons in the messenger RNA (or cDNA) are indicated.

active transactivator (27,28). The use of PCR and rapid mismatch detection methodology should make possible a more thorough investigation of the role of AR in prostatic cancer progression.

Proof that a mutation is involved in an altered response, such as androgen resistance in prostate cancer, requires recreation of the mutation from the wildtype sequence and expression to demonstrate altered AR function. Site specific mutagenesis in M13 bacteriophage (29) was recently used to express the mutation causing androgen insensitivity in the rat (3). An alternative method for recreation of mutations involves the use of the polymerase chain reaction (30,31). The internal oligonucleotide primers of about 25 residues in length are designed to have wild type sequence except for a centrally located mutation of choice. When possible, the outside primers have unique restriction sites included on their ends. Two separate amplification reactions allow the production of an AR DNA segment containing the specific base change. Following cloning of the cassette back into wild type AR cDNA in an expression vector such as pCMV (32), the biochemical properties of the expressed mutant can be correlated with the disease state. Analysis of ligand or DNA binding properties of mutated AR can be done by expressing the protein in COS cells. Transcriptional activation can be assessed by cotransfection of the mutant receptor with a reporter gene such as chloramphenicol acetyltransferase coupled to a suitable promoter and hormone response element. Such an approach was used to analyze an AR defect associated with human androgen insensitivity (2).

Thus, the cloning and sequencing of the AR gene has made possible the application of powerful new methodologies to search for alterations in the AR gene in association with prostate cancer.

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DISCUSSION

Dr. Matusik:

On your transduction studies looking at the androgen response element in a C3 gene, can you tell us something about that? What areas of the C gene? What is the sequence? Does it look like a glucocorticoid response element?

Dr. Wilson:

Yes, it is very similar. It is not a perfect palindrome, but it is very similar to a GRE.

Dr. Matusik:

Have you tried transfecting with the glucocorticoid receptor expression vector to see if that will also regulate the C3 gene?

Dr. Wilson:

No, we haven't done that experiment.

Dr. Matusik:

I'm going to present some data tomorrow identifying an ARE in the probasin gene. It also appears to be a GRE, and they do look very similar.

Dr. McPhaul:

I really have difficulty conceptualizing what kind of effect that point mutation could be having. You mentioned phosphorylation. I just wondered if you could expand on that.

Dr. Wilson:

Yes, I know. We've also had a hard time trying to conceptualize what's going on. We see a large amount of receptor protein produced in the transfection studies, yet we have a low binding capacity. Right now we imagine there may be a low amount of phosphorylation occurring on that serine or threonine position in the Tfm rat receptor, so that we get a low amount of binding which has the same high affinity. Loss of arginine could reduce the level of phosphorylation and thus lower the binding capacity.

Dr. McPhaul:

One other question along that same line. Have you looked to see whether it's in the same place in the cell when you transfect that into your cells? In other words, is it possible that 99% of it is not folding right, and only 1% is folding correctly?

Dr. Wilson:

I don't believe we've done immunocytochemistry on a transfected mutant Tfm rat. But we've done immunohistochemistry of the androgen receptor in the pituitary, and it is a nuclear protein clearly in the Tfm rat.

Dr. McPhaul:

We've looked at a number of patients with AIS as well. We have yet to detect in up to 20 different patients now, a single deletion in the receptor negative category. I want to point out that I think it really does reflect to some degree the patient population you're drawing on. In fact, in different patient populations, the frequency of the type of different mutations as far as deletions are concerned may be completely different.

Dr. Lee:

I was very much interested in seeing your real beautiful immunohistochemistry staining of human prostate tissues. Have you or anybody else had any opportunities to realize if the basal cell in human prostate is also stained positive for androgen receptor?

Dr. Wilson:

I don't know if the basal cells stain for the androgen receptor.

Dr. Barrack:

We've done both androgen receptor autoradiography and immunocytochemistry using Dr. Wilson's androgen receptor antibody. We find that basal cells in human BPH are AR negative.

Dr. Wang:

On your interesting arginine mutant, GR hormone binding site also interacts with HSP 90 and that's supposed to compete with hormone to regulate a receptor function. Have you looked in a system where you have lower sites by seeing the mutant proteins are totally bound up by another regulatory protein. Have you sized the mutant in a wild type receptor in your binding assay?

Dr. Wilson:

No, we haven't done that, but we've thought about that as being a potential site for the binding of the 90kDa heat shock protein or perhaps another protein. That certainly is another possibility that we should consider. But, maybe I should ask you what you think of that site as a possible phosphorylation site. Does it seem to fall into the sequence pattern for phosphorylation recognition sequences?

Dr. Wang:

Well, that looks like a cyclic A or protein kinase C site, but it's kind of difficult to think of phosphorylation not changing affinity, but only changing the total number of sites. It looks like kind of more inaccessibility of the hormone to the receptor.

Question from the floor:

I've been very impressed in looking at these immunohistochemical studies how these receptors appeared to almost exclusively in the nucleus except for one earlier talk. I'm just wondering, is this an artifact of the system where the receptor bound to the steroid may have one immunoreactive and basically where the unbound protein may have a different immunoreactivity. And basically are there studies that show that there is a good correlation between the immunohistochemical studies and radioautography studies. I mean, do the radioautography studies show the same finding that these receptors are almost exclusively in the nucleus?

Dr. Wilson:

Autoradiography studies are done in the presence of radiolabeled hormone so they really don't address the question of where the receptor is localized in the absence of hormone. This particular antibody does not recognize the native receptor well in immunocytochemistry in the absence of hormone. All of the slides that I've shown were done in the presence of hormone. In the case of the Tfm rat, very high levels of androgen were used.

Question from same:

Well, has it ever been done say *in vitro* to take the receptor protein by itself, and stain it with an antibody and then just add the hormone and restain it? Does it stain in an identical fashion?

Dr. Wilson:

I'm not quite sure how you would do that, but we have done immunoprecipitation to see if the antibody would react with the receptor in the absence of hormone by immunoprecipitating and then trying to label it. In that case it indicated that the antibody could recognize the receptor without the hormone.

SPECIFIC RECEPTORS FOR VITAMIN D₃ IN HUMAN PROSTATIC CARCINOMA CELLS

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INTRODUCTION

Approximately 103,000 men will be diagnosed as having prostatic cancer during 1990 [1]. Of these, more than one half will have locally advanced or metastatic disease at the time of diagnosis [2]. For many of these men, hormonal manipulation will become their chief form of therapy. However, although 70% of tumors will initially respond to androgen withdrawal, the majority will eventually progress to a hormone refractory state. These grim statistics have spurred the search for the basic explanation of the phenomenon of androgen "independent" growth.

To a great degree, exploration of the cell biologic mechanisms which produce androgen independence have been hampered by a paucity of appropriate human cell models. Recently, the LNCaP cell line originally established by Horoszewicz et al. [3] has been confirmed as being responsive to androgens when grown in the presence of charcoal stripped fetal calf serum (CSS) which reduces endogenous steroid concentrations [4-7]. These cells have been found to contain specific, high affinity receptors for androgens (both 5 α -dihydrotestosterone and methyltrienolone). In addition, the cells fail to grow in the presence of CSS unless exogenous androgens are added. Their growth appears to be mediated, at least in part, by androgen induction of epidermal growth factor receptors [6,8,9]. Estrogen, progesterone and glucocorticoids also bind to the androgen receptor and produce growth effects which are proportional to their respective affinities. Although these alternative ligands can induce growth, specific receptors for steroids other than androgens have not been detected. Such "promiscuity" of the androgen receptor for other ligands could in part, however, explain androgen independence.

An alternate explanation is that receptors could exist for growth stimulatory ligands which have not yet been examined. At present, a number of steroids are recognized as being part of a large family of ligands which produce their biologic effects through similar receptors [10,11]. These include seemingly diverse hormones such as androgens, thyroxine, aldosterone, and vitamin D₃.

Since receptors for the active metabolite of the latter, 1 α -,25-dihydroxycholecalciferol [1,25 (OH)₂ D₃], have been identified in breast carcinoma cells [12], we decided to examine the three currently available human prostatic carcinoma cell lines (LNCaP, DU145 and PC-3) for their presence. Although preliminary, our results indicate that unique, high affinity receptors for 1,25 (OH)₂ D₃ exist in human prostatic carcinoma cells. 1,25 (OH)₂ D₃ also appears to be capable of stimulating the growth of LNCaP cells in the absence of androgens.

MATERIALS AND METHODS

1 α -,25-Dihydroxycholecalciferol (RO 21-5535) was a generous gift of Dr. M. Uskokovic, Hoffman-LaRoche, Inc., Nutley, NJ. 5 α -Dihydrotestosterone (5 α DHT), testosterone, and triamcinolone acetonide

were obtained from Sigma Chemical Co., St. Louis, MO. Hydroxyflutamide (SCH 16423) was a generous gift from Dr. R. Neri, Schering Corp., Brookfield, NJ. 1α -,25-Dihydroxy (26,27-methyl- ^3H) cholecalciferol Sp Act 180 Ci/mmol was obtained from Amersham Corp., Arlington Heights, IL. Methyltrienolone (17- α -methyl- ^3H -R1881) Sp Act 87 Ci/mmol and unlabeled R1881 were obtained from NEN Research Products, Boston, MA.

Cell Culture: The LNCaP cell line, originally derived from a lymph node metastasis [3] was a gift from Dr. Horoszewicz (Roswell Park Memorial Institute, Buffalo, NY). The DU145 cell line [13] was obtained from Dr. M. Webber (University of Colorado Health Sciences Center, Denver, CO). The PC-3 [14] cell line was obtained from the American Type Culture Collection, Rockville, MD. All of the cells were maintained in RPMI-1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, UT) and antibiotics. The medium was routinely changed twice per week. All of the cell lines were maintained as monolayers, grown at 37°C , in a humidified atmosphere of 5% CO_2 in air. Verification of all cell lines was carried out by cytogenetic analysis.

Receptor Assays: Receptor/ligand binding assays were carried out using whole cell suspensions. Cells are trypsinized from monolayer cultures and washed with supplemented media. A cell suspension containing 1×10^6 cells is then aliquoted to replicate polypropylene microcentrifuge tubes. The tubes are centrifuged at 1000xg at 4°C for 5 minutes, and the media is removed. The cells are resuspended in media containing 1% fetal calf serum (FCS) in addition to varying concentrations of labeled steroids. For competition studies, a 200-250 fold excess of unlabeled ligand is added to a duplicate set of tubes. All points are performed in triplicate. The cells are incubated at 37°C for 1 hour with vortexing at 15 minute intervals. To stop the incubation the tubes are placed into an ice bath for 15 minutes followed by centrifugation at 1000xg at 4°C for 5 minutes. Cell pellets are then washed once with media containing 1% FCS followed by phosphate-buffered saline. Following resuspension, the cells are lysed in 0.5% Triton X-100 in 0.02M Tris pH7.8, 0.25M sucrose. Resultant nuclei are rinsed with 0.25M sucrose and pelleted as above. The pellets are resuspended in $100\mu\text{l}$ of 0.25M sucrose and are transferred directly to scintillation vials containing 4ml of Ecolume (ICN Biomedicals, Inc., Irvine, CA) for scintillation counting.

Growth Curves: For the study of *in vitro* stimulation of cell growth, 2.5×10^4 cells are plated into six-well plates. They are grown for 3 days in the presence of 1640 + 5% CSS as previously described [7]. On day 3 the appropriate concentrations of unlabeled steroids are added to the media. The media is changed every 2 days, thereafter. At desired intervals, the cells are trypsinized in 0.5ml of 1X Trypsin-EDTA (Life Technologies, Inc., Grand Island, NY). After 1-2 minutes, 0.5ml of 1640 + 5% CSS is added. An aliquot is then counted using a hemocytometer.

RESULTS

Karyotypic analysis of the three cell lines revealed unique abnormalities which were comparable to those previously reported [3,15,16]. LNCaP cells were found to contain two copies each of 1p- and 15q+ marker chromosomes. DU145 cells contained an 11q-, a 4q+ and were trisomic for chromosome 7. The PC-3 line was characterized by the presence of 34 marker chromosomes including an 11p+ and two large metacentrics. All of the lines could, therefore, be easily distinguished from one another.

Since LNCaP is the only line of the three examined which exhibits androgen dependent growth, we carried out most of our studies with these cells. The ability of LNCaP cells to bind $1,25(\text{OH})_2\text{D}_3$ is shown in Figure 1. It can be seen from the figure that LNCaP cells bind $1,25(\text{OH})_2\text{D}_3$ in a saturable fashion which can be nearly completely competed with a 200 fold molar excess of unlabeled ligand. Scatchard rearrangement of the data reveals a K_d of 1.7×10^{-10} with approximately 1,000 receptors per cell. However, when a 200 fold excess of unlabeled 5α DHT is added, no statistically separable competition was detected.

Since LNCaP cells are known to contain high affinity androgen receptors, we also investigated the ability of $1,25(\text{OH})_2\text{D}_3$ to compete with 5α DHT. Figure 2 represents the results of a binding experiment in which increasing concentrations of labeled 5α DHT were added to LNCaP cells. Here, it can be seen that while binding occurs, a much higher molar concentration of 5α DHT is required to reach saturation. Scatchard analysis of this data revealed a K_d of only 1.7×10^{-8} with approximately 25,000 receptors per cell. Attempting to compete the binding of 5α DHT with a 250 fold molar excess of $1,25(\text{OH})_2\text{D}_3$ also failed to produce significant competition.

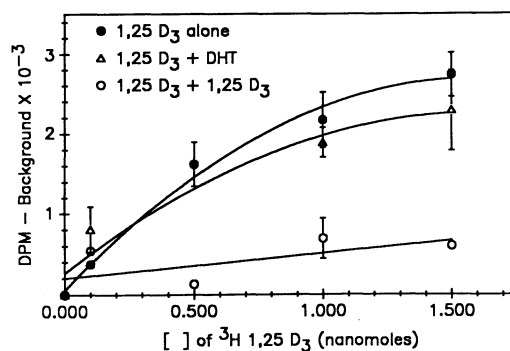


Figure 1: Binding of 1,25 (OH) $_2$ D $_3$ (1,25D $_3$) to LNCaP cells.

5 α DHT is quickly metabolized by LNCaP cells [4] and thereby alters the apparent K $_d$ of this ligand for its receptor. To eliminate this problem, we also studied the binding of the nonmetabolized androgen analog, R1881 to the androgen receptor. In addition, to eliminate the potential effects of androgen binding to glucocorticoid receptors, 1 μM unlabeled triamcinolone acetone was added to all reactions carried out in the presence of R1881.

Again, saturable binding occurs (Figure 3) but this time, at a much lower ligand concentration. Scatchard analysis revealed a K $_d$ for R1881 of $4.8 \times 10^{-10}\text{M}$ with approximately 10,000 receptors per cell. As with 5 α DHT, a 250 fold molar excess of 1,25 OH $_2$ D $_3$ was ineffective in competing with R1881 for the androgen receptor.

As previously reported [4-7] the growth of LNCaP cells in CSS (Figure 4a) was minimal. Growth was clearly stimulated over the course of 1 week with the addition of either $1 \times 10^{-8}\text{M}$ testosterone or $1 \times 10^{-8}\text{M}$ 5 α DHT. Hydroxyflutamide (OHFL) also stimulated the growth of these cells (Figure 4b) in a paradoxical fashion as previously reported [7]. It would seem, therefore, that the LNCaP cell line, as carried in our laboratory, is indeed androgen dependent and behaves in a manner similar to that reported by others.

Addition of $1 \times 10^{-9}\text{M}$ 1,25 (OH) $_2$ D $_3$ to LNCaP cells in the absence of androgens produced the results seen in Figure 5. Initially the growth in the presence of 1,25 (OH) $_2$ D $_3$ actually exceeded that produced by a 10 fold higher concentration of 5 α DHT. With time, however, while the 5 α DHT continued to be stimulatory, 1,25 (OH) $_2$ D $_3$ addition produced a smaller response. At the end of 11 days in culture, the number of cells were about 4 fold greater in the presence of 5 α DHT and 2 fold greater in the presence of 1,25 (OH) $_2$ D $_3$ when compared to those grown in CSS without exogenous steroids.

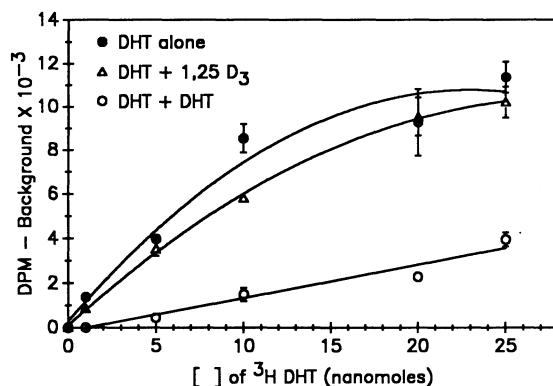


Figure 2: Binding of 5 α DHT (DHT) to LNCaP cells.

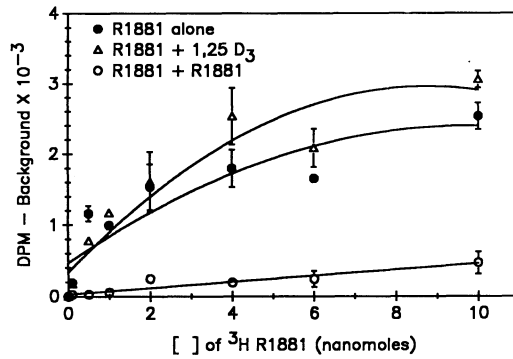


Figure 3: Binding of R1881 to LNCaP cells.

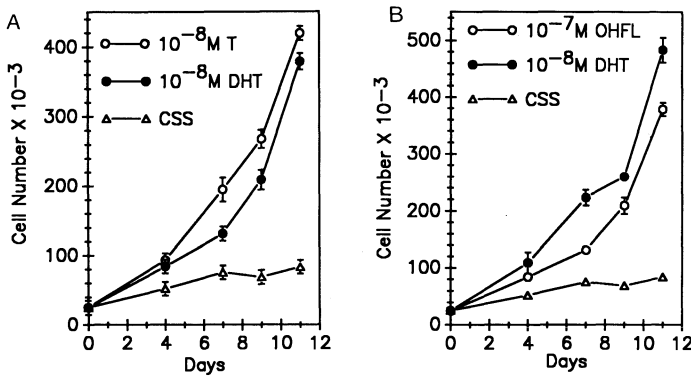


Figure 4: A. Androgen dependent growth of LNCaP cells in the presence of 10^{-8}M testosterone (T) or 10^{-8}M 5α dihydrotestosterone (DHT). Note the lack of growth in charcoal stripped serum (CSS). B. LNCaP growth stimulated by 10^{-7}M hydroxyflutamide (OHFL). Again, compare to the growth in CSS.

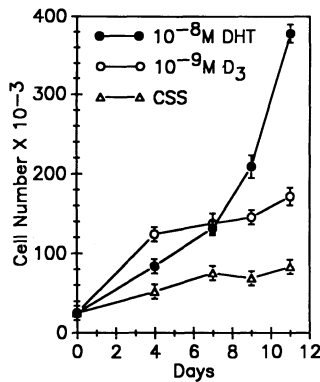


Figure 5: LNCaP cell growth stimulated by 10^{-9}M $1,25(\text{OH})_2\text{D}_3$ (D_3), compared to 10^{-8}M 5α -dihydrotestosterone (DHT) or charcoal stripped serum (CSS).

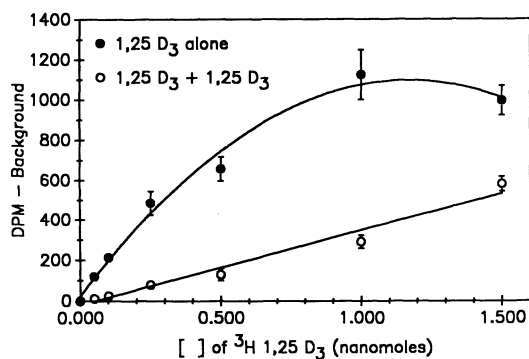


Figure 6: Binding of 1,25 (OH) $_2$ D $_3$ (1,25D $_3$) to DU145 cells.

Finally, to investigate whether or not receptors for 1,25 (OH) $_2$ D $_3$ exist only in LNCaP, we carried out binding studies with DU145 and PC-3 cells. The results are presented in Figures 6 and 7. Each of these cell lines demonstrated saturable binding of 1,25 (OH) $_2$ D $_3$ which could be competed using unlabeled ligand. The amount of binding per cell, however, was about 50% of that seen with LNCaP for DU145 cells and about 30% for PC-3 cells. In addition, the nonspecific binding to PC-3 cells was considerably higher than that seen with LNCaP or DU145, indicating that PC-3 cells may contain additional lipophilic substances not present in the other two cell lines.

DISCUSSION

Independent investigations by many workers over the past 10 years have indicated that the active metabolite of vitamin D $_3$ (1 α -25-dihydroxyvitamin D $_3$) mediates a large number of biologic functions in addition to its classically recognized role in mineral homeostasis [for review see 11]. Specific receptors have been found in over twenty different tissues of wide embryologic origin. The receptor for this secosteroid has been confirmed to be a DNA-binding protein which shares structural homology with various other steroid hormone receptors [17]. The biologic role of 1,25 (OH) $_2$ D $_3$ in various cells and tissues includes regulation of growth factor expression [18,19], oncogene expression [20,21], peptide hormone synthesis [22,23], and its own metabolism [24]. Any of these mechanisms could play potential roles in the phenomenon of androgen independent growth. Of particular note, however, is the fact that in rat calvarial cells [18] 1,25 (OH) $_2$ D $_3$ is known to upregulate EGF receptors in a manner similar to the regulation of EGF receptors in LNCaP cells by androgens [6,8,9]. The possibility that 1,25 (OH) $_2$ D $_3$ induces the expression of EGF receptors in LNCaP cells is currently being pursued in our laboratory.

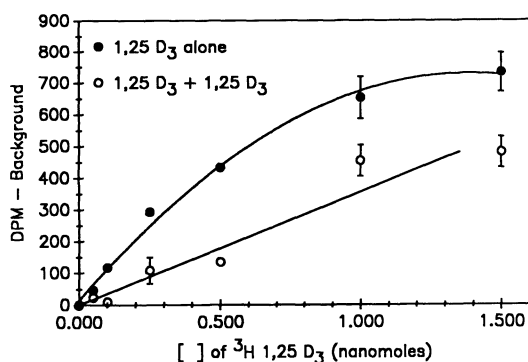


Figure 7: Binding of 1,25 (OH) $_2$ D $_3$ (1,25D $_3$) to PC-3 cells.

The results presented here clearly indicate that LNCaP cells contain a separate receptor for 1,25 (OH)₂ D₃ in addition to androgen receptors. The effect of 1,25 (OH)₂ D₃ on the growth of these cells, while not as great as those of androgens, introduces the possibility that steroid hormones other than androgens could support malignant proliferation. Continued study will be necessary to elucidate the role of 1,25 (OH)₂ D₃ in the progression of prostatic carcinoma cells from an androgen-dependent to an androgen-independent state.

ACKNOWLEDGEMENT

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DISCUSSION

Dr. Coffey:

One of the earlier descriptions the Dunning tumor is that it would form bone occasionally. Have you looked at the Dunning tumors to see these receptors and what do you think these things might do to calcium levels inside the cells, and what type of histological changes do you see when you treat with Vitamin D?

Dr. Miller:

One of the things we wanted to do right away was to see if we could develop an animal model that we could use to study the progression to an androgen independent state. John Isaacs was good enough to send us two lines of Dunning cells. At the present time, we have only looked at one of them; the AT3 cells. They absolutely do not contain D3 receptors. In fact they have served as a control in some ways to show that not all cells have receptors. The AT3 cells do not.

Dr. Coffey:

Have you looked at the H-tumor when it forms the bone?

Dr. Miller:

We have not looked at the H tumor yet. A variety of urothelial cells have the ability to induce bone formation. One of the first descriptions of heterotopic bone induction was that normal transitional epithelium put into an ectopic site would form heterotopic bone.

Dr. Rowley:

Have you looked at any potential effects of D3 on the growth of PC3 or DU145?

Dr. Miller:

No, we have not. I can tell you one other piece of data is that increasing concentrations of D3 actually become growth inhibitory in these cells. That is something that has also been reported in other cells with D3. There's a sort of biphasic distribution of growth response, and that appears to be true in LNCaP cells as well.

Dr. McPhaul:

This is reaching a little bit, but is prostate development thought to be normal in patients who have complete Vitamin D resistance?

Dr. Miller:

I don't know the answer to that. Actually, I was amazed coming here and hearing Dr. Chiarodo say that there is an epidemiological study trying to link Vitamin D deficiency with an increased incidence of prostate cancer. We then heard Dr. Linehan talk about the fact that D3 levels are modulated in animals that bear PC3 tumors. I have not seen anything relating to the phenomenon you're asking about.

Dr. Matusik:

I think I recall data saying that the Vitamin D receptor is high in bone and high in a region in the brain that is also high in zinc content. Is that true?

Dr. Miller:

Yes, I believe at least in bone, that's true. I don't know about the brain.

Dr. Matusik:

O.K. The reason I raise that is that in the prostate there is high zinc content. That's specifically true in the rat. There are three lobes, and the dorsolateral lobe has the highest zinc. The ventral has very little zinc. So I would suggest you look at lobe distribution with the Vitamin D receptor too.

Dr. Miller:

The zinc distribution in those glands is secretory, and is clearly under the influence of an energy dependent pump which concentrates it in the lumen at very high concentrations compared to the extracellular fluid. I don't know whether intracellular concentrations of zinc are as clearly elevated. But the luminal secretions have tremendous amounts of zinc.

Dr. Matusik:

Are you saying in the prostate?

Dr. Miller:

In the prostate.

Dr. Matusik:

In the prostate there is a high concentration of zinc within the cell and the nucleus. That's old data by Gunn.

Dr. Miller:

This is a difficult area to address because of course when you start dealing with ions like that you have to be very careful about how they distribute when you try to actually take cells or tissues apart. As soon as you disrupt the system, ions are going to redistribute. That's been a real problem in trying to interpret such findings.

Dr. Barrack:

Have you measured Vitamin D receptors in normal prostate?

Dr. Miller:

No, we have not.

Dr. Barrack:

The reason I ask that obviously is the connection between Vitamin D and bone and prostate cancer bone metastasis.

Dr. Miller:

Yes, there's some very obvious connections that one can start to draw about what this might have to do with the osteoblastic potential of prostatic carcinoma cells.

Dr. Linehan:

I referred just very briefly to Mark Dresner, and Kennie Lyles paper, which came out in 1981. They looked at patients with advanced prostate carcinoma and found low 1,25 levels and normal 25(OH)-cholecalciferol levels and they hypothesized that prostate cancer might form a substance which inhibited 1 alpha hydroxylase, the enzyme which converts from 25 to 1,25. In the murine model we saw elevated 1,25 levels, but normal 25 levels. Of course, it's not clear whether the hypophosphatemia, which is a big drive of course for 1,25 production, was associated with that or whether it was a tumor produced factor.

Dr. Miller:

Apparently, I have raised as many questions as I have answered. Again, 24,25(OH)₂O₃ in some cells is actually stimulatory to a variety of processes, whereas in other cells it seems to be a way that the cell gets rid of 125 from an active to an inactive form. So, the possibilities here go on and on.

PANEL DISCUSSION

Steroid Receptors

Panelists: Michael Freeman, Frank French, Robert Getzenberg, Tomohiko Ichikawa, Shutsang Liao, and Wyske van Weerden

Dr. van Weerden:

You briefly mentioned that at a high concentration of Vitamin D3 LNCaP cells are inhibited. Did you see any morphological changes in the histology of the cells?

Dr. Miller:

No, we have not. The cells grown under either the influence of 10^{-9} M up through 10^{-7} M D3 morphologically look very similar to each other. But the growth is quite different.

Dr. van Weerden:

And did you see *c-fas* expression in the nuclei, i.e. whether it was decreased or not?

Dr. Miller:

We have not done that.

Panelist:

I just want you to conjecture a little bit. You have these androgen receptors that you think are the same, or seem to be the same when you are looking at your 8 probes. I wonder if you can think of any reason why you would get different reactions even though they seem to be the same in your system? Do you have any hypothesis on why you would get these different reactions with what seem to be similar androgen receptors? You haven't tried any DNA binding assays or have you?

Dr. McPhaul:

Are you referring to the cell lines themselves?

Panelist:

Yes.

Dr. McPhaul:

In every cell line that we've looked where we can identify specific androgen binding, this binding is correlated with both the presence of the androgen receptor message and the protein. Every cell line, including two other cell lines, in addition to the DU145 and PC3, does not have specific androgen binding, does not have demonstrable androgen receptor protein and does not have demonstrable androgen receptor mRNA. I don't see any discrepancy in the data as far as the cell lines are concerned. With respect to the immunohistochemical analysis, we can show the presence of the receptor in the nuclei of target cells in the presence or absence of hormone. Although in the absence of hormone, we have to use prolonged incubation times with the primary antibody to demonstrate immunoreactivity using the anti-C terminal antibodies. That's the only discordance that exists.

Panelist:

Right, but didn't you say that the DU145 and PC3 had an intact androgen receptor gene?

Dr. McPhaul:

They have intact androgen receptor genes, but they don't express mRNA, and they don't express protein nor specific DHT binding. At this point I can't address whether the genes are transcriptionally

inactive or the RNA is turned over more rapidly, or if the genes are transcriptionally inactive. If I had to bet, I would bet that more likely than not that they are transcriptionally inactive.

Dr. Freeman:

Would speculate if possible on the significance of your observation of increased androgen receptor staining in BPH stroma and whether or not that's likely to correspond with any increase in any other gene activity? Also, how do you reconcile your results on androgen induction of C3 regulatory region with Malcolm Parker's observation that the C3 regulatory region is not androgen inducible?

Dr. Wilson:

In the stromal localization studies, we see androgen receptor primarily in fibromuscular regions of BPH. In the more glandular areas of BPH, the stromal staining is much less. The significance of that I really couldn't comment on. That's why it would be higher in those regions. Malcolm Parker I believe used the C3 gene with an endogenous androgen receptor. It wasn't a co-transfected receptor. We used transient transfection to express the receptor together with the reporter plasmid. So perhaps the high level of receptor that we get might account for the difference.

Comment from the floor:

In the Shionogi cells the androgen receptor is functional, and it's not likely to be a defect in the AR.

Dr. Wilson:

There are some groups that have evidence that there may be a defect in the androgen receptor of the Shionogi tumor.

Dr. French:

In one of the cell lines you had, was it the LNCaP cells that had the 2 X chromosomes?

Dr. Miller:

That's right.

Dr. French:

I wondered if both of those are active. Could you get an idea, or do you know if those cells have bar bodies, which would indicate that one of them is late replicating, and probably inactive?

Dr. Miller:

I don't know. That's a very interesting question. It certainly is easy enough to look and see if one of them appears as a bar body. I hadn't thought to look.

Dr. French:

Have you looked at possible synergistic effects of DHT and Vitamin D3 in these cells?

Dr. Miller:

Yes, we have. The results have been a little variable and consequently I was hesitant to discuss them. It does appear, at least in a few experiments, that they've been synergistic. That is, the growth seen in the presence of 10^{-8} M DHT with 10^{-9} M $1,25(\text{OH})_2\text{D}_3$ is greater than that seen with DHT by itself. I believe we're at or near the optimum for the growth with DHT by itself.

Dr. Coffey:

I wondered if you could tell us what's known in other systems about these orphan receptors and whether that's the type of thing Dr. Chang is seeing with his T2.

Dr. Tindall:

The term, "orphan receptors" has been coined from a group of genes which were isolated using a consensus sequence, such as the DNA binding region. This is a region that is conserved among all of the steroid receptors and the thyroid hormone and retinoic acid receptors. By using this consensus sequence, there have been more genes cloned than there have been ligands to account for them. Because they're genes in search of ligands, they've been termed orphan receptors. This is obviously an orphan receptor gene that Dr. Chang has uncovered. These receptors were created for a purpose. I think that it's going to be an extremely important finding to impart a function for this protein. We're looking forward to his results of those experiments.

Dr. Chang:

One possibility is that these new receptors genes are just pseudo genes. But as I showed in the slide, the message expressed in the human prostate. Now we are able to raise monoclonal antibody, which stains this protein in the nuclei. So at the gene level, at the message level, and at the protein level, there was such a protein. So we are extensively trying to identify the kind of ligand, but there is always the possibility that it is an orphan receptor.

Dr. Chung:

Dr. Tim Thompson and I did some experiments earlier on Tfm/y mouse kidney AR. We found that mouse kidney does not contain androgen receptor during isolation if sodium molybdate was not added to the homogenizing medium. However, if sodium molybdate was added, you can actually detect a low level of high affinity androgen receptor in the preparation. Dr. Wilson, in your transfection experiment, are you isolating the receptor under sodium molybdate conditions or not? Also, I wonder if the phosphorylation state might affect affinity and total binding of androgen receptors you have put in?

Dr. Wilson:

Let me mention that the mutation in the Tfm mouse is different from that of a Tfm rat, in that there is a very low level of androgen receptor message detected in the mouse. As far as the endogenous receptor in the Tfm rat, we can measure binding, for example, in kidney or liver, and certainly adding molybdate helps in detecting binding. I didn't show you a binding curve on the endogenous receptor, but it looks very similar to the one that I showed on the reconstructed receptor. So there is activity but it was so low that we couldn't do a Scatchard analysis on the endogenous receptor. The counts were just too low. I'm not sure if that's answering your question.

Dr. Chung:

Yes, I guess if you speculate on that receptor you put in, if it's phosphorylated or dephosphorylated, would this affect the binding affinity?

Dr. Wilson:

I just can't answer that question yet. There was a recent paper in Molecular Endocrinology by Auricchio's group where they were studying the estrogen receptor and showed that in the absence of tyrosine phosphorylation, that they observed a greatly reduced amount of binding capacity, but the binding affinity remained exactly the same. The Scatchard plots were almost superimposable with our results. Actually, that was one of the experiments that led us to look more closely at the sequence and see that serine was highly conserved. But I don't have any direct evidence yet that that is a phosphorylation event.

Dr. Catalona:

An interesting clinical observation is that patients with metastatic prostate cancer who are castrated or who have their testosterone suppressed with estrogens, which work primarily on the pituitary gland by shutting down LH secretion, become impotent. On the other hand, patients who are treated with either flutamide, which blocks the binding of DHT to the receptor, or patients who are treated with the new Merck drug, Merck 906, which is a 5-alpha reductase inhibitor, retain their sexual potency. So, in these patients treated with flutamide or the 5-alpha reductase inhibitor, what you're doing is blocking the conversion to DHT or you're blocking the binding of DHT to the receptor. In this setting, the patients retain their sexual potency. It would seem to me that the explanation for this may be that DHT is important for stimulating the prostate cancer cells, but testosterone is what's really important for potency. If these receptors are the same, if the DHT receptor and the testosterone receptor are the same, it really doesn't make sense why these patients would be able to maintain their potency. Why does eliminating testosterone knock out sexual potency, but eliminating dihydrotestosterone does not?

Dr. Barrack:

I would be willing to predict that the androgen receptor gene is going to be DNase 1 sensitive in cells where it's expressed. I wonder if anybody has looked at that. Also, if the androgen receptor gene is DNase sensitive in cells where it's expressed, is it also DNase 1 sensitive in cells that are androgen receptor negative, such as Mike McPhaul's cell lines where he sees no AR expression. Have you looked at any DNase sensitivity in those cells?

Dr. McPhaul:

As far as the preceding question is concerned, I think that there is clearly a number of androgen receptor mediated events in sexual development that are dependent on 5 α -reductase, but all androgen-mediated events in sexual development are dependent on the presence of an intact 5 α -reductase enzyme. If

you have a functional androgen receptor present, certain things will happen, both in the presence and absence of 5 α -reductase. This enzyme being an intracellular modulator, I think the fact that you can inhibit 5 α -reductase could easily affect the availability of the more potent androgen, 5 α -reduced dihydrotestosterone, to a particular cell while not affecting the availability of testosterone which appears to bind to the same receptor to other cells that are not as dependent on 5 α -reduced metabolites for the action of the androgen. The difference may be that some events require the more potent 5 α -reduced dihydrotestosterone. So, I understand your puzzlement, but if you look at external genital verilization during development, the remainder of the androgen dependent events will occur normally. But in the absence of a 5 α -reductase enzyme, those events that effect external verilization in development don't occur. It's not necessarily that puzzling if you accept the fact that certain events might require higher levels of androgen to activate particular genes.

Dr. Catalona:

So what you're saying here is that testosterone gives you a weaker signal, but preserves potency. Dihydrotestosterone gives you a stronger signal but does not preserve potency.

Dr. McPhaul:

In that situation, you're talking about potency which is just an endpoint of many steps. All that would be required to say is that those steps are not dependent on the action of androgen in cells that are dependent on the 5-alpha reduced metabolites for the androgen response.

Dr. Coffey:

Dr. Catalona's question is a very central question clinically and physiologically that we have to address as receptor people. If you are looking at developmental biology, where the timing of things is important, that's one thing. I think that the testosterone and DHT difference in their action has to be looked at as either different receptors or the same receptor acting differently. Already there are papers showing TGF alpha and EGF working at the same receptor and causing all sorts of different situations on how you could inhibit them or make them work, and it looks like it's an allosteric-like thing. They may get on different sites or do different things. We've already heard strange things happen with flutamide that goes into the cell and brings about things that don't make sense to us. We have heard in this meeting that the C part, the carboxyl terminal end, won't react with antibodies because it's folded a certain way. We know that in the estrogen field, you can take off the estrogen receptor steroid binding part and its active without it, like it's folded. We'll have to work all those things out.

My question is about the phosphorylation of this receptor and about taking off the terminal end and whether it can be active. Concerning CDC 2 kinase, (cycle dependent controlled 2) in the cell cycle, there are very dependent proteins and some of them are kinase enzymes. The CDC 2 kinase doesn't change the amount of the enzyme across the cycle, but its activity is changed. It does so by interacting with a control factor, just like the cyclic AMP system is controlled by regulatory units, by a protein called cyclin, which comes up at these places and goes down and turns on phosphorylation. What Dr. Wang showed us this morning is that one of the targets of that phosphorylation is this retinoblastoma gene. What I want to know is, is there any evidence for any of these steroid receptors that are getting phosphorylated that they are phosphorylated in response to a signal, like a cyclin in the cell cycle signal? Has anybody worked on seeing if the cell cycle receptor phosphorylation pattern controlled by cyclin or something? Secondly, when you take off that terminal end of the C, does that change the activity of androgens as a responsive element without steroids? Has anybody looked at that yet, like they did for the estrogens? It's not true for all steroid receptors. It's just some that you can clip off, but that's very important for our argument on why a cell becomes hormone insensitive. If you just clipped off that part, and if it's like estrogens, it would just function without any steroid there.

Dr. French:

That's a very interesting question in relation to the androgen insensitivity syndrome. We've found a couple of deletions now. The steroid binding domain, but those patients don't have detectable levels of mRNA, which means there is something about this deletion that's probably causing the message to degrade very rapidly. But I think when we look at individuals with partial androgen insensitivity, it's quite possible that we may find one that has a deleted steroid binding domain, but has a receptor that remains somewhat active constitutively.

Dr. McPhaul:

I just wanted to say, in our patient population, we've not found any deletions in the androgen receptor. I don't know if this is a function of sample size or racial background of the population or what.

We have found one patient that I discussed at the Endocrine Society meeting last year that had a termination code on exon 6. Interestingly enough, this patient does not have the development of prostate, but she did present with metastatic seminoma. I don't know if that has any relationship at all, but I think it's interesting. While such malignancies do occur in patients with complete androgen resistance as they get older, this patient was 14 when she presented and she did have a C terminal truncation. In our assays that is not functional in stimulating the response elements that we have had. But then again, our responses in transfection assays were not very good with the androgen receptor at this point anyway.

Dr. Smith:

I'd like to address the T and DHT apparent discrepancy, because it's one that doesn't bother me too much. I think it's based on the studies that we've done with the chicken estrogen receptor. In that situation it is very clear that you can convert the receptor between three different states. One which binds with high affinity, one with lower affinity, and another which doesn't bind the steroid at all. Now, in that situation, there's an ATP dependent event which converts with the highest to a high affinity binding form. The change appears to reflect the conformational change in the receptor because the molecular weight of the receptor stays the same, but it sediments slightly different on a sucrose gradient. I think you could also explain the T & DHT effect by receptor conformation because equilibrium binding studies indicate that the conformation is different between the two receptor hormone complexes. It isn't difficult to explain the two effects with the same receptor because you've stabilized two different conformations. Now the data isn't in yet on androgen receptors as to whether you can actually stabilize those two conformations and tell whether the conversion is an ATP dependent event. But certainly in the estrogen receptor field you can stabilize one with an antiestrogen and it will still bind to DNA. The other form is stabilized by estrogen, which also binds to DNA. In one case you've got well characterized activation of gene transcription; in the other case you've got activation of transcription of some genes, but not the ones that are commonly looked at. There's some data beginning to appear in the literature now where hydroxytamoxifen estrogen receptor complexes activate transcription of some genes. So, I think it's very clear that you can have two different conformations of the same receptor, and see different biological endpoints. With respect to hormone binding, the estrogen receptor, in contrast to the androgen receptor when it's cDNA is transcribed and the resulting RNA translated *in vitro*, the protein doesn't bind steroid. If it's expressed in *E. coli*, again almost none of it binds steroid. Now, that's not true of the androgen receptor. My suggestion is that the androgen receptor in its hormone binding form is probably phosphorylated, and the estrogen receptor is dephosphorylated. Whether it's phosphorylated or dephosphorylated will depend on the receptor. But what I think is very clear, and very important, is that phosphorylation can effect the conformation of the receptor and can effect the hormone binding characteristics. What we see in the estrogen receptor are three different states. One which binds with K_d in the high picomolar range, one which binds in the nanomolar range, and one which doesn't seem to bind the ligand at all. This process is completely reversible. With the androgen receptor, all we've seen at the moment is a single steroid binding form of the androgen receptor, except perhaps with some of these mutant forms of the receptor that bind with a different affinity. What I saw on Dr. Wilson's data today was an indication that if you start to alter a serine, or alter the potential for binding sites, and so on, and you I look carefully at those Scatchard plots of the wild type receptor, you can see a biphasic binding curve. Certainly with the form of the receptor that has the mutation, you apparently have only one binding form of the receptor. However, because of the low concentration of binding sites it's very difficult to be sure of its K_d .

Dr. Coffey:

That's exactly what I was trying to get at on the allosteric change. Now if that's brought about by phosphorylation and when you add your ATP, is there an estrogen receptor kinase? Has anybody found a specific kinase for a phosphorylase, or can the receptor itself complement itself?

Dr. Smith:

The only data is from Aurrichio's lab and from our lab with the estrogen receptor. Unfortunately we don't completely agree. We do agree that the steroid binding form of the receptor is not phosphorylated on a serine residue. Our data suggest that's because of a specific protein phosphatase. What is confusing is that a subunit of the phosphatase must be phosphorylated in order to activate it. The activated phosphatase then dephosphorylates the estrogen receptor. Now Aurrichio believes there's a very specific tyrosine kinase which phosphorylates the estrogen receptor and that's responsible for hormone binding. Our disagreement is that I don't believe that tyrosine phosphorylation is essential for steroid binding.

Dr. Matusik:

The essence of the question about testosterone and DHT is one of the looking at how we activate genes. We're not thinking in terms of whether testosterone can bind to receptor or DHT and inactivate

them. In the glucocorticoid receptor, we know it has the GRE or glucocorticoid response element, and a negative glucocorticoid response element exists. So, likewise, we might envision that there is a negative androgen response element, and that will allow for differential regulation of genes. The question is for Mike McPhaul. When you transfect the cells, you show that there was a large AR receptor, as well as a protein at 64Da. You talked about the idea of splicing. In the transfection, are you using the full length coding region? So, if it's the coding region, then it's unlikely that it has interons. Rather, it must be using an artificially created interon.

Dr. McPhaul:

That's what I said. I said that in a lot of these vectors, when splicing was first looked at, people thought that you had to have splice donor and splice acceptor sites to have efficient expression of a gene. The people who have propagated these CMV vectors have not put in these kind of signals. While it has a growth hormone terminator, the CMV promoter, an origin of replication for S40, it doesn't have any artificial splicing sequences. So, when you go through and do the same analysis using different antibodies, the pattern that you get suggests that some of those smaller bands have both the amino terminal and carboxyl terminal ends of the molecule, and that the middle half of the molecule is missing. These results imply that it's not just simply an internal methionine where translation is beginning.

Dr. Matusik:

My second question is whether you've done a Northern analysis?

Dr. McPhaul:

I've never done a Northern on these transfected cells.

Dr. Liao:

Maybe I should make one comment on mutation. The mutation in the androgen receptor so far reported from North Carolina, Texas, and our group, and a couple of other groups, seems to be mainly due to G to A mutation, which results in either change in amino acid or deletion or truncated receptor. I just wonder whether this is true for other steroid receptors? Does anyone know about this? Because the G to A mutation, when it's mutated, needs correction for repair mechanism, if it can be done. The so called GT protein is presumably involved in correction of the repair of a G to A mutation. If there is something wrong with that protein expression, it could lead to mutation. Another point I'd like to make is that we really didn't get into the understanding of how receptor works. We now have very nice studies about understanding structure, but we don't really know, for example, the intracellular and the nonintracellular steroid receptors, relative to the function of the receptor itself. Another thing, in many cases when expression of a particular gene is stimulated by androgen, you see an increase in mRNA, maybe mainly due to stabilization of mRNA, and how the receptor is acting to help this I don't know. I've been thinking about this for twenty years, since my first paper on receptors. I'm quite disappointed that not many people are working on this.

Dr. French:

I have just one comment regarding the mutations in other steroid receptors. With the exception of the Vitamin D receptor, and there are some reports of some mutations in glucocorticoid receptors that are natural mutations in humans, I don't think these have really been worked up. But one of the great things about the androgen receptor is that there are so many different mutations out there to be looked at. I think it's going to provide us with some interesting information in future years.

ROLE OF ANDROGENS AND EXTRACELLULAR MATRIX IN THE GROWTH AND DIFFERENTIATION OF BENIGN AND MALIGNANT PROSTATE EPITHELIAL CELLS

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ABSTRACT

Normal tissues maintain a balance between growth and differentiation. During normal adult life, the prostatic epithelium undergoes little net growth but synthesizes and secretes proteins such as prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) which are markers of differentiated function in the prostate. In prostatic neoplasia, regulation of prostate epithelial cell growth is diminished resulting in abnormal proliferation of the prostatic epithelium. The intra- and extracellular factors involved in promoting prostate epithelial growth and differentiation are poorly understood. Studies were undertaken in our laboratory to develop an *in vitro* system to selectively promote prostate epithelial cell growth or differentiation. Cultivation of freshly isolated epithelial cells from benign prostate on mesenchymal extracellular matrix (ECM) resulted in the formation of acinarlike structures as opposed to the characteristic monolayers observed on plastic. Differentiated morphology was further demonstrated by electron microscopic analysis showing the presence of apical microvilli, numerous secretory vesicles and hemidesmosome-like structures in epithelial cells cultured on ECM. Cultivation of epithelial cells on plastic resulted in a 10 fold increase in cell growth compared to cells grown on ECM. However, a four-fold increase in PSA and PAP secretions were noted following cultivation of epithelial cells on ECM compared to plastic in the presence of 10-nM dihydrotestosterone (DHT) and 1 mg/ml of prostate stromal cell conditioned medium (SCM). Similarly, cultivation of the prostate cancer cell line LNCAP on prostate stromal ECM resulted in promotion of differentiated function as indicated by acinar-like morphology, reduced growth and enhanced PSA/PAP secretion. Addition of DHT and SCM further promoted the ability of ECM to enhance differentiated properties in LNCAP. On plastic, addition of DHT stimulated an 8-fold increase in LNCAP growth compared to cells grown on ECM but had little impact on PSA/PAP secretion. These data show that: 1) cultivation of benign or malignant prostate epithelial cells on plastic will promote cell growth but not PSA/PAP secretion; 2) prostate epithelial cells grown on ECM exhibit differentiated function as indicated by acinar-like morphology, reduced growth and increased PSA/PAP secretion and 3) addition of androgens and prostate stromal secretory products will greatly enhance PSA and PAP secretion by prostate epithelial cells on ECM.

DISCUSSION

Panelist:

Which components of the matrix do you think are most important in this effect?

Dr. Sherwood:

At this point we don't know for sure. I can tell you that culturing the same cell types on collagen or laminin alone will not induce increased secretion and reduced growth. However, we haven't gone through and dissected all of the different components of extracellular matrix to determine which ones are the most important.

Panelist:

Have you looked at heparin sulfate proteoglycans?

Dr. Sherwood:

No, we haven't specifically tested heparin sulfate proteoglycans or many of the other components of extracellular matrix.

Panelist:

A somewhat related question. Have you tried matrix from other fibroblasts or other stroma? Do you know whether that specificity is there?

Dr. Sherwood:

That's a good question. We haven't tried extracellular matrix from other stromal cell sources, but we plan to conduct these experiments to determine if this is a prostate-specific effect. I suspect that the prostate matrix-induced effect will not be specific, because we have also tested extracellular matrix from the murine EHS sarcoma and observed an effect similar to that induced by prostatic stromal extracellular matrix.

Dr. Buttyan:

You slowed or stopped the growth of the tumor cell by using this extracellular matrix. Dr. Leland Chung presented data in which he took a cell, which was not very tumorigenic, and when he put it in the mouse in the presence of the Matrigel, it was much more tumorigenic. How would you explain this difference?

Dr. Sherwood:

I think an experiment that we should do to try to answer your question is to grow the different cell types, particularly the tumor cells, on living prostate stromal cells. At this point we still have a very artificial environment, considering that we are only adding the stromal extracellular matrix and secondary products. I would think that by using the live stromal cells, we would be able to get more of an indication of the dynamic interactions between stroma and epithelia. Although our results and those of Dr. Chung may be conflicting in some ways, I don't think our *in vitro* system has looked at the whole story. The observed differences may be due to differences between the *in vitro* and *in vivo* environments.

Question from the floor:

Could you comment on the doubling time of the cells in particular the LNCaP cells and the presence and absence of matrix?

Dr. Sherwood:

I can't tell you the actual doubling time in hours or days, but I can tell you that we observed a 4-fold increase in LNCaP growth in response to androgen on plastic compared to cells grown on extracellular matrix. The doubling time appears to be about 3-4 days on extracellular matrix compared to about 24 hours on plastic.

Dr. Heston:

What was the effect of hydroxyflutamide in this system?

Dr. Sherwood:

We haven't looked at any anti-androgens in our system.

Comment from the floor:

LNCaP will respond to anti-androgen by undergoing proliferation the same way as responding to androgen.

Dr. Sherwood:

That's true. A recent paper published in Cancer Research by the Tufts group headed by Carlos Sonnenschein found that anti-androgens will stimulate the growth of LNCaP. This phenomenon has also been reported by George Wilding and coworkers.

Dr. Heston:

I was wondering if the matrix would impact on that?

Dr. Sherwood:

That's possible, and is something we should look at.

Comment from the floor:

It might also be of interest to find if the proliferation is still universally related to gene expression by anti-androgen. That might be a very interesting comparison.

Dr. Sherwood:

We have only touched the surface of the potential utility of this system. Hopefully, by using this system, we will be able to answer the questions you ask as well as a variety of other questions.

TISSUE SPECIFICITY AND CELL DEATH ARE ASSOCIATED WITH SPECIFIC ALTERATIONS IN NUCLEAR MATRIX PROTEINS*

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ABSTRACT

In the same animal, the ventral prostate and the seminal vesicle are both sensitive to androgen activation of tissue specific genes. We have reported that the nuclear matrix is the DNA organizing structure and is the location of actively transcribed genes as well as an acceptor site of the androgen receptor. In the present study, we have isolated nuclear matrices from seminal vesicle and ventral prostate of both normal and castrate rats, and compared each nuclear matrix preparation by high resolution two dimensional gel electrophoresis. We found that tissue specificity of the ventral prostate and the seminal vesicle resides within the protein composition of the nuclear matrix. The nuclear matrix is not only a determinant of tissue specificity but it also undergoes characteristic alterations during hormone action and cell death. In addition to novel proteins, phosphorylation of the nuclear matrix proteins is important in the determination of the tissue specific gene expression.

Nuclear Matrix Proteins

kD	96	95	38	31	95	52	50	48	50	50	110	59	52	105
pI	6.3	6.8	5.1	6.0	6.8	6.5	6.4	6.4	6.3	6.1	6.4	6.4	6.9	6.6

V.P.

int	-	-	-	-	+	+	+	+	-	-	-	-	-	-
cast	-	-	-	-	+	+	+	+	+	+	-	-	-	-

S.V.

int	+	+	+	+	-	-	-	-	-	-	+	+	+	-
cast	+	+	+	+	-	-	-	-	-	-	-	-	-	+

V.P. - ventral prostate; S.V. - seminal vesicle; cast.- 23 hour castrate; int. - intact

*Supported by HHS NIDDKD DK22000

DISCUSSION

Dr Lee:

I think the problem with 2-D gels is that the tool is too powerful. There may be a few spots in there specific to the prostate and castration and it takes you 10 years to identify exactly what they are.

Dr. Getzenberg:

That's true. This work is certainly in the beginning steps, so there is a lot more work ahead. I think it is going to be very interesting.

Question from the floor:

I would like to ask you a theoretical question. I wonder whether you think these matrix differences is a reprogrammable event or is something set there that would never change. In other word, what is the neighboring cells effect on that protein? Do you think it is a stable element?

Dr. Getzenberg:

I think by looking at castration you can see it is not a totally stable element, that there are changes that occur even when you castrate and probably when you do other things. It is just a part of the cell signalling pathway. I think that you start out in the extracellular matrix and you can go through with the actin and the microtubules and the intermediate filaments. Somehow you are transmitting information to the DNA that in turn changes the conformation. This change might be caused by the changes in nuclear matrix proteins, which causes the DNA to take another configuration.

Question from the floor:

So if you put the organ in organ culture, do you think the organ culture isolating matrices would be different?

Dr. Getzenberg:

I have done a number of preliminary phosphorylations by incubating them for four or five hours in the tube and I think you definitely see changes by even incubating for a short period of time, once you take the tissue out. That is a problem in trying to do those studies.

EFFECT OF TRANSFORMATION ON RAT PROSTATIC FIBROBLASTS: ALTERATIONS IN EXTRACELLULAR MATRIX AND CYTOSKELETON GENE EXPRESSION WITH RETENTION OF ANDROGEN RESPONSIVENESS AND ANDROGEN RECEPTOR EXPRESSION

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ABSTRACT

The NbF-1 fibroblast cell line was derived in our laboratory from normal adult rat ventral prostate (Chung et al., *Int. J. Cancer* 43:1179, 1989). Between the 25th and 30th passage the cell line became transformed spontaneously and late passage cells are capable of forming rapidly growing fibrosarcomas in syngeneic animals. In this communication we present the results of experiments designed to determine the effects of spontaneous transformation on NbF-1 cells with regard to karyotypic stability, biochemical properties, gene expression and hormonal sensitivity. Gross chromosomal differences, as assessed by G-banding analyses, between pre-transformed and tumorigenic NbF-1 cells were minor. Tumorigenic NbF-1 cells maintained a normal modal chromosome number and a trisomic chromosome 4 was the only chromosome abnormality that was acquired with tumorigenicity and persisted in the culture with later passages. Transformation, however, resulted in major phenotypic alterations. Extensive analysis of extracellular matrix (ECM) expression revealed profound changes correlating with the acquisition of tumorigenesis. In comparison with early passage NbF-1 cells, tumorigenic NbF-1 cells expressed decreased levels of mRNAs for fibronectin, collagens I, III, and IV, and laminins B1 and B2. The greatest alternation in mRNA expression, a ca. 20-fold decline in transcript levels, was observed for $\alpha 2$ collagen IV. Similarly, synthesis and expression of chondroitin sulfate proteoglycan and glycosaminoglycan declined markedly (ca. 90%) with transformation. Notably, in contrast to ECM, expression of vimentin and actin cytoskeletal protein mRNAs increased (2-2.5 fold) and androgen receptor (AR) mRNA and high affinity androgen receptor, measured by ligand exchange assay, increased slightly (ca. 1.5-fold increase). Transformed NbF-1 cells remain sensitive to mitogenic stimulation by dihydrotestosterone (DHT) in vitro and tumor growth in vivo is androgen responsive. Mitogenic effects of DHT correspond with a rapid induction of ornithine decarboxylase mRNA expression.

These results indicate that: 1. A prostatic cell type can retain androgen responsiveness, AR expression, and apparent AR function despite substantial transformation-associated pleiomorphic change. 2. A highly tumorigenic phenotype can be acquired rapidly and with minimal genetic alteration. 3. In contrast to AR expression, extensive change in ECM and cytoskeletal protein gene expression may occur early in tumorigenic transformation. Inverse changes in ECM and cytoskeletal mRNAs may reflect a regulatory interdependence between the ECM and the cytoskeletal protein systems and is likely to be associated directly with gross cell structure changes that accompany tumorigenicity.

DISCUSSION

Panelist:

We were talking earlier about the expression of type 4 collagen. What evidence do you have that these are mesenchymal cells?

Dr. Freeman:

Well they express interstitial collagens and that is a marker for mesenchyme and on the basis of their tissue culture morphology.

Question from the floor:

Can you differentiate these cells?

Dr. Freeman:

Morphologically there is a tremendous difference. You can just see it in the light microscope.

Question from the floor:

Can you induce differentiation and restore the production of the various factors?

Dr. Freeman:

We haven't really tried those experiments. We have talked about doing experiments like that, but we really haven't done anything like that.

Panelist:

You are going to see a lot more experiments on cell to cell interaction. It is a fascinating subject. I think you have a unique system. You probably have experienced difficulty, after you mix them, to separate them to study individually the mesenchymal and epithelial cell. Do you have any way to get around that?

Dr. Freeman:

We have done experiments where we have combined the epithelial and the mesenchymal cell types in culture under conditions where they are not in contact or are not in contact. So mitogenetic activity of DHT is one parameter that we can measure under conditions where we can separate the epithelial and mesenchymal compartments. We can do that *in vitro*. We also have taken both epithelial cells and mesenchymal cells out of the experimental tumors that have been developed in the lab. We can analyze those to see what effect proliferation in the mixed tumor has on either compartment. So we can put the cells together and then separate them out and look at the effects of cell interaction.

A POTENTIAL ROLE FOR THE MDR-1 GENE IN THE DEVELOPMENT OF ANDROGEN-INDEPENDENT TUMORS

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ABSTRACT

To date, the most powerful weapon in the fight against metastatic prostate cancer is the activation of a spontaneous death process in the tumor cells by androgen withdrawal therapy. Inevitably, however, some of the prostate tumor cells will be unresponsive to the androgen-repressed signals for cell death. These cells continue to proliferate in the absence of androgens, are refractory to further hormonal manipulation and ultimately, lead to the death of the cancer patient. The possibility of preventing the development of this population of cells or of determining the mechanisms involved in preventing these cells from undergoing "androgen-programmed" cell death might increase the survival of patients with prostate cancer.

Prior research from our laboratories have demonstrated that the molecular events during androgen-programmed prostate cell death are remarkably similar to the molecular events associated with the chemotherapeutic-induced programmed death of tumor cells. Recently, a large body of data has shown that a membrane-bound protein, the P-glycoprotein (180 kd) can interfere with chemotherapeutic-induced cell death, resulting in a drug resistant phenotype in tumor cells. The P-glycoprotein is encoded by the MDR-1 gene (for multi-drug resistance) and its expression can enhance the ability of tumor cells to survive in a noxious environment containing cytotoxic agents. Therefore we have initiated a study to determine whether the expression of the MDR-1 gene might be an important factor allowing for the survival of benign and malignant androgen-dependent cells in the androgen-depleted milieu of a rodent following castration. Simple quantitation of the relative mRNA transcript levels for the MDR-1 gene (approximately 4.5 kb) in androgen-dependent tumor cells compared to androgen-independent tumor variants showed significant elevation of the transcript in the androgen-independent tumor lines. The androgen-independent Mat LyLu subline of the Rat Dunning prostate carcinoma model expressed almost 20-fold more mRNA for MDR-1 than the androgen-dependent line, R3327-H while a recurrent Shionogi mouse carcinoma growing in a castrated host demonstrated over 5-fold more MDR-1 transcript than the corresponding androgen-dependent Sc115 tumor growing in an androgenically intact host. Both of these measurements would support a potential role for the MDR-1 gene in the development of androgen-resistance in tumors.

To determine whether this gene product might have relevance to the biology of human prostate cancer, we have initiated a study of human prostate tissue for MDR-1 P-glycoprotein expression with the use of flow cytometric procedures. Fluorescein-labeled anti-P-glycoprotein antibody staining allows the quantitation of cells expressing this antigen in a prostate dispersion. By this technique, 2 specimens of benign human prostate tissues obtained from surgical procedures demonstrated less than 1% positive cells

while an individual specimen of primary prostatic adenocarcinoma contained up to 37% cells positive for P-glycoprotein expression. More extensive evaluation of this parameter should determine its relevance to human prostate cancers.

Of more interest, we also probed mRNA extracted from rat ventral prostate glands at daily intervals after castration to determine whether the MDR-1 transcript might be enriched in the vestigial prostate tissue that survives castration. While we never detected the canonical 4.5 kb transcript in any of the ventral prostate tissues analyzed, we did find that a novel transcript of 2 kb in size, homologous to the MDR-1 probe, was intensely and transiently induced in the ventral prostate following castration. No trace of MDR-1 related transcript could be detected in RNA extracted from normal mature rat ventral prostate while peak induction of a novel 2 kb transcript occurred on the third day following castration. We have not determined whether this smaller transcript is capable of encoding a protein related to the P-glycoprotein, however, its intense induction during the cell death phase of prostate regression prompts further study of this novel MDR-1-related transcript and its potential relationship to androgen-programmed cell death.

DISCUSSION

Panelist:

I have a question regarding to this animal cell line or animal model you use to investigate MDR. That's a gene expression. Have you looked at the protein expression to see what is higher.

Dr. Katz:

Again, this is the pioneer work and we have only looked at the expression of the gene and the next step is to determine whether the protein as well as the gene is elevated in these cells.

Dr. McPhaul:

This gene, the glycoprotein, is a family of genes, is it not?

Dr. Katz:

It is a family, however the main gene that appears to be amplified in coding for the p-glycoprotein and the drug resistance is the MDR1 gene.

Dr. McPhaul:

So it is not at this point that other members of the family play any role in drug resistance. If so, is there a possibility of doing the same studies with the other members of this gene family?

Dr. Katz:

It is certainly possible. Again, this is the gene that has been associated with drug resistance and this is what we employed at this time.

Dr. Isaacs:

Have you seen if there is any resistance to the various chemotherapeutic agents? With that level of expression one might have assumed that they would be fairly refractal. Have you tested that?

Dr. Katz:

No, we haven't looked at that yet, but that's a good point.

Dr. Isaacs:

Do you have any evidence for amplification or was it just increased expression that you've seen?

Dr. Katz:

As of right now we are just seeing increased expression; we haven't done any Southern blot analyses to look at whether the gene is amplified. We don't have any data on that yet.

RELEVANCE OF LOW ANDROGEN LEVELS AND ADRENAL ANDROGENS IN THE GROWTH OF TRANSPLANTABLE HUMAN PROSTATIC CARCINOMAS

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ABSTRACT

The objectives of this study were to investigate the necessity of total androgen ablation therapy in prostate cancer patients and to study the effect of adrenal androgens on prostate tumor growth. The androgen dependent human prostatic tumor lines PC-82 and PC-EW were used as models. Female and castrated male nude mice were transplanted with the PC-82 or PC-EW tumor and simultaneously substituted with Silastic implants containing testosterone (T). When tumors were exponentially growing, T implants were removed for a short period of time (12 and 5 days, respectively) in order to deplete the tumor tissue from androgens. Subsequently, mice were supplemented with Silastic capsules containing cholesterol mixed with different proportions of T. These implants resulted in plasma levels of androgens varying from 0.2 to 20 nmol/l.

The PC-82 tumor started to regress when plasma T levels were below 0.3 nmol/l, whereas reduction of PC-EW tumor burden was induced at a slightly higher plasma T level (0.5 nmol/l). The regression of the PC-EW tumor was significantly faster (t-test $P < 0.005$) than the decrease in PC-82 tumor volume ($74 \pm 10\%$ and $42 \pm 13\%$ in 28 days, respectively). Intratumor concentrations of T and DHT in the various groups of PC-EW tumor bearing mice were significantly smaller than the androgen levels in the PC-82 tumors of the corresponding T supplemented groups, except for the 0 %T-substituted mice. However, the threshold level for T stimulated tumor growth was found to be the same for both tumor lines, i.e. an intratissue DHT concentration of approximately 3 pmol/g tissue (intratumor T levels of 5 to 10 pmol/g tissue). These androgen levels, which are well above the concentrations found in castrated mice, did not result in any net growth of the tumors, although a maximal regression of the tumors was not induced either. From these results it is concluded, that a total withdrawal of androgens is not necessary to prevent growth of the human prostate cancer models PC-82 and PC-EW.

In addition, the same protocol was used for investigation of the growth of the PC-82 tumor in mice supplemented with Silastic implants filled with the main adrenal androgens androstenedione (A) and dehydroepiandrosterone (DHEA). The resulting plasma levels of A and DHEA were 13.5 ± 1.3 and 9.2 ± 1.7 nmol/l, respectively, which is higher than the peripheral levels found in castrated men. Substitution with A led to a plasma T level of 10.7 ± 1.6 nmol/l and accordingly to an increase in PC-82 tumor burden. DHEA substitution did not result in tumor growth stimulation. It is concluded that high levels of A, causing androgen levels exceeding the threshold level for DHT stimulated tumor growth, can play a role in growth activation of the human prostatic carcinoma PC-82.

ACKNOWLEDGEMENT

This study was supported by the Dutch Cancer Society through grant IKR 87.8.

DISCUSSION

Dr. Isaacs:

Does any level of androgen stimulate growth? In other words, is there a continuous dose response curve where there is a critical threshold above which you get growth and below which you don't?

Question from the floor:

Did you inject these human tumor cells into the nude?

Dr. van Weerden:

No, we didn't inject tumor cells, but we transplanted tumor fragments subcutaneously into the mice.

Question from the floor:

Suppose you leave the pieces in the animal without any hormone for a year and then you challenge the animal with hormone. Do you think it will still grow?

Dr. van Weerden:

If we carried out a delayed androgen substitution experiment as we call it, the PC-82 tumor can be stimulated to grow even after long term androgen deprivation (up to 70 days) following tumor transplantation. In contrast, similar experiments with the PC-EW tumor showed that this tumor can not be stimulated to grow after initial androgen deprivation, and thus delayed androgen substitution could not induce any PC-EW tumor growth in these mice.

Question from the floor:

In some systems DHEA is a differentiating hormone. Did the PSA level in these animals respond to DHEA more than testosterone? Have you analyzed them?

Dr. van Weerden:

We didn't analyze them and we didn't look at differentiation of the tumors.

GROWTH-STIMULATING EFFECT OF GROWTH FACTOR(S) FROM ANDROGEN
INDEPENDENT TUMOR CELLS (CS 2-CELL) ON ANDROGEN RESPONSIVE TUMOR CELLS
(SC 115-CELL)

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ABSTRACT

An androgen-responsive cloned cell line (SC 115-cell) from androgen-responsive Shionogi Carcinoma 115 (SC 115), and an androgen-independent cloned cell line (CS 2-cell) from an androgen-independent subline (CS 2) from SC 115 were established. When SC 115-cell and CS 2-cell were co-cultured in the serum-free medium, SC 115-cell grew similarly irrespective of the presence or absence of testosterone (T), although SC 115-cell alone did not proliferate without T. Conditioned medium (CM) from CS 2-cell cultured in the serum-free medium without T, stimulated growth of SC 115-cell and CS 2-cell. The CM showed proliferative activity on SC 115-cell and CS 2-cell in dose-dependent manner. When the concentrated CM was applied to a heparin Ultrogel column, the growth promoting activity was eluted at approximately 1.0 M NaCl. The activity of the partially purified CM was rather resistant to heat and acidic treatments, although it was lost by trypsin treatment. Effect of various growth factors on DNA synthesis of SC 115-cell was examined. Acidic fibroblast growth factor (FGF) stimulated DNA synthesis of SC 115-cell, and much greater effect was observed with basic FGF. The growth promotion rate of basic FGF was similar to that of the CM. Other growth factors examined did not show such stimulative activities. In conclusion, androgen-independent CS 2-cell secreted a growth promoting factor which controlled the growth of CS 2-cell itself and also of parent SC 115-cell in autocrine or paracrine manner.

THE CELLULAR FORM OF HUMAN PROSTATIC ACID PHOSPHATASE MAY FUNCTION AS A PHOSPHOTYROSYL PROTEIN PHOSPHATASE IN CELLS

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INTRODUCTION

Prostate cancer, next to lung cancer, has the highest incidence of any form of cancer affecting men in this country. About 100,000 new cases are diagnosed each year, accounting for more than 17% of all cancer in males. It has been proposed that steroid hormones, chemical carcinogens, viral oncogenes, and immunodeficiency of the aging may play roles in the development of the cancer. However, there is no conclusive causal correlation between these factors and prostate cancer (1,2).

Human prostatic acid phosphatase (PAcP) is a differentiation antigen in prostate epithelial cells (2,3). PAcP has been reported to have several forms in prostate cells, including cellular and secretory forms. Although the secretory form of PAcP serves as an important marker for the diagnosis of cancer, most prostate carcinomas are poorly-differentiated and have a repressed expression of cellular PAcP (2,3).

Tyrosine phosphorylation of proteins has been believed to play a crucial role in the control of cell proliferation (4,6). Tyrosine phosphorylation levels are regulated by a combination of tyrosine kinase activity levels and phosphotyrosyl (p-tyr) protein phosphatase activity levels (7,8). Our studies demonstrated that PAcP has p-tyr phosphatase activity. Therefore, we proposed that the cellular form of PAcP may affect cell growth. In this communication, we present our recent observations which may indicate that the cellular form of PAcP regulates cell growth by interacting with the tyrosine phosphorylation system in prostate cells.

RESULTS AND DISCUSSION

Clinical Observation

Most prostate carcinomas are poorly differentiated and have decreased expression of PAcP (9). Quantitatively, the PAcP activity per unit of normal prostate tissue is approximately 10 times more than that of carcinoma tissues (10). The low level of PAcP could be due to the poor differentiation of cells. It may indicate the possible correlation of the PAcP expression with the prostate cancer.

PAcP has p-tyr Phosphatase Activity

Li *et al.* (11) biochemically co-purified the major p-tyr phosphatase activity with the cellular form of PAcP from prostate tissues. We further confirmed that the purified PAcP has p-tyr phosphatase activity (12). PAcP removes phosphates from tyrosine residues in proteins effectively. This is the first clue that PAcP may play a role in growth regulation.

An Inverse Correlation of Cellular PAcP Activity With the Cell Growth Rate

Using prostate carcinoma cell lines, LNCaP, DU145, and PC-3, as model systems, we observed an inverse correlation of cellular PAcP activity with the growth rate and p-tyr level.

As shown in Table 1, LNCaP cells contain higher PAcP activity with lower p-tyr levels in proteins and slower growth rate than that of DU145 and PC-3 cells.

Using LNCaP cells as the model system, stimulation of cell growth causes the decrease of cellular PAcP activity. For example, androgen, a classical stimulator of the prostate cell growth, caused the increase of the p-tyr level and the growth rate of LNCaP cells (data not shown). Nevertheless, androgen caused the decrease of the specific activity of PAcP in cells. In contrast there was no effect on the activity of another group of acid phosphatase, the tartrate-insensitive acid phosphatase. The androgen effects on the cellular PAcP activity and the growth rate were observed within the 24 h-treatment with dihydrotestosterone (DHT, 10^{-8} M) (Lin, M.F. and DaVolio, J., unpublished observation). While the regular generation time of LNCaP cells is about 60 hrs (Table 1).

The epidermal growth factor (EGF), a recently identified growth stimulator, also caused the decrease of the cellular PAcP activity that correlated with the increase in the growth of LNCaP cells. The EGF effect on the cellular PAcP activity was obtained within a 16 h treatment (data not shown).

A Potential Substrate of PAcP in Cells

For understanding the possible function of PAcP, we tried to identify the putative substrate for PAcP in cells. DU145 cells that express lower PAcP activity, contain higher p-tyr level and higher tyrosine kinase specific activity than LNCaP cells (13). Pre-incubating purified PAcP with DU145 cell lysates caused the decrease of tyrosine kinase specific activity. However, PAcP has no effect on tyrosine kinase specific activity in LNCaP cell lysates. Furthermore, the decrease of tyrosine kinase activity is not due to the dephosphorylation of tyrosine kinase products during the kinase reaction by PAcP (13).

Androgen causes the decrease of the cellular PAcP activity with a 40% increase of tyrosine kinase specific activity in LNCaP cells. The increased tyrosine kinase specific activity in the androgen-treated cell lysate was partially (60%) inhibited by pre-incubated lysates with purified PAcP *in vitro* (Table 2 and ref. 13). Furthermore, there is no effect on tyrosine kinase specific activity by pre-incubating PAcP in control cell lysates. The data, (Table 1 and Table 2), may imply that cellular PAcP interacts with tyrosine kinases and regulates their kinase specific activities.

For understanding the possible interaction of PAcP with tyrosine kinases, LNCaP cells were grown in the presence or absence of L(+)-tartrate. Inhibition of cellular PAcP activity by growing LNCaP cells in the presence of L(+)-tartrate, a classical inhibitor of PAcP, causes the stimulation of tyrosine kinase specific activity and the phosphorylation pattern in cells (Lin, M.F., unpublished observation). The data further suggests that the cellular PAcP interact with tyrosine kinases in cells.

Table 1: The correlation of cellular PAcP activity with the p-tyr level and generation time

Cells	PAcP ^a (nmol/mg/min)	p-tyr (%)	Generation Time (hr)
DU145	4.0	0.10	29
PC-3	6.0	0.07	37
LNCaP	27	0.04	60

^ap-Nitrophenyl phosphate (PNPP) was used as the substrate. The PAcP activity in LNCaP cells may contain both cellular form and secretory form.

Table 2: PAcP effect on tyrosine kinase specific activity in androgen-treated LNCaP cells

PAcP	³² P-Angiotensin(cpm/100 μg) -DHT	+ DHT	Increment
-	5,430(100%)	7,550(100%)	2,120(100%)
+	5,410(99.6%)	6,250(82.8%)	840(40%)

Of several proteins phosphorylated in the plasma membrane from DU145 cells, PAcP preferentially dephosphorylated the phosphorylated EGF receptor at pH 7.2 *in vitro* (14). In addition, the EGF receptor was not the major protein nor the only major phosphoprotein in the membrane preparation. Furthermore, the optimal rate of dephosphorylation of the autophosphorylated EGF receptor complex occurred at neutral pH, the physiological pH. The dephosphorylation of the EGF receptor correlates with a decrease in the tyrosine kinase specific activity (14). This may be a clue to the manner in which PAcP may function inside the cell.

Subcellular Localization of PAcP Isozymes

The subcellular localization of an enzyme affects its function in cells. For example, only the plasma membrane-associated pp60^{v-src} can cause the transformation of cells (15). For understanding whether PAcP may function inside the cell, the subcellular localization of PAcP is a critical issue.

Most tyrosine kinases including growth factor receptor tyrosine kinases are plasma membrane-associated proteins. We therefore examined the possible membrane association of PAcP. PAcP has been reported in association with the plasma membrane. For example, a form of PAcP isoenzyme with a pI range of 5.5 to 5.8 in comparison with 4.8 to 5.0 of the secretory form of PAcP was obtained only by the addition of detergent Tween 80 or Triton X-100 in the purification of PAcP from tissues (16). The requirement of detergent for the PAcP isoenzyme purification indicates the membrane association. In addition, a tartrate-sensitive acid phosphatase activity was observed in the fractionated plasma membrane from LNCaP cells (Lin, M.F., and Clinton, G.M., unpublished observations). The membrane-associated isoenzyme of PAcP has not yet been fully characterized.

Immunofluorescence studies to determine the topology of cellular PAcP in LNCaP cells was performed. Immunofluorescence studies showed no significant staining of viable cells unless they were permeabilized. In permeabilized low density cells, the strong fluorescence staining was observed at the Golgi apparatus. However, in permeabilized high-density/confluent cells, there was an additional staining band appearing in association with the inner surface of plasma membrane (Lin, M.F., Clinton, G.M. and Kabat, D., unpublished observations; Mark Noble, personal communication). The data demonstrated that the subcellular localization of PAcP isoenzymes isoforms is cell-density-dependent. The data that PAcP can only be detected when cells are permeabilized, further indicates that PAcP functions inside the cells.

CONCLUSION

The regulation of protein phosphorylation is a crucial element of cell regulation. This is a cyclical process of phosphorylation and dephosphorylation, which can be regulated at either step. Phosphoprotein phosphatases have been reported to be involved in the signal transduction, cell proliferation, and cell cycle regulation (8).

We have established a novel system that is the prostate cell growth control and the cellular PAcP activity in LNCaP cells. The strong inverse correlation of cellular PAcP activity with the p-tyr level and the cell growth rate indicates that PAcP may play an important role in regulating the p-tyr level. Therefore, the elevation of p-tyr levels in rapidly growing cells may be a direct result of the repressed levels of cellular PAcP activity (7).

The data, taken collectively, indicated that the cellular form of PAcP may function as a negative regulator in cell growth control. The mechanism of this control is by regulating the p-tyr expression in cells (7).

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DISCUSSION

Dr. Jones:

Have you tested the pH optimum of the enzyme-substrates other than the EGF receptor?

Dr. Lin:

No. We tried to identify what could be the *in vivo* substrate first. Otherwise, you might test a whole bunch of artificial substrates and might still get an artificial result. We intend to identify the *in vivo* substrate of PAP for its possible function. The optimum pH for PAP to dephosphorylate the EGF receptor is between 7 to 8. It's an unexpected pH optimum.

Question from the floor:

Has anybody cloned and expressed PAP inside of a cell and seen differential growth effect?

Dr. Lin:

PAP has been cloned by two independent groups, but they haven't got a full length cDNA yet. Therefore, they are unable to express it currently.

EXPRESSION OF PSA IN LNCaP CELLS IN CULTURE

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ABSTRACT

The human prostate cancer cell line LNCaP (lymph node obtained carcinoma of the prostate) was used to study the effects of androgens on the expression of PSA. The PSA protein was measured using the Hybritech Immunoenzymetric Assay, and PSA mRNA levels were measured using a 428 base pair [³²P]-labeled Sty 1 fragment cDNA probe from the noncoding 3' region of the PSA gene with Northern Blot hybridization. Dihydrotestosterone (DHT) and the synthetic androgen, mibolerone, produced a significant stimulatory effect on PSA expression that was independent from any increases in overall DNA levels. This response was significantly reduced when the antiandrogen, hydroxyflutamide, was added to the media. There was no significant effect on PSA expression by diethylstilbestrol (DES) or hydroxyflutamide when compared to controls. These cells remained viable, with low levels of PSA expression, in the absence of androgen. PSA mRNA levels also were stimulated by androgens. These data demonstrate that PSA levels are strongly dependent on the levels of androgens present. Therefore, a normal serum PSA in patients that have received prior hormonal therapy for prostate carcinoma may not represent the volume of viable tumor but rather may reflect the current hormonal milieu.

DISCUSSION

Dr. Lee:

I have no problem accepting your hypothesis that these cells are in a state of quiescence in the absence of androgen. Can you explain in clinical situations where patients are undergoing continuous androgen depletion treatment, why eventually the serum PSA level goes up in the absence of androgen.

Dr. Bilhartz:

Usually the first sign of tumor progression is an elevated PSA, even before you will see a positive bone scan. An elevated PSA may be seen as early as 6 months before clinical progression of the tumor. Prostate carcinoma consists of a heterogeneous tumor population with some androgen dependent cells, androgen responsive cells and androgen independent cells. The androgen sensitive cells and responsive cells produce more PSA than androgen independent cell populations. When you treat a patient with endocrine therapy you selectively kill off the majority of the androgen dependent cells and the PSA falls in direct response to this. The androgen independent cells continue to grow, eventually reach a large enough

volume to where they do produce an elevated serum PSA. Another possible mechanism for elevated serum PSA after endocrine therapy is that PSA production is regulated by androgens at the cellular level. After androgen ablation, the regulatory mechanisms of PSA gene expression may be altered allowing PSA to once again be produced in the absence of androgen.

Panelist:

I have a question about the level of PSA in the blood and in tissues. Dr. Lin mentioned about the PAP in the blood is high and in the tissue is low in tumor situations. Is the PSA going in the same direction? Could you tell us about the transport mechanism of PSA vs. PAP.

Dr. Bilhartz:

I don't think that anyone has worked out the detailed transport mechanisms for PSA. I know that they've found it in high levels in the seminal fluid and it has been shown to have some proteolytic activity, breaking down proteins in the semen. Maybe this is important in semen liquefaction. I don't think that anyone has shown for sure that it is only excreted into the seminal fluid. I have looked at the immunohistochemical staining of PSA in several prostate cancers, but I have not been able to correlate this with serum PSA and to quantitate the expression in the primary tumor with the serum PSA. Androgens may be only one of the factors that turn on PSA secretion. It may be stimulated by other factors such as growth factors in androgen insensitive cells. There is some evidence to suggest that in prostate cancer cells the polarity of PSA secretion towards the acinar lumen is lost. It's not only secreted at the luminal border but it leaks out into the blood stream. Therefore, prostate cancer cells produce less PSA but they are very leaky cells and more of it ends up in the bloodstream.

ALLELIC EXPRESSION OF THE MOUSE *REN-1* GENES IN THE ANTERIOR PROSTATE
(COAGULATING GLAND)

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ABSTRACT

Renin is an aspartyl protease involved in blood pressure homeostasis and electrolyte balance. Inbred strains of mice have either one of two alleles fixed at the *Ren-1* locus on chromosome 1, *Ren-1^c* or *Ren-1^d*. *Ren-1^d* is found in association with a tightly linked duplicated locus, *Ren-2*. While RNA derived from both *Ren-1* alleles and *Ren-2* are detected at equivalent levels in the kidney, locus- and allele-specific expression differences are observed in several extra-renal tissues.

The anterior prostate (coagulating gland) in mice shows dramatic differences in the expression of the highly similar *Ren-1* alleles. The anterior prostate is an abundant source of renin RNA in mice carrying the *Ren-1^c* allele, while strains that harbor *Ren-1^d* (and *Ren-2*) do not accumulate detectable levels of renin RNA in this tissue. The results of genetic analyses, in which the respective *Ren-1* alleles were present on alternative genetic backgrounds, indicate that the *Ren-1* expression patterns observed in the anterior prostate are most likely regulated by closely associated sequences in *cis*. *In situ* hybridization analysis, using a renin cRNA probe, demonstrates that the accumulation of *Ren-1^c* RNA is restricted to the epithelial cells of the anterior prostate. SI nuclease analysis of anterior prostate RNA populations reveals that transcription initiates at the major transcription start site utilized in the kidney and other renin expressing tissues. In addition, a series of upstream initiation sites were also observed, several of which map within a 0.5 kb transposable-like element located 5' of the mouse renin genes. Interestingly, the upstream start sites used in anterior prostate *Ren-1^c* transcription are similar to a series of start sites previously mapped in submandibular gland (SMG) derived *Ren-1^c* and *Ren-2* RNA.

At present we are producing transgenic mice with chimeric constructs containing *Ren-1^c* flanking sequences linked to the reporter gene, SV40 T-antigen. Examination of T-antigen expression in these mice should help to localize *cis*-acting regions which regulate *Ren-1^c* expression in the anterior prostate. Furthermore, since T-antigen is capable of transforming many different cell types, renin directed T-antigen expression may lead to tumor formation and thus may provide an animal model for tumorigenesis in the anterior prostate region.

GENOMIC ALTERATIONS IN PROSTATIC CANCER

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It has become apparent that the development of cancer is associated with, and quite probably the result of, a series of genetic alterations in the cell. These alterations are very specific, as in the case of a point mutation leading to the activation of an oncogene, or the deletion of specific genetic information coding for a suppressor of tumor formation. The great progress that has been made in this area in recent years has provided us with an emerging picture of the molecular events responsible for the development of a number of human tumors (for reviews see 1 and 2). Perhaps the best example of this is the study of colorectal cancer. The pioneering studies by Vogelstein and coworkers (3-5) have delineated a series of genetic events, including DNA hypomethylation, *ras* gene mutations and allelic loss, which are associated with both the initiation and progression of this disease. These and other critical studies indicate that collaboration between oncogene activation and anti-oncogene (suppressor gene) inactivation plays a central role in human carcinogenesis.

We are attempting to identify changes in the genome which are associated with the development and progression of prostatic neoplasia. We have begun by looking for both mutations (of the *K-ras* and *H-ras* genes) and allelic deletions (of sequences on the long arm of chromosome 10). In addition, we have measured the 5-methylcytosine content of the genome of prostatic cancer cells to see if consistent reductions are observed, as has been reported for a variety of human cancers. Finally, we have employed the ³²P postlabeling assay, a highly sensitive method for the detection of modified (adducted) DNA bases, as a means to evaluate the level of DNA damage in prostate cells. Each of these ongoing studies are discussed below.

MATERIALS AND METHODS

Tissue Collection and DNA Preparation

All tissue analyzed was obtained from patients undergoing radical retropubic prostatectomy for clinically localized (B₁ or B₂) prostatic adenocarcinoma. All pathological evaluations of normal and neoplastic samples were performed by one of us (J.I.E.). To prepare samples suitable for analysis (i.e. tumor samples containing >75% tumor cells and the normal samples containing the same proportion of normal cells) the cryostat sectioning method of Goelz et al (6) was used. Briefly, the prostate gland upon surgical removal is bisected and 1-2mm sections of grossly normal and cancerous tissue are cut from the gland and frozen in O.C.T. embedding compound. Cryostat sections are then cut from each tissue piece molecular weight DNA was prepared from these samples following digestion of the tissue with proteinase

K in sodium dodecyl sulfate as previously described (6). DNA was quantitated by the diphenylamine assay, using calf thymus DNA as standard. In some cases, hyperplastic central zone tissue was processed for comparison to normal peripheral and cancer tissue collected from the same gland.

RFLP Analysis of Allelic Loss

10 μ g normal and tumor DNA was digested with a 5 fold excess of either BamHI or MspI according to the manufacturer's instructions and subjected to electrophoresis in adjacent wells in 1.0% agarose gels. After electrophoresis, DNA in the gel is depurinated by incubation in 12 mM HCl for 30 min, followed by transfer onto nylon membrane using 0.4 M NaOH, 0.6 M NaCl as the transfer buffer. Following overnight transfer, DNA is immobilized on the membrane by baking at 80 °C for 0.5 hr. Probes (11UK-8 and HOAT1, obtained from ATCC and as a gift from Dr. V. Ramesh, respectively) were labelled by the oligolabeling technique of Feinberg and Vogelstein (7). Nylon membranes are prehybridized for 1 hr at 42 °C in 6x SSPE, 10x Denhardt's solution, 0.5% SDS, and 100 μ g/ml denatured, sheared, salmon sperm DNA. Labeled probe is hybridized for 18 hr at 42 °C in the same solution described above with 50% formamide and without Denhardt's. The membranes are then washed in 6x SSPE/0.1% SDS at room temp., then in 1x SSPE/0.5% SDS at 37 °C, and finally in 0.1x SSPE/1% SDS at 65 °C for 30 min. The membranes are then exposed to x-ray film with intensifying screens at -70 °C.

Mutation Analysis of Codons 12 and 13 of the K-Ras and H-ras Genes

Samples of genomic DNA (500 ng) from tumor specimens and benign prostatic hyperplasia specimens were amplified using a modification of the polymerase chain reaction as described by Saiki et al (8). 20-mer oligonucleotide primers, and heat-stable Taq polymerase were obtained from Clontech Inc. These primers flank codons 12 and 13 of the K-ras and H-ras genes and allow the amplification of an approximately 100 bp fragment of genomic DNA containing the codons of interest. The PCR synthesis consisted of thirty cycles of denaturation (30 seconds at 96 °C), annealing (15 sec at 56 °C), and extension (30 sec at 74 °C). After the PCR amplification the reaction products were examined on a 4% agarose gel and the DNA was slotted onto nylon membranes which were air dried and exposed to 1 min of UV short wave irradiation. Oligonucleotide probes were labelled and hybridized exactly as described by Rochlitz et al (9). Each of the filters was hybridized to a panel of ³²P end-labeled synthetic oligonucleotide probes corresponding to the wild type and to all possible mutated sequences at codons 12 and 13 of the K-ras and H-ras genes. The hybridization is performed under temperature conditions such that only fully matched hybrids are stable and single base pair mismatches fail to hybridize. The procedure permits the identification of the exact sequence of the mutations.

Measurement of Genomic 5-Methylcytosine Content

Samples of genomic DNA (30 μ g) were exhaustively digested with RNases A and T₁ and hydrolyzed in 88% formic acid for 45 min at 180 °C. The resulting free bases were separated by HPLC, using a Partisil 10SCX column as previously described (10). 5-methylcytosine values were calculated as percent of total cytosine content.

³²P-Postlabeling Assay for DNA Adducts

The postlabeling assay as described by Gupta et al (11), and modified by Reddy and Randerath (12) was carried out as described. Briefly, the method involves digestion of purified genomic DNA to the free nucleotides with a mixture of spleen phosphodiesterase and micrococcal nuclease. Adducted DNA nucleotides are then enriched by an incubation with nuclease P1 which dephosphorylates the normal nucleotides at the 3' position, rendering them inactive as substrates in the subsequent kinase reaction. The adducted DNA molecules, which are not substrates for nuclease P1, are then labeled with gamma-³²P ATP at the 5' position with T4 polynucleotide kinase. The DNA adducts are then separated by 4-dimensional thin layer chromatography on PEI-cellulose plates, and detected as spots by autoradiography.

RESULTS

RFLP Analysis of the Long Arm Of Chromosome 10 in Prostate Cancer Cells

Since confirmation of Knudsen's two hit hypothesis in retinoblastoma by a number of groups (13-15), chromosomal deletions leading to reductions to homozygosity at specific loci have now been detected in a wide variety of human tumors. These include Wilm's tumor, osteosarcoma, bladder cancer, renal cell

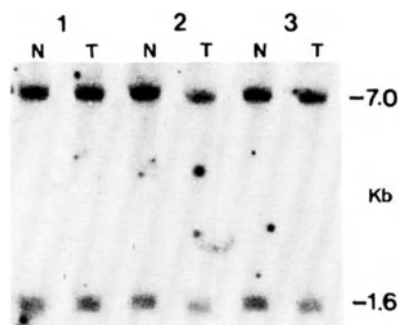


Figure 1: RFLP analysis of normal (N) and tumor (T) DNA from 3 patients with B₁ prostatic adenocarcinoma. Genomic DNA samples were digested with BamHI and probed with pHUK-8 which detects a RFLP on the long arm of chromosome 10 (10q24). These three patients are informative (i.e. have both the 7 kb and the 1.6 kb allele). No deletions are detectable in these tumor samples.

carcinoma, breast cancer, small cell lung cancer, cervical cancer, and colorectal cancer (3,4,16-23). A number of these cancer types are associated with allelic loss at multiple sites. In colorectal cancers, deletions at both 17p and 18q are observed in over 70% of the tumors analyzed. Mapping of the deletions on 17p to the region between 17p12 and 17p13.3 has led to the identification of the p53 gene as a tumor suppressor gene which is inactivated in colorectal cancer (5). Thus, the paradigm established by the study of retinoblastoma may hold for a wide variety of human cancers, with the number of tumor suppressor genes quite possibly being rather large - some being tumor specific and others being inactivated in a number of different tumor types.

To date there have been no reports of allelic loss in prostate cancer. There does exist, however, a series of reports describing karyotypic abnormalities in human prostate cancers and cell lines derived thereof (24-28). The most extensive of these studies was performed by Atkin and Baker (25) who demonstrated that a deleted chromosome 10, del (10)(q24), was found in all four primary prostatic adenocarcinomas examined, leading the authors to suggest that this deletion may be a specific marker for this tumor. A similar deletion had been previously observed by Gibas et al in the LNCaP cell line (24), and has been subsequently observed by Konig et al in the PC-82 cell line (27) as well as by Lundgren et al in a primary prostatic adenocarcinoma (28). Consistent deletion of this region of the genome of prostate cancer cells implicates this site as the location of a tumor suppressor gene whose inactivation is an important step in prostatic carcinogenesis.

To test the hypothesis that sequences located at or near q24 on chromosome 10 tumor were frequently deleted in primary adenocarcinomas of the prostate, we performed restriction fragment length polymorphism (RFLP) analysis of this region of DNA prepared from normal and prostatic cancer tissue. High molecular weight DNA from normal and tumor tissue was digested with either BamHI or MspI, and probed with either HUK-8 or HOAT1, respectively. HUK-8 is a cDNA of the human urokinase gene localized between band 10q24 and q-terminus which reveals a two-allele BamHI RFLP (29). HOAT1 is a cDNA of the human ornithine aminotransferase (OAT) gene localized at band 10q26 which reveals a two-allele MspI RFLP (30). Examples of patients which are informative (heterozygous) with the HUK-8 probe are shown in Figure 1. The data obtained using these probes are summarized in Table 1. Three of 10 patients who were heterozygous at the urokinase locus showed allelic loss. One of these three patients also showed allelic loss at the OAT locus, indicating loss of sequences distal to 10q24. The other three patients who were heterozygous at the OAT locus showed no loss with either probe.

Ras Gene mutations in Prostate Cancer and BPH

We have examined DNA from prostatic adenocarcinoma and BPH tissue from 18 patients for mutations at codons 12 and 13 of the K-ras and H-ras genes. We have employed differential hybridization to detect wild type and mutant alleles in genomic DNA amplified using the polymerase chain reaction. Using probes for all mutations which have been reported to occur in human tumors, we have detected only two mutations - one in codon 12 of K-ras in a BPH sample; the corresponding cancer tissue from this gland did not have the mutation; the other was in codons 12/13 of the H-ras gene in a sample of prostate cancer.

Table 1: Summary of RFLP Analysis

	<u>Probe (map position)</u>	
	pHUK8(10q24)	HOAT(10q26)
Number of patients analyzed	18	18
Number of patients informative	10	4
Number of patients with allelic loss	3	1

DNA Hypomethylation in Prostate Cancer and BPH

To determine if hypomethylation of cytosine residues is associated with development of benign prostatic hyperplasia or carcinoma of the prostate, we have measured the 5-methylcytosine content in 7 sets of prostate tissues. The tissue was divided into normal (peripheral zone [N]), BPH (central zone [B]), and cancer [C], at the time of processing of the surgical specimen and the percent cytosine methylation was determined by HPLC. Mean values for 5-methylcytosine expressed as percent of total cytosine are: N 4.32 % .22; B 4.12 % .14; and C 3.98 % .08. This represents a mean reduction of 5-methylcytosine of 8% in cancer and 5% in BPH. A reduction of 5% is equivalent to replacement of approximately 2.5×10^6 5-methylcytosine residues with cytosine residues per haploid genome. We are currently using the methylation sensitive enzyme HpaII and its insensitive isoschizomer MspI to probe these samples for the presence of hypomethylated sites in specific genes.

DNA Adducts in Prostatic Tissue

DNA damage due to exposure to environmental carcinogens has been shown to play a critical role in the development of a number of human cancers. Carcinogenic compounds are converted by metabolic or chemical activation into electrophiles which can react with nucleophilic centers in DNA, resulting in the formation of covalent adducts. Such adducts, if not eliminated via repair or cell death, may result in a variety of genomic alterations such as point mutations at the time of DNA synthesis. We have employed the highly sensitive ^{32}P postlabeling assay, developed by Randerath and coworkers (11,12), to detect and quantitate DNA adducts in prostatic tissue. We have also examined DNA adducts in human seminal vesicle as a control for tissue which is relatively immune to conditions of abnormal growth. Extensive studies by Randerath's group and others using the postlabeling assay have demonstrated that 1) untreated rats develop DNA adducts which increase in an age-related, tissue specific manner (31); these DNA adducts form in the absence of exposure to exogenous carcinogens, and are presumably the consequence of normal cellular metabolism; 2) chronic estrogen treatment stimulates the formation of DNA adducts in the hamster kidney (32)- a site of estrogen-induced neoplasia; and 3) the level of DNA adducts in the lung tissue of smokers as measured with this assay correlates with the number of cigarettes smoked (33). In an effort to evaluate the possible role of DNA damage in the development of prostatic neoplasia, we have applied the postlabeling technique to analyze DNA samples from prostatic and seminal vesicle tissue. Examples of DNA adducts found in various regions of the prostate are shown in Figure 2. The results obtained using this assay can be summarized as follows:

Normal prostatic tissue from men between the ages of 55 and 65 contains at least 10 distinct adduct spots which range in abundance from 0.1 to 200 adducts per 10^9 nucleotides.

The level of adducts can vary 25 fold between prostates (e.g. from 20 to 500 adducts per 10^9 normal nucleotides - this corresponds to 240 to 6,000 adducts/cell).

Peehl et al previously demonstrated, using the 3T3 transfection assay, that only one of eight prostate tumors contained an activated K-ras gene (35). We have been unable to detect mutations in codons 12 or 13 of

The pattern from the seminal vesicles shares two of the same adducts as the prostate, and there are numerous spots which are unique to each tissue. The overall level of adducts in the prostate is ≈ 2 fold greater than in the seminal vesicles.

The pattern of adducts in both BPH and cancer shows a number of quantitative and qualitative differences when compared to age-matched control tissue.

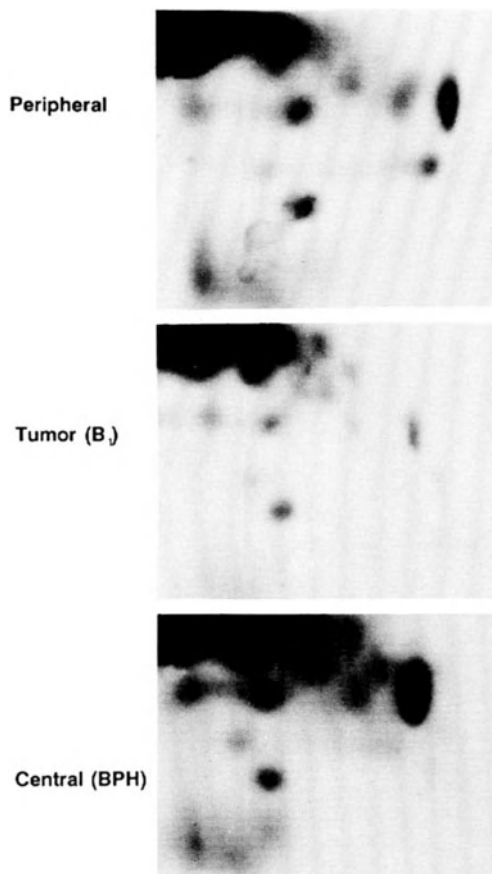


Figure 2: Comparison of DNA adducts in three different regions of a prostate removed from a 64-year-old-man with prostatic adenocarcinoma. The origin is at the upper left. The chromatograms were exposed to x-ray film for 12hrs at -70°C .

We are currently in the process of cataloging the various spots in these samples to examine their correlation with the development of prostatic neoplasia. In addition we are examining the relationship between age and adduct formation to gain insight into the high age relatedness of abnormal prostatic growth. It is possible that age associated increases in DNA damage play an important role in loss of growth control.

SUMMARY

We have taken a variety of approaches to evaluate genetic changes associated with the development of prostate cancer. Preliminary RFLP analysis of sequences on the long arm of chromosome 10 has demonstrated that allelic loss could be detected in approximately 30% of the prostatic cancers analyzed. It is possible that the frequency of allelic loss at this site increases with tumor progression, as has been observed for glioblastoma (34). It is also likely that allelic loss occurs frequently as an early event in prostatic carcinogenesis, but not at the sites we have examined. We are currently pursuing both these questions.

Peehl et al previously demonstrated, using the 3T3 transfection assay, that only one of eight prostate tumors contained an activated *K-ras* gene (35). We have been unable to detect mutations in codons 12 or 13 of the *K-ras* gene in PCR amplified DNA from prostate cancer tissue. Only one mutation has been detected in these codons of the *H-ras* gene. These results suggest that events other than *ras* gene mutations are involved in providing the growth stimulus for prostate cancer cells.

Analysis of the total genomic 5-methylcytosine content of prostate cells indicates that both BPH and carcinoma of the prostate are associated with hypomethylation of the DNA, when compared to

normal peripheral prostate tissue. The association of hypomethylation and neoplasia has been observed in a wide variety of human tumors (36,37). Although there is a wide body of evidence to suggest that this alteration might result in changes in gene expression, the significance of hypomethylation in cancer is unclear at present. One intriguing consequence of hypomethylation of the genome is the possible destabilization of chromosomal structure, leading to karyotypic aberrations at mitosis, as has been suggested by the work of Schmid et al (38).

We have demonstrated that both normal and cancer tissue from the prostate are characterized by the presence of significant quantities of adducted (modified) DNA bases. We plan to examine the relationship between aging and the formation of DNA adducts in the prostate to determine whether the accumulation of these adducts parallels the onset of neoplasia.

ACKNOWLEDGEMENT

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DISCUSSION

Dr. Smith:

Concerning adduct formation, are there any hot spots of adduct formation that are associated with specific DNA sequences that might be predictive of an increased probability of a specific mutation.

Dr. Isaacs:

We have not addressed that question, although that would be a very interesting set of experiments to do. One difficulty in looking at specific sequences is that you need to have a very large amount of tissue to start with. Even in a situation where you have several thousand adducts per cell, you are still talking about adducts that are present on the order of 100 adducts per 10^8 nucleotides, so it becomes somewhat of a technical problem.

Dr. Miller:

Regarding the seminal vesicle data that you presented, are the number of adducts in seminal vesicles paired with prostates from the same patients?

Dr. Isaacs:

Yes. These are all taken from prostates. The ones that we have looked at up to this point have all been from prostates that have been removed via prostatectomy for prostate cancer.

Dr. Miller:

So, in a given gland, the number of adducts that you would see in a given patient's seminal vesicles is less than you would see in his prostate.

Dr. Isaacs:

Right. As far as we can tell.

Dr. Miller:

That's very interesting in view of the age related changes which occur in seminal vesicle nuclei. The nuclei of the involutinal seminal vesicle are often more pleomorphic and aneuploid than their prostatic counterparts.

Dr. Isaacs:

We originally chose seminal vesicle as a control tissue since this organ doesn't develop either hyperplasia or cancer with a very high frequency. I was very surprised to learn that in the aging seminal vesicle you have these severe changes in the nuclei. So we don't really know what the correlation between those are in the development of these adducts.

Dr. Miller:

It might be worth at least going back and looking at the seminal vesicles that you examined to see if they really were involutinal, since your age distribution is relatively low.

Dr. Tindall:

How reproducible is this technique? Have you taken an animal model or a cell line and normalized your values so that you've convinced yourself that you see the same spots each time?

Dr. Isaacs:

We are still in the process of optimizing this technique. Because the assay is operationally defined in the sense that the adducts that you visualize are a function of the solvents that you use, we have been changing the solvents in order to get a large number of adducts in an area in which we could quantitate them. There are changes in mobilities and we cannot, for example, assign a consistent RF to each spot. Some spots we see are very reproducibly, other spots have variability with respect to their mobility in the system. At the present time we just haven't done enough to have a strong feeling for the reproducibility. One of the slides that I didn't discuss was the same sample run four different times and the pattern is virtually identical in each case. That's one of the things that we are concentrating on right now.

Dr. Catalona:

Of your prostate cancer patients where you're looking for the chromosomal deletions, do you know whether those patients had a family history of prostate cancer?

Dr. Isaacs:

I do not know. Dr. Walsh and Dr. Steinberg at Hopkins, along with Bob Carter, have undertaken a large study of familial prostate cancer and have some very interesting results with respect to familial clustering of prostate cancer. We are very interested to look at those particular cases with respect to both deletions and adduct formation, but we have not at this point started this study.

Dr. Thompson:

How extensively have you probed for *K-ras* mutations, what codons, what transitions?

Dr. Isaac:

What we have looked for is all the known mutations that have been reported for codons 12 and 13 of *K-ras*. We have not looked at 61 and we haven't looked at anything else but *K-ras*, so at this point we certainly cannot make a definitive statement about *ras* genes in general or even at 61, but the preliminary evidence, certainly with codons 12 and 13, would suggest that *K-ras* mutations are not commonly seen in prostate cancer.

REGULATION OF GENE EXPRESSION IN THE PROSTATE

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INTRODUCTION

Cloning of the steroid receptors has demonstrated that they belong to a family of transcription factors that contain a highly conserved "zinc finger" motif responsible for DNA binding (1). Using DNA probes to this conserved DNA binding domain, a large number of receptors have been cloned, creating a superfamily of transcription factors. Among the receptors cloned are those for the classic steroid hormones (glucocorticoid, progesterone, estrogen, aldosterone, and androgen) and the related hormones (thyroid, vitamin D₃, and retinoic acid). Additionally, approximately 10 (published) and 15 (unpublished) receptors have been identified whose functions remain unknown (2). These receptors have strong homology to the classic receptors, thus specific ligands are predicted. These new transcription factors have been termed "orphan receptors" (2) in search of a ligand with a specific function.

Once the steroid binds to the nuclear receptor, target genes are expressed. Estrogen and progesterone regulation of chick oviduct genes are the classic examples for steroid regulation of gene expression (3,4). Regulation by glucocorticoids is defined for a large number of genes (5,6,7,8). However, androgen regulation of gene expression remains an enigma. The best model systems for androgen action have not elucidated its mechanism for regulation of a cellular gene (9,10,11). The recent cloning of the androgen receptor (12,13) and a related orphan receptor (14), their clear homology to the other steroid receptors, and their zinc finger motif necessary for binding to DNA, all indicate that they can regulate gene expression through interactions with DNA elements. Androgens do induce transcription via *cis*-DNA elements in the Long Terminal Repeat (LTR) of the Mouse Mammary Tumor Virus (MMTV,15,16), but the DNA sequence for a cellular androgen response element has not been identified. Androgen receptor binding domains within intron DNA (17,18,19) and changes in accumulation of mRNAs due to post-transcriptional (20,21) effects offer a second mechanism of action. Finally, translational regulation of mRNA by androgens (22) may account for a third mode of action.

THE RAT DORSOLATERAL PROSTATE

The prostate is a classic androgen target tissue. Within the rat prostate, four different lobes are defined: ventral, dorsal, lateral, and anterior (coagulating gland). Each lobe is anatomically distinct but the dorsal and lateral lobes, because of size and proximity, are often examined collectively as the dorsolateral prostate. Biochemically, the ventral lobe differs the greatest from the other lobes (19,21) since its protein products are quite distinct. The anterior lobe produces many of the androgen regulated proteins seen in the dorsolateral prostate (23). Isolated mRNA from the dorsal and lateral lobe shows a distinct profile of three abundant and two abundant mRNAs (21), respectively (Fig. 1). Translation of this mRNA in the rabbit reticulocyte cell-free system demonstrates similar protein profiles except for Dorsal Prostate I (DPI;23) which appears as dorsal specific (Fig 1). We have cloned two abundant mRNAs present in both the dorsal and lateral lobe. The androgen regulated RWB mRNA has been identified as coding for the previously describe Seminal Vesicles Secretion II (SVS II), is equally abundant in the seminal vesicles, and is androgen regulated in both organs (24). The M-40 mRNA has been identified as coding for the dorsolateral protein probasin (25,26), which is androgen (27) and zinc regulated (21). We have focused our research on regulation of probasin.

We have developed the probasin (PB) gene as a new model system to study transcriptional regulation by androgens and glucocorticoids (28) and translational regulation of a mRNA (25). We have detected a *cis*-acting androgen response element (ARE) and glucocorticoid response element (GRE) within the same DNA segment of the PB 5'-flanking region. This is the first demonstration that an *in vivo* androgen regulated cellular gene can be regulated transcriptionally by androgens. Further, we have demonstrated that glucocorticoids regulate PB expression in the castrated rat, suggesting a role for this steroid in normal prostatic function. The PB model system also offers a unique bifunctional mRNA that enables a cell to target translation products to secretions within the prostatic ducts and to the nucleus. This supports the notion that two proteins can be produced from a single eukaryotic mRNA by utilization of different translational start codons and that regulation at the initiation site determines intracellular protein distribution. Since PB is a new member of a ligand carrier family of proteins (25), it suggests that the dual location functions to transport a ligand to the prostatic nuclei and seminal fluid.

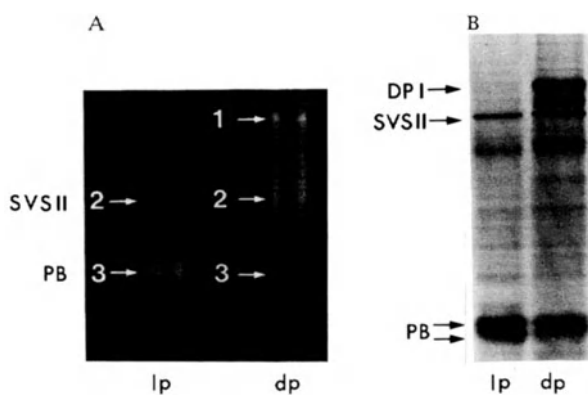


Figure 1: Gene expression in the dorsolateral prostate. The poly(A) + RNA was electrophoresed on a 2% agarose gel containing 10 mM methyl mercury hydroxide (21) and visualized by ethidium bromide staining and UV illumination. Poly(A) + RNA from rat lateral (lp) and dorsal prostate (dp) showed distinct staining patterns. Bands 2 and 3 represent 1550-nucleotide RWB mRNA (SVS II) and 880-nucleotide M-40 mRNA (PB), respectively. Band 1 represents a highly abundant dp mRNA (panel A). Poly(A) + RNA from lp and dp was *in vitro* translated in the rabbit reticulocyte cell-free system containing [³⁵S]methionine and electrophoresed on 15% polyacrylamide - SDS gels (21). The autoradiograph shows the similar nature of translated products between lp and dp. The SVS II and PB protein counterparts are identified. The dp sample contains two high molecular weight proteins one of which represents the DPI protein not seen in the lp sample (panel B).

Transcriptional Regulation of PB Expression

We have demonstrated that after castration the PB mRNA initially decreases in the dorsolateral prostate as expected for an androgen sensitive gene (21,27), but then returns to the precastrated level between 12 to 14 days (29). A second androgen dependent gene, SVS II, does not rebound (29). Using *in situ* hybridization, we determined that PB rebounded after castration in all the epithelial cells of the lateral lobe (29). Our data demonstrates an androgen-independent, yet responsive nature of certain prostatic cells regarding their differentiated function. This androgen-independent nature of certain prostatic cells is manifested in terms of growth since in the absence of testicular androgens the prostate retains 20% of its original size (30). The paradox of how these epithelial cells survive independent of androgens yet require androgens to return the gland to its normal state never has been resolved.

In order to explain the PB mRNA rebound after castration, it is necessary to understand how androgens initially regulate the PB gene and what other agents can replace androgen regulation. Previous attempts to identify a *cis*-acting ARE in mammalian genes have been unsuccessful largely due to the lack of an appropriate androgen responsive recipient cell line. However, it has been possible to characterize androgen response elements within the MMTV long terminal repeat by transfection into ZR-75-1 cells which contain endogenous androgen, glucocorticoid and progesterone receptors (16). Ham et al., demonstrated the MMTV-LTR contained four 15 base pair (bp) DNA elements, that share homology with the consensus GRE. These MMTV hormone response elements induce preferentially by progesterone, then glucocorticoid, and lastly by androgen (16). A GRE in the tyrosine aminotransferase gene also functions as an ARE *in vitro* (31). In addition, differential expression of steroid receptors can govern which steroid regulates the target cell. Expression of the progesterone receptor in a rat hepatoma cell line reprogrammed the endogenous glucocorticoid regulated genes to respond to progesterone (32). Thus, identical responsive elements can act as enhancers for several steroid receptors. *Cis*-DNA elements which are similar in sequence to these hormone response elements can also bind the receptor and function to negative regulate or repress the gene (33,34,35).

Martikainen et al. (36), reported that cortisol is as effective as testosterone in maintaining expression of the prostatic binding protein in rat ventral prostate organ culture. Continuous cortisol treatment of castrated rats retards the process of prostatic involution. This treatment results in a decrease of ventral prostatic weight loss (37), an inhibition of the castration induced increase of plasminogen activators (37), as well as TRPM-2, hsp-70, and c-fos mRNAs, and a retardation of the decrease in C1 prostatic binding protein mRNA (38).

This evidence that glucocorticoids can affect prostatic gene expression and the promiscuous nature of *cis*-DNA elements for both androgens and glucocorticoids was the basis for us to test whether both steroids could influence PB expression. First, to examine the effect of glucocorticoids on the prostate, seven days after castration a group of rats were treated with either dexamethasone (DEX) or testosterone (TEST). The prostatic tissue was removed 48 hours later. Prostatic tissue also was removed from rats seven and nine days post-castration or from intact rats. Seven days after castration, PB mRNA levels decreased to 25% of that observed in intact animals ($P < 0.05$), but by day 9 had rebounded to 44% ($P < 0.05$) compared with controls (Fig. 2). Treatment of 7 day castrated rats by either steroid increased the PB mRNA steady state levels within 48 hours to 82% with TEST and 67% with DEX when compared to intact control levels. For both steroids, this induction was statistically significant from the PB mRNA levels seen at 9 days after castration ($P < 0.05$). A significant drop in dorsolateral tissue weight was observed after castration ($P < 0.05$) and an equally significant increase in tissue weight ($P < 0.05$) was observed after castrated rats were treated with either TEST or DEX (Table 1). The weight of the seminal vesicles, another androgen target tissue, decreased with castration and significantly increased after TEST treatment of the rat. If DEX functioned like an androgen *in vivo*, then all androgen target tissues should increase in weight after treatment of castrated rats with this steroid. DEX treatment of the castrated rat did not affect seminal vesicle tissue weight (Table 1).

Next, to determine if both androgens and glucocorticoids activated PB gene transcriptionally, it was necessary to isolate the 5'-flanking DNA. Presently, only limited choices of cell lines exist which carry endogenous androgen receptors. These cell lines are either difficult to grow or transfect. Thus, it was necessary to develop an androgen receptor containing cell line that was 1) easy to grow, and 2) easy to transfect. Using the complete coding region of the rat androgen receptor (39), we constructed a eukaryotic rat androgen receptor (rAR) expression vector (28) for transfection in human cervical carcinoma (HeLa) cells. We have co-transfected a hybrid PB 5'-flanking region fused to a chloramphenicol acetyltransferase

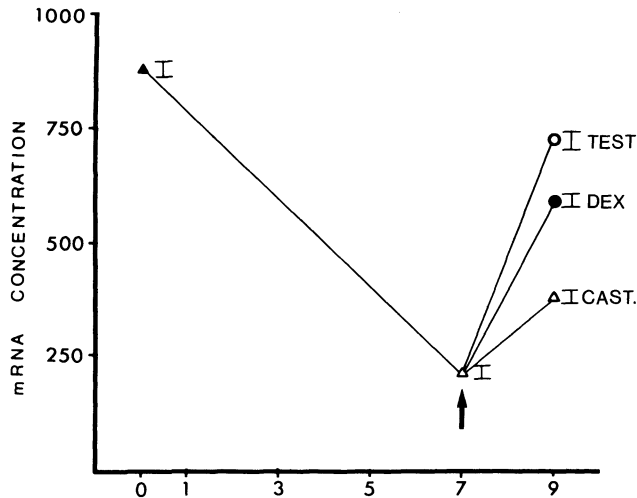


Figure 2: The effect of castration followed by androgen or steroid administration on the dorsolateral prostate. The RNA was isolated from individual dorsolateral prostates (5 rats/group) removed from either intact normal rats (Δ), 7 day castrated rats (Δ), 7 day castrated + 48 hr testosterone ($\circ$), 7 days castrated and 48 hr dexamethasone ($\bullet$) or 9 day castrated (Δ) rats. The isolated RNA was dotted onto nitrocellulose, in duplicate, over a series of dilutions and hybridized with ^{32}P -labeled pM ^{40} cDNA clone (PB cDNA). The counts bound per RNA concentration were analyzed by linear regression. The PB mRNA concentration was expressed as the slope of this line. The decrease in PB mRNA levels at 7 day and 9 day post castration were statistically significant as were the 48 hr increase by TEST or DEX.

Table 1: The effect of steroid treatment on androgen target tissue weight

Treatment	Dorsolateral	Ventral	Seminal Vesicles
Intact	185 $\pm$ 19	318 $\pm$ 25	245 $\pm$ 18
7 C	65 $\pm$ 3	53 $\pm$ 6	103 $\pm$ 6
9 C	60 $\pm$ 0	40 $\pm$ 5	93 $\pm$ 13
7 C + DEX	93 $\pm$ 8*	50 $\pm$ 0*	88 $\pm$ 6
7 C + TEST	115 $\pm$ 12*	68 $\pm$ 8*	175 $\pm$ 17*

After castration (C) tissue was removed 7 (7 C) or 9 (9 C) days later or treatment with either DEX or TEST was begun on the 7th day and tissue was removed on the 9th day. The results are expressed as the mean of tissue weight (mg) $\pm$ standard error of the mean (n=4). * Indicates a significant ($P < 0.05$) increase in weight of treated groups vs. 9 C, using the Student's test.

Table 2: Potential hormone response elements in probasin

Consensus GRE (+)	GGTACAnnnTCTTCT
Probasin GRE/ARE	-240...ATAGCAnnnTGTTCT...-226
Consensus GRE (-)	ATYACNnnnTGATCW
Probasin GRE (-)	-358...TTCAAAAnnnTGATCA...-344
Probasin GRE (-)	-312...CATCTAnnnTGATTG...-299

The consensus GRE (+) and GRE (-) are as reported (1). The numbers indicate the location of the sequences within PB 5'-flanking DNA relation to the start site of transcription.

(CAT) gene with the rAR vector or the glucocorticoid expression vector, rGR (40). Either androgens or glucocorticoids induced CAT activity. This demonstrated that the PB 5'-flanking DNA has a functional ARE and a GRE that transcriptionally activated the gene. Deletion mapping has located both hormone response elements within the same DNA region (28). Examination of the PB 5'-flanking DNA reveals a consensus glucocorticoid receptor binding sequence located within the hormone responsive region of the PB 5'-flanking DNA (Table 2). This consensus sequence is the likely element responsible for both androgen and glucocorticoid regulation.

Further, PB 5'-flanking deletions appear to demonstrate a difference in the manner by which the androgen and glucocorticoid receptors regulate the PB gene. Deletions do not affect the androgen induced CAT activity until the consensus GRE sequence is split. These same deletions demonstrate that glucocorticoids regulate CAT activity increased until the consensus GRE sequence is split. These profiles for CAT regulation by PB 5'-flanking DNA with steroids suggest the presence of repressor sequence(s) specific for glucocorticoid receptor function but not for androgen receptor regulation. Examination of the PB 5'-flanking DNA revealed the presence of consensus sequences for a negative (n) GRE (1,33,34,35) located in the first deleted and second deleted DNA segment that relieved repression (Table 2). This suggests that the PB gene is regulated by repressor elements, perhaps nGREs, and enhancer elements for the androgen and glucocorticoid receptor.

By *in vivo* experiments and transient transfection assays, we have demonstrated the presence of a cellular ARE and GRE in the prostatic PB gene (28). This is the first demonstration that an androgen responsive cellular gene is transcriptionally induced *in vivo* by androgens. Further, it demonstrates a role for glucocorticoids to regulate prostatic function. These data raise the provocative question as to whether glucocorticoids can replace androgens as growth regulators in prostate cancer allowing for the progression of this disease to the androgen-independent state.

TRANSLATIONAL REGULATION OF PB EXPRESSION

Eukaryotic translational initiation usually occurs at the first AUG codon in frame which is in an optimal context, CCACCAUGG (41). However, if the first AUG codon in frame is not in an optimal context, dual initiation may occur from this site in addition to the more favorable site further downstream. This would result in a long and short protein that differ only in the sequence between the these two AUG start sites. The additional amino acids would be N-terminal, a domain often associated with signals for intracellular distribution (42,43). The first report in eukaryotic cells indicating two proteins were synthesized based upon alternate initiation of two in-phase AUG codons was the Ia antigen-associated invariant chain (44). In this instance the relative amount of the two proteins favors the second initiation site as predicted by the favorable context of this AUG. Since then, this mechanism of "leaky scanning" is documented for a number of oncogenes, hormone receptors, and growth factors (42); but, does not explain how initiation occurs when the first AUG is in a good context and the second AUG is in a weak context. The latter group of mRNAs often have a termination codon after the first AUG resulting in a separate short

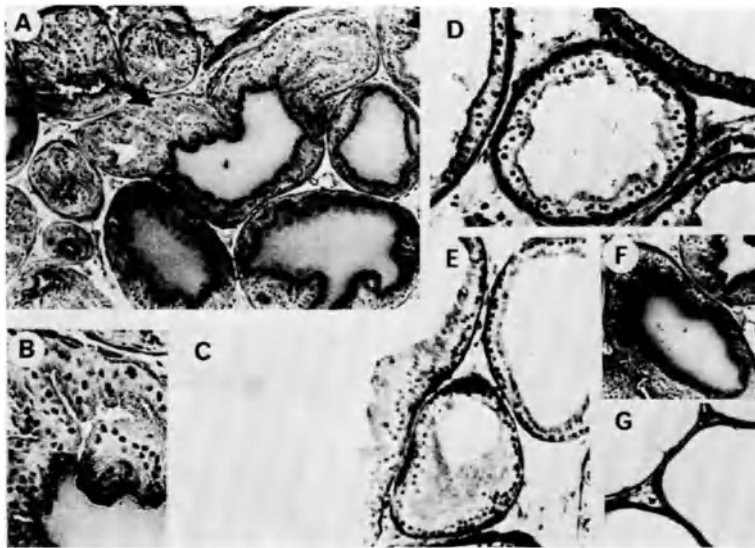


Figure 3: Immunohistochemical localization of a nuclear and secreted probasin product. Photomicrographs to localize probasin were performed with both polyclonal and monoclonal antibody detected by peroxidase-anti-peroxidase complex. Staining is qualitative with the purpose of showing cellular distribution of probasin. Polyclonal antibody to PB shows as follows: Panel A, lateral prostate nuclear and secretory staining, 120X. Arrow indicates a region of two different epithelium within the same duct. Panel B, Enlargement of region noted by arrow in Panel A, 260X; Panel C, control prostate, 80X. Monoclonal antibody to PB shows as follows: Panel D, dorsal prostate nuclear staining, 225X; Panel E, ventral prostate nuclear staining, 225X; Panel F, lateral lobe secretions and nuclear staining, 115X; Panel G, control prostate 100X (reprinted from Ref 25).

peptide from the 5' leader sequence as well as a major protein resulting from reinitiation at the second AUG codon. However, the principle of more than one protein initiating from different AUG codons in higher eukaryotic mRNAs has been established for the Ia (44), and the progesterone receptor (45), while a second initiation site at a non-AUG codon has been reported for the c-myc mRNA (46). The mechanism by which favorable initiation occurs is not fully understood.

We have demonstrated that the M-40 mRNA codes for a secreted protein (21,25) and corresponds to the nuclear protein, PB (25,26). We have unraveled this apparent conflict in M-40/PB localization within ducts as a secretion and within the nucleus by demonstrating that the PB mRNA is a bifunctional mRNA that is translationally regulated *in vivo* (25). Initially, Matuo et al., (47,48) reported that PB was an abundant prostatic nuclear protein. Subsequently, they reported that immunofluorescence staining with an anti-probasin monoclonal antibody localized the protein within prostatic ducts with hardly any staining in the nuclei (49). This limited nuclear staining has been enhanced by achieving efficient nuclear penetration with the antibody (Fig. 3). Dual cellular localization within the ducts and nucleus has been confirmed with the polyclonal and monoclonal antibodies. Perfused fixed and fresh frozen prostatic tissues were sectioned, and immunoperoxidase stained using primary antibody to PB. Both nuclear and cytosolic staining were visualized. Within the lateral lobe epithelium, staining was concentrated along the border of cells revealing secretory granules, within the secretions of the ducts, and in the nucleus (Fig. 3A). Epithelial cells showing nuclear staining, but lacking secretory granules, could be seen in the same ducts and were adjacent to cells showing nuclear and secretory staining (Fig. 3B). The morphology of the lateral epithelial cell layers correlated with PB localization in the nucleus and/or secretions. For example, in cells which formed parts of ducts that have highly convoluted structures, the nuclear protein was present, while cells lining ducts that are not convoluted expressed both nuclear and secreted PB (Fig. 3A). In the dorsal (Fig. 3D) and ventral (Fig. 3E) lobes nuclear staining is seen while secretions are negative.

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      -30      -20      -10      1      10
      *        *        *        *        *
C UUG UCA GUG AGG UCC AGA UAC CUA CAG AGC UCA CAC ACG AUG AGG GUC AUC CUC CUC
                               Met Arg Val Ile Leu Leu

      20      30      40      50      60      70
      *        *        *        *        *        *
CUG CUC ACA CUG GAU GUG CUA GGU GUC UCC AGU AUG AUG ACA GAC AAG AAU CUC AAA
Leu Leu Thr Leu Asp Val Leu Gly Val Ser Ser Met Met Thr Asp Lys Asn Leu Lys

      80      90      100      110      120      130
      *        *        *        *        *        *
AAG AAG AUU GAA GGG AAU UGG AGA ACC GUU UAC UUA GCU GCC AGU AGC GUG GAG AAG
Lys Lys Ile Glu Gly Asn Trp Arg Thr Val Tyr Leu Ala Ala Ser Ser Val Glu Lys

      140      150      160      170      180
      *        *        *        *        *
AUA AAU GAA GGC UCA CCA UUG AGG ACC UAC UUC CGU CGC AUU GAG UGU GGG AAG AGA
Ile Asn Glu Gly Ser Pro Leu Arg Thr Tyr Phe Arg Arg Ile Glu Cys Gly Lys Arg

190      200      210      220      230      240
      *        *        *        *        *        *
UGC AAC CGA AUC AAU CUC UAC UUU UAU AUU AAG AAA GGG GCC AAG UGC CAA CAG UUU
Cys Asn Arg Ile Asn Leu Tyr Phe Tyr Ile Lys Lys Gly Ala Lys Cys Gln Gln Phe

      250      260      270      280      290      300
      *        *        *        *        *        *
AAA AUC GUG GGA AGG AGA UCC CAA GAC GUU UAC UAU GCA AAG UAU GAA GGG AGC ACG
Lys Ile Val Gly Arg Ser Gln Asp Val Tyr Tyr Ala Lys Tyr Glu Gly Ser Thr

      310      320      330      340      350      360
      *        *        *        *        *        *
GCA UUC AUG UUA AAG ACA GUG AAU GAG AAG AUA UUG CUG UUU GAU UAU UUU AAC AGA
Ala Phe Met Leu Lys Thr Val Asn Glu Lys Ile Leu Leu Phe Asp Tyr Phe Asn Arg

      370      380      390      400      410
      *        *        *        *        *
AAC AGA AGA AAU GAC GUU ACA CGA GUG GCU GGA GUU UUG GCG AAA GGC AGG CAA CUG
Asn Arg Arg Asn Asp Val Thr Arg Val Ala Gly Val Leu Ala Lys Gly Arg Gln Leu

      420      430      440      450      460      470
      *        *        *        *        *        *
ACU AAG GAU GAG AUG ACA GAA UAC AUG AAC UUC GUG GAA GAA AUG GGC AUU GAG GAU
Thr Lys Asp Glu Met Thr Glu Tyr Met Asn Phe Val Glu Glu Met Gly Ile Glu Asp

      480      490      500      510      520      530
      *        *        *        *        *        *
GAG AAU GUA CAA CGU GUC AUG GAC ACA GAC ACC UGU CCA AAC AAG AUC AGA AUU AGA
Glu Asn Val Gln Arg Val Met Asp Thr Asp Thr Cys Pro Asn Lys Ile Arg Ile Arg

      540      550      560      570      580
      *        *        *        *        *
UGA CAU CAG GAA UUU UCC ACU AUA UUC UUC CUG GAA CCU GAA ACA UCA AUA UGA AGA
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590      600      610      620      630      640
      *        *        *        *        *        *
UGA AGC AAU CUU UUC UUU CAG AUC AUA UCU UCC UAU UUG CUG CAA AUU ACA AUU CUU

      650      660      670      680      690      700
      *        *        *        *        *        *
GUC UCC AUA CUU UCU CUU UCA UUC AUA CUU UCC CAU GUU CUA AUU GGU AUU AGU ACA

      710      720      730
      *        *        *
UCU UUG AAU GUU UAA AUA AAU CUA UUU CAC UUG C

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Figure 4: Sequence of probasin. The coding nucleotide sequence of probasin mRNA was derived from the cDNA clone (25) that started the sequence at -8 while the remaining 5'-untranslated sequence was obtained from the genomic clone. Initiation of translation at the first in-frame AUG codon (+1) starts a protein containing a hydrophobic sequence that would be absent if initiation occurred at the second in-frame AUG codon (+52). The remaining amino acid sequence and termination codon (--) are the same for both predicted proteins. Sequencing of the N-terminal region of purified probasin demonstrated an a.a. sequence identical (boxed sequence) to the mRNA predicted sequence starting with the second AUG codon (reprinted from Ref 25).

The presence of probasin in two cellular locations could be achieved by translation from two different mRNAs. However, by primer extension and S1 protection analysis we demonstrate the presence of only one PB mRNA (25). Further, differences in expression of a secreted or nuclear protein between epithelial cells is not due to altered cellular PB mRNA levels. This was confirmed by *in situ* cDNA hybridization histochemistry where we show that all epithelial cells of the lateral lobe uniformly express the PB secreted and nuclear mRNA (29). Therefore, the presence of a secreted and nuclear protein must arise from one PB mRNA. This can occur if initiation of translation occurs at two sites within the mRNA. In PB mRNA, the first AUG codon is located at nucleotide +1, the second and third AUG are at nucleotides +52 and +55, respectively (Fig. 4). All three AUG's begin long open reading frames. For this mRNA, the first, then the second AUG codon appear in the most favorable context for initiation of translation. These two reading frames code for proteins of 177 and 160 amino acids with calculated molecular weight of 20,760 and 18,597, respectively. Predicted amino acids 1 to 17, starting at the first AUG codon, are predominantly hydrophobic. Previously, we have demonstrated the processing of a probasin translation product in the presence of microsomal membranes (21), consistent with the presence of a signal peptide for secretion. By the (-3,-1)-rule of von Heijne (50), the predicted site of signal peptide cleavage would be at Ser 17, which would leave a mature protein product beginning with two Met residues.

To demonstrate if a single mRNA can code for two PB proteins, synthetic mRNA transcripts of the pM-40 cDNA from a bacteriophage promoter system were produced with various deletions of the 5' end (25). The synthetic PB mRNA transcribed from a series of these constructions and total dorsolateral prostate mRNA were *in vitro* translated in rabbit reticulocyte lysate (Fig. 5). Synthetic PB mRNA that includes the first, second, and third AUG initiates to produce two proteins. Synthetic PB mRNAs which lack the untranslated region and first AUG, and various portions of the signal sequence, produced a protein which initiates at the second set of AUG codons. Synthetic PB mRNA lacking the second set of AUG codons did not produce an *in vitro* translation product (data not shown). These constructions confirm that the two proteins are not the result of a possible frameshift.

This regulation occurs due to secondary structure within the PB mRNA sequence, as demonstrated by *in vitro* translation of synthetic mRNA (Fig. 5). Protein interactions may be involved in this secondary structure. For example, the ferritin mRNA contains a stem-loop iron response element in the 5' untranslated region. Translational regulation occurs in ferritin mRNA via the binding of a specific cytosolic protein to this stem-loop structure (51,52). When iron is added, this protein is removed and the ferritin mRNA is translated (52). This has been proven by adding the ferritin stem-loop leader to the CAT gene. Transfection into cells of this chimaeric gene produces a CAT mRNA that is iron regulated (51,52). Binding of a cytosolic protein to the stem-loop structure will inhibit translation of the CAT mRNA in the absence of iron. Addition of iron will increase translation of this hybrid CAT mRNA. A mutation of one nucleotide in the six that form the loop structure prevents binding of the protein and results in a loss of regulation by iron (52). Thus, the binding of a cytosolic protein to the stem-loop structure acts as a repressor which prevents translation; this protein is removed in the presence of iron allowing mRNA translation. Since translational regulation occurs in ferritin mRNA due to the presence of a stem-loop structure in the leader sequence (51,52), we have examined the PB mRNA untranslated region for a similar structure. Additionally, PB is a member of a ligand carrier protein family of proteins (25), so we examined the untranslated region of the two closest related members, rat alpha-2-microglobulin, A2u-G (53), and the mouse analog, major urinary protein, MUP (54). These three mRNAs contain sequences within the 5'-untranslated region that could form a stem-loop element (Fig. 6). This raises the possibility that the stem-loop structure is involved in translational regulation of these mRNAs. This is consistent with recent report that androgens increase A2u G protein synthesis without any change in mRNA levels (22). Further, no increased nuclear androgen receptor was detected but a cytosolic androgen binding protein was necessary for A2u-G mRNA expression (22,55). This raises the possibility that translational regulation for the A2u-G mRNA occurs via this cytosolic androgen binding protein.

Our data presents compelling evidence for *in vivo* translational regulation of the PB mRNA. It supports the notion that 1) two proteins can be produced from a single eukaryotic mRNA by utilization of a different translational start codon; and 2) that regulation at the initiation site of translation plays a role in intracellular protein distribution. Further, the presence of PB as a nuclear and secreted protein indicates that this bifunctional mRNA is producing two biologically important ligand carrier proteins. The nature of the common ligand and its' function in the secretions and the nucleus raise provocative questions.

Figure 5: *In vitro* translation of synthetic probasin mRNA. By Kozak's rules, initiation of translation for the bifunctional probasin mRNA could occur at Met AUG codons at +1, +52, and +55 (see panel A). The first two AUG codons appear in the most favorable context and sequencing of the purified protein confirm both Met 18 and 19 are present in the mature protein. Total dorsolateral prostate mRNA translated in rabbit reticulocyte lysate resulted in two probasin proteins (see panel B, lane 1, arrows) as previously defined by hybrid arrested translation (27). Synthetic PB mRNA which contained G's from the 5' end cloning site starting at nucleotide -8 produced two probasin proteins (lane 2) where initiation of translation at the second start site was markedly higher than lane 1. Synthetic PB mRNA which lacked the 5' end G-tail and starts at nucleotide -12 produces two PB proteins (lane 3) similar in ratio to lane 1. Synthetic PB mRNA which remove the first Met residue and including up to amino acid 8, or 11, or 13, (Fig. 4), initiated translation at the second start site (lanes 4, 5, and 6, respectively).

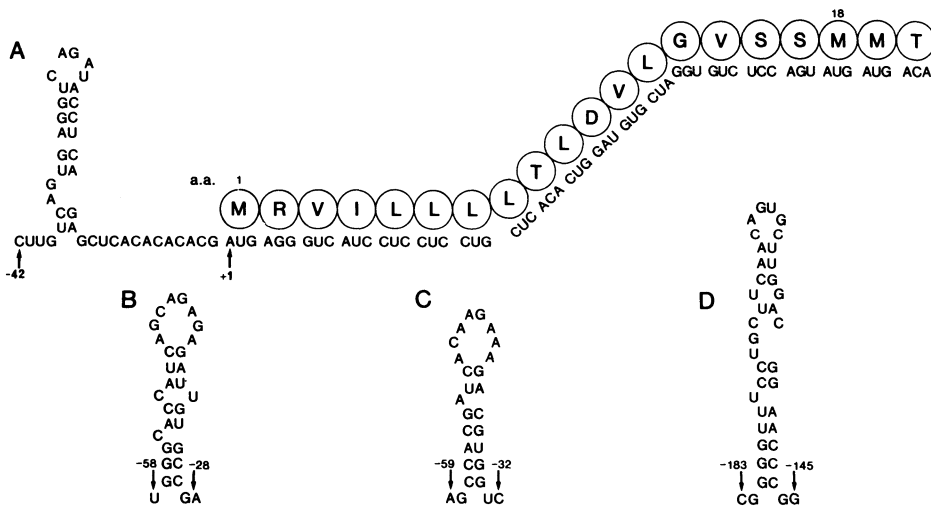


Figure 6: Possible stem-loop structure within the 5'-untranslated region of PB. The 5'-untranslated region of PB was examined to determine if RNA could form a stem-loop structure. A RNA stem-loop structure with the PB-mRNA is possible (panel A). Similar structures could form in A2u-G (panel B) and the mouse analog MUP (panel C). This predicted stem-loop structure is similar to the stem-loop structure in ferritin mRNA (panel D) which regulates translation of that mRNA.

PB	MRVILL---LTLDVLGVSS	MTDKNLK-KKIEGNWRTVYLAASSV	42
A2u-G	MKLLLLLCLGLTL-VCQHA	EEASSTRGNLDVAKLNGDWFSIIVVANSKR	
OBP	M-VKFLILVIALVSC	AHHENLDISPSEVNGDWRTLYIVADNV	
BLG		LIVTQIMKGLDIQKVAGTWYSLAMAASDI	
PB	EKINEGS-PLRTYFRRIECGKRCNRINLYFYIK-KGAKCQQFKIVGRRSQ-		90
A2u-G	EKIEENG-SMRVFMQHIDVLENSLGFK--FRIK-ENGECRELYLVAYKTPE		
OBP	EKVAEGG-SLRAYFQHMECGDEQQLKIIPNVK-LDSEC-QTHTVVGKHED		
BLG	SLLDAQSAPLRVYV-EELKPTPEGDLLEILLQ-KWENGECAQKKIIAENTKI		
PB	DVYYAK-YEGSTAFML--KTVNEKILLFDYFN-RNRRNDVTRVAGVL-ANG		136
A2u-G	DGEYFVEYDGGNTFTIL-KTDYDRYVMFHLINFKNGETFQIMVL---YGRT		
OBP	-GRYTTDYSGRNYFHVLLKTTDDIIFHNVNVDSEGRNQCDL-VAG----KR		
BLG	PAVKIDALNENKVLVL-DTDYKYYLLFCMENSAEPEQSLACQCL---VRT		
PB	ROLTKDEMTEYMNFEEMGIEDENVQVRMDT-DTCPNKIRIR		177
A2u-G	KDLSSDIKEKFAKLCEAHGITRDNIIIDLTKT-DRQLQARG		
OBP	EDLNKAQKQELRLKLAEEYNIPNENTQHLVPT-DTCNQ		
BLG	PEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI		

Figure 7: Amino acid sequence alignment with the ligand carrier family. Protein similarity was analyzed by the DFASTP program (56) and alignment to three members of the ligand carrier family are presented in ranking order of statistical significance. Rat probasin (PB) is compared to rat alpha-2-microglobulin (A2u-G; Ref 59,60), rat odorant-binding protein (OBP; Ref 57), and bovine beta-lactoglobulin (BLG; Ref 61). The signal peptides are separated from the mature proteins by a space. Inserted gaps (-) are indicated. The number of amino acids in PB are listed at the right. The shaded area represents highly conserved amino acids. Abbreviations of the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Conserved amino acid changes are as follows: acidic, D or E; basic, K or R; aromatic, F or Y; hydrophobic, I=L=M=V or T=S (reprinted from Ref 25).

PB IS A NEW MEMBER OF LIGAND CARRIER FAMILY

The predicted amino acid sequence of PB was used to search a protein database to determine if it was related to any known proteins. Statistical analysis by the DFASTP program (56) revealed a significant similarity of PB when compared to a ligand carrier family of proteins (57,58). PB was significantly related to A2-uG (59,60) and the mouse analog MUP (54), odorant binding protein, OBP (57) and to bovine beta-lactoglobulin, BLG (61; Fig. 7). Other identified ligand carrier family members that scored lower but showed striking conservation in structure with PB, are apolipoprotein D (62), androgen-dependent secretory protein (63), retinol binding protein (64), protein of heterogeneous charge (65), tobacco hornworm insecticynin (66), alpha-1-acid glycoprotein (67), pregnancy-associated endometrial alpha-2-globulin (68), purpurin (69), and protein of Bowman's gland (70). These proteins are low molecular weight (18 to 25 kDa), form dimers, and bind small lipophilic molecules (<300 Da) such as retinol, retinoic acid, steroids, cholesterol, odorants, and heparin (57). The naturally occurring ligand(s) for PB has not been identified; however, PB can be purified by heparin-agarose chromatography (26).

Careful analysis on the structure of this family of proteins have been reported (71,72,73,74). The tertiary structure of BLG and retinol binding protein consists of a alpha-helix, eight antiparallel beta-sheets that form a barrel core which encapsulates the ligand, and a beta-strand that interacts with the same strand in another protein to form a dimer. Even though we lack the predicted tertiary structure of PB as determined by crystallographic techniques, it is likely that the topology of all family members is conserved. Hydropathy plots between PB and other family members demonstrate the same charge distribution with an unusual difference. When PB is compared to other ligand carrier proteins, basic amino acids are substituted for most of the acidic acids except in the alpha-helical domain. This results in PB having a pI>11. The very basic nature of probasin places it in sharp contrast within this family. Evolutionarily, this demonstrates that a basic or acidic charged protein can function as a ligand carrier; however, the acidic domain of the alpha

helix remains highly conserved. This alpha-helix is not directly involved with ligand binding, but its conservation suggests an important function. Among all family members certain amino acids are highly conserved. For example in probasin, the Gly-X-Trp (residues 30 to 33) and the cystine's (residues 79 and 170) align with at least 13 family members. In the first ten amino acids of the mature PB, a lysine rich area (K-N-L-K-K-K) exists. This area is not conserved to the other ligand carrier family members (Fig. 7). This core of positively charged amino acids is similar to the nuclear transport signal of the SV40 large T-antigen (P-K-K-K-R-K-V, 75) and polyoma virus T-antigen (V-S-R-K-R-P-A, 76) which have been documented as essential for nuclear transport. These positively charged cores of amino acids act in nuclear transport for a number of proteins (77).

The retinol binding proteins, which are members of this ligand carrier family (78,79,80), appear in the serum, cytosol, and nucleus. Differences in target tissue distribution, nuclear binding sites, and specificity for retinol and retinoic acid indicate that cellular retinol-binding protein and cellular retinoic acid-binding protein have distinct functions. Cellular retinol and cellular retinoic acid binding protein carry retinol or retinoic acid into the nucleus where it transfers to some chromatin factor(s) which now activates gene expression (81,82,83,84). Specific transcription factors that are members of the receptor superfamily have been identified for retinol and retinoic acid (85, 86) and are the likely targets for the ligand transport system.

Previous "dogma" stated that cytoplasmic receptors were activated by the steroid to enter the nucleus and activate transcription (87). Only the glucocorticoid receptor appears as a cytoplasmic receptor that enters the nucleus through activation by the ligand (87). Present "dogma" now states that most of the steroid receptors are nuclear in location and the steroid or steroid-like ligand enters the nucleus by passive diffusion. However, the possibility that the steroids simply diffuse to the nucleus seems unorthodox for such an important function. In contrast to the retinoid transport system, no parallel shuttle system has been defined to explain how ligands enter the nucleus for other members of this superfamily of receptors (1,2). This known system of ligand carrier proteins raises the possibility that specific carrier proteins for receptor ligands exist that transport these ligands to the nucleus. The presence of PB in the nucleus suggests this transport function. This shuttle system would provide a new level for control of gene expression, offering an opportunity to synthesize agonist or antagonist that would affect this ligand carrier.

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DISCUSSION

Dr. Smith:

You've shown very clearly that both androgens and glucocorticoids can increase expression. What happens when you use the two together?

Dr. Matusik:

No we haven't. The adrenalectomy and the castration experiment I showed are results that are only a week old and we have not done those experiments. Those are obvious ones in addition to looking at adrenalectomized animals alone, castrated animals getting back glucocorticoids, etc. We have not done any of these experiments.

Dr. Coffey:

Is the gene androgen sensitive really because it only drops 50% with castration and you can easily restore that 50% back with glucocorticoid?

Dr. Matusik:

No. We give dexamethazone. It will restore it back to the intact level, but androgens will restore it more rapidly, so androgens are more potent to activate the gene.

Dr. Coffey:

You talked about this being a nuclear transport factor. You showed your anti- PB staining there was some in the cytoplasm. When you put glucocorticoids on it do you see translocation?

Dr. Matusik:

No. That is a working hypothesis. What we can show is secretions and in the nucleus, the staining that you see in the cytoplasm could very well be due to the synthesis of protein there. The protein in the lateral prostate is probably about 10% of the total protein and the dorsal about 3%. It is very abundant. No, we have not tried giving a steroid to see if we could alter the location of the protein. I don't think that will work because of the sheer abundance of the protein. It's going to be impossible to make a judgement call as to where it's moving or if it is moving.

Dr. Lee:

Have you looked at prolactin?

Dr. Matusik:

We have done some work with Gail Prins which we presented at the Endocrine meeting two years ago and we have to write up. What we have demonstrated is that if you put a graft into the kidney capsule, a pituitary graft, you find that the pituitary will produce some substance which will increase expression of mRNAs in the prostate. That can be blocked by bromocryptine. The interesting thing about those experiments was that the ventral prostate prostatic-binding protein mRNA's, which are very, very low in the dorsolateral prostate, were turned on in the dorsolateral prostate by the prolactin to the extent that the dorsolateral message went up to about 4% of the total. This is about one-third of the level for each individual prostatic binding protein mRNA's compared to the ventral. So prolactin does have a very profound effect and I would suspect that as hypophysectomy removes prolactin, this is also affecting the genes, but we haven't looked this.

Dr. Thompson:

You said that there were several days between the period of androgen regulation and the onset of glucocorticoid regulation. What do you think is happening during that time?

Dr. Matusik:

I think it's similar to what happens in prostate cancer. We don't understand how the cancer progresses. There are a lot of inconsistencies in the data and hypothesis. We find that with the cis DNA elements of the probasin gene, there is a negative glucocorticoid responsive element and then it looks like we have a positive one at the same time. Perhaps the negative glucocorticoid responsive element is functioning in the intact animal so the glucocorticoids do not regulate the gene but androgens do, and then for some reason when we castrate an animal we now switch to the positive glucocorticoid element to reinduce the gene. Now I don't know that that is really happening in an animal. I mean that's based upon the cell culture work, the presence of an ARE-GRE, some of the animal work, and we are trying to put together a working hypothesis, but that's the most plausible one that I can think of right now.

There was a paper by Darbre and King about two years ago, in which they demonstrated a reprogramming event basically in terms of regulation of the C3 gene, but what they also reported and previously have reported is that if you maintain the Shionogi cell lines on glucocorticoids, you diminish their progression to the androgen-independent state. They will become androgen independent but it slows them down, suggesting that glucocorticoids can impact on that phenomenon of going to the independent state.

Dr. Tindall:

Did you look at stability of the message to rule out that as a possibility?

Dr. Matusik:

We haven't looked at half-life directly. We have done nuclear run off experiments and they are difficult to interpret. Basically, (I'm contradicting my own data now) if we castrate the animal we see that the rate of transcription decreases about 8-fold, which is fairly dramatic, at least I think it is in this case. If we give back androgens, we can't detect a change in the rate of transcription. That probably isn't surprising because if you look at the prostatic binding protein mRNA's the same problem exists. The nuclear run off experiments don't show much of an effect and perhaps what we are seeing is that we are getting a little effect. I actually agree with the statement Dr. Liao made earlier that one of the prime purposes of androgen regulation of gene expression is mRNA stability. However, now I'm demonstrating that we have an androgen response element that regulates transcription, which is contrary to the previous statement. It must be both.

ANDROGEN REGULATION OF HBGF-I (aFGF) mRNA AND CHARACTERIZATION OF THE
ANDROGEN-RECEPTOR mRNA IN THE HUMAN PROSTATE CARCINOMA CELL LINE -
LNCaP/A-DEP

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SUMMARY

A low serum media was developed for growing LNCaP/A-dep cells. It was demonstrated that testosterone at 10^{-9} M can stimulate growth 5-fold. The anti-androgens, cyproterone acetate and hydroxyflutamide, also stimulates growth of LNCaP/A-dep cells at an optimum concentration of 10^{-7} M. Estradiol 17β stimulates growth at an optimum concentration of 10^{-8} M. Both aFGF/HBGF-I and EGF have an additive affect on growth when added together with androgen, anti-androgens or estrogen. By Northern analysis and *in situ* hybridization the steady state level of aFGF/HBGF-I mRNA is increased 5-20 x after a 72 hr to 5 day treatment with testosterone (10^{-8} M), R1881 (10^{-8} M) or estradiol 17β , either in the presence or absence of EGF. A cDNA to LNCaP/A-dep aFGF/HBGF-I mRNA was isolated and shown to be identical to the normal human aFGF/HBGF-I in the coding region. The unusual positive growth response of LNCaP/A-dep cells to anti-androgens and estradiol- 17β suggested that the androgen receptor may be altered in these prostate carcinoma cells. Several cDNA (AR) clones to the LNCaP/A-dep androgen receptor were isolated and the DNA sequence was determined. A point mutation in the steroid binding domain at amino acid 877 converts a threonine to an alanine in the LNCaP/A-dep AR. This mutation was confirmed by PCR techniques and sequencing the relevant region in LNCaP/A-dep DNA and normal human liver DNA. The role the Thr to Ala mutation plays in steroid-dependent transactivation is now being tested by co-transfection of wild-type AR and LNCaP/A-dep AR expression vectors with MMTV-CAT and rat SVS IV-CAT reporter genes.

INTRODUCTION

The development of model *in vitro* culture systems, in combination with *in vivo* tumor growth, has provided a wealth of information documenting the effects of steroids on growth, and, more importantly, how these steroid-sensitive cells change when grown with or without steroids over extended periods. The study of the mechanisms of development of hormone independence in androgen-responsive systems, may provide clues on how to treat prostate cancers, which eventually develop hormone independence and become lethal to the patient (1).

The human prostate carcinoma cell line, LNCaP and its derivative LNCaP/A-dep (androgen-sensitive), may offer an androgen-responsive *in vitro* system (2,4). LNCaP cells have provided evidence of androgens affect on growth factor production and growth factor receptor number. It has recently been shown that R1881 can increase the number of EGF receptors 2 fold after 6 to 12 hrs exposure to androgens (3). This higher level of EGF-R is maintained for up to 6 days. Also, it has been shown that TGF- β 1 can partly inhibit the stimulatory effect of EGF on proliferation of LNCaP cells in the presence or absence of androgen (5). However, TGF- β 1 did not affect androgen-responsive growth directly. This suggests that androgens may be affecting other growth factors/receptors in the LNCaP model. Recently, Wilding (8), has demonstrated that androgens affect the level of TGF- α produced and secreted by LNCaP cells into the conditioned media (8). TGF- α mRNA levels may also be affected by both androgens and estrogens (8) and Wilding (this symposium).

The role that the HBGF or FGF family of growth factors play in promoting proliferation of normal prostate, prostate carcinoma epithelial cells and mesenchymal cell is just beginning to be appreciated (6,7). Recent evidence indicates that normal and cancerous prostate epithelial cells and prostate epithelial cells grown in culture from young animals express aFGF/HBGF-I mRNA and contain aFGF/HBGF-I activity in cell extracts (9).

The hormone-independent rat Dunning tumor derived AT-3 prostate carcinoma cell line produces extremely high levels of aFGF/HBGF-I mRNA, as well as bFGF/HBGF-II mRNA. *In vivo*, the tumor is androgen-independent and grows rapidly compared to the androgen-dependent R3327PAP Dunning tumor grown *in vivo*. aFGF/HBGF-I mRNA expression from the androgen-responsive solid tumor R3327PAP is mainly in the stromal or mesenchymal elements (9).

No information is available on the role of aFGF/HBGF-I plays in androgen-dependent growth of LNCaP/A-dep cells. This report will begin to address this question.

Recent results also indicate that progestins, estradiol 17 β (but not diethylstilbesterol), and the anti-androgens - hydroxyflutamide (HOFLU), RU23908, and cyproterone acetate (CY) stimulate growth of LNCaP-FGC (10,11). LNCaP cells have approximately 30,000 androgen binding sites with a $K_d = 10^{-9}$ M and all three anti-androgens inhibited specific uptake of radioactive R1881 (11). These results suggest the anti-androgens acting through the androgen-receptor, are stimulating growth of the LNCaP cells.

Sonneschein, et al., again using LNCaP-FGC, demonstrated the cells also respond to progesterone (3×10^{-10} to 3×10^{-8} M) and estradiol 17 β (3×10^{-9} to 3×10^{-7} M), but LNCaP cells do not respond to diethylstilbestrol(DES) (10). They demonstrated that ^{3} H-estradiol 17 β binding could be displaced by androgens and progestogens, but not by DES. R5020 binding was also displaced by androgens and estradiol 17 β . Using monoclonal antibodies against human estrogen receptor, there was no detectable estrogen receptor in the LNCaP-FGC cells (10).

These results suggest that the androgen receptor in LNCaP cells may contain interesting mutations in the steroid binding domain or other regions which allow the androgen receptor to activate growth factor/receptor pathways (positive transactivation) with anti-androgens, progestins and estradiol 17 β . This report will also detail our initial search for mutation in the LNCaP androgen receptor gene.

MATERIALS AND METHODS

Materials

aFGF/HBGF-I and EGF were purchased from Upstate Biotechnology Inc., Lake Placid, NY. DMEM with glutamine, HAM's F12 and other general tissue culture reagents were purchased from Gibco-BRL. ZAPII, packaging extracts and pBlue-script SK plasmid were purchased from Stratagene. Most DNA sequence reagents were purchased from United Biochemicals Inc., Cleveland, OH. A Gene Amp kit for PCR reactions was purchased from Cetus Inc. Oligonucleotides were prepared on an ABI 381A DNA synthesizer. Hydroxyflutamide was a gift from Schering, NJ and cyproterone acetate was a gift of D. Lamb, Baylor. Other compounds were from Sigma Chemicals.

Cell Culture

LNCaP/A-dep cells were obtained from Sheila Judge, University of Chicago and Don Tindall, Mayo Clinic. The LNCaP/A-dep cells were routinely maintained in 0.2% 2 x charcoal-stripped fetal calf

serum in DMEM: F12 (1:1) with insulin (5 µg/ml) transferrin (5 µg/ml) and selenium (10⁻⁸M) supplemented with 10⁻⁸M testosterone (T) and EGF (10 ng/ml). This media is referred to as 0.2% CS/FCS DFITS plus EGF + T.

For large scale growth (10⁸ cells), 20-135 mM dishes were used with 25 ml of media per dish. For cells used in -T (androgen withdrawal) experiments, the cells were first grown for 8-10 days from an initial plating of 10⁶ cells/25 ml. Media was changed every 4-5 days. For -T experiments the media was first removed and replaced without testosterone for 3 days. The media was again removed and replaced for another 3 days. Cells were then harvested for RNA or protein extraction.

In the growth assays, 24-well/1.0 ml/well plates were utilized. After preparing fresh LNCaP cells by mild trypsinization and washing 2 x with DFITS media, the cells were plated at 5,000 cells/well (1.0 ml). On day 1, the first plate was fixed with 4% paraformaldehyde (after 5 min air drying) and stained with 0.1% crystal violet. Growth was then monitored at 6, 11 and 14 to 15 days by the above fixative-staining procedure. The plates were then scanned with an Artec cell density scanner for estimation of cell density (7). Media was carefully changed every 3-4 days without completely removing all of the media from the previous change. Differential cell loss can occur if the cells are exposed to air for even a brief period.

Northern Analysis

Total RNA from LNCaP cells was isolated using the RNazol method (Cinna/Biotech Laboratories, Friendswood, TX). Poly (A⁺)-RNA was prepared by passage over oligo dT cellulose (2 x) and was free of ribosomal RNA as visualized on formaldehyde-formamide agarose gel using ethidium bromide (12). 5 µg of poly (A⁺) from -T, +T and +E2 treated LNCaP/A-dep cells was electrophoresed on denaturing gels and transferred to cellulose-nitrate filters. Antisense RNA probe to human aFGF/HBGF-I was made using a pBlue-script plasmid with the 518 bp ECOR1/Bgl II fragment containing human aFGF/HBGF-I coding region (17) and the RNA transcription kit supplied by Stratagene, San Diego. Hybridization and washes with the RNA probe and filter were carried out following the Stratagene protocol.

In Situ Hybridization

LNCaP cells were plated in DMEM media plus 10% Nu-Serum in Lab-Tek glass slide chambers at 10,000 cells/1.5 ml. After 24 hrs, the media was removed and replaced with just DMEM ± R1881 (10⁻⁸M) for 72 hrs. The details for fixation, hybridization, etc. are given in reference 13.

Screening LNCaP cDNA Library

Poly (A⁺)-RNA from LNCaP/A-dep cells (+T) was used to prepare double-stranded cDNA using an Invitrogen Copy Kit, San Diego, CA. The cDNA was sized selected (600 bp) by gel electrophoresis after addition of ECOR1/NOT 1 adaptors (Invitrogen). The cDNA was ligated to ZAPII ECOR1 arms and packaged using materials and instructions from Stratagene, San Diego.

The library (2 x 10⁶ independent recombinants) was screened with a human aFGF/HBGF-I cDNA probe (518 bp) and with the 713 bp Hind III/ECOR1 fragment from the human androgen receptor cDNA. Positive plaques were purified and the pBlue-Script (pBS) plasmid containing the appropriate insert was prepared using the automatic excision protocol supplied by Stratagene for use with ZAPII clones.

DNA Sequencing

The inserts in the pBS plasmids were sequenced using the reverse and forward primers and Sequenase II supplied in the UBI DNA Sequencing II Kit, (Cleveland, OH). All plasmids were purified by 2 x banding in CsCl (12) or using a Qiagen column (Qiagen, Studio City, CA).

PCR of LNCaP DNA and Human Liver DNA

Normal human liver DNA was purchased from Clontech, Palo Alto, CA. LNCaP DNA was purified by phenol extraction, followed by banding in CsCl (12).

Specific oligo nucleotide primers (24 to 26 base) encompassing exon H (or 8) of the androgen-receptor gene was prepared and purified using OPC columns (ABI, Foster City, CA). A specific 347 bp AR-exon H containing fragment was amplified using the protocol supplied by Cetus in their Gene Amp Kit.

The fragments were gel purified and blunt-end (Hinc II) ligated into pBS plasmid. The DNA sequence was then determined as above.

RESULTS

LNCaP/A-dep cells

The original LNCaP cells were established from a metastatic lesion of human prostate carcinoma previously residing in a lymph node (2). A fast growing colony (FGC) has been in culture for approximately 10 years (LNCaP-FGC). The LNCaP-FGC are androgen responsive, contain androgen receptor and generally grow slow in 10% serum containing media with a doubling time of 3-4 days (2).

Horoszewicz recloned the LNCaP-FGC cells and tested various clones for their androgen responsiveness. Several androgen-"dependent" clones were found to grow very slowly or not at all in charcoal-stripped serum media without steroids. One of these androgen-"dependent" clones, referred to as LNCaP/A-dep, we originally obtained from Dr. Sheila Judge, University of Chicago, and we have been investigating its growth properties and developing a low-serum media for analysis of its androgen-responsive growth factor production, growth factor sensitivity and possible mutations in the androgen receptor.

Low Serum Growth Assay for LNCaP/A-dep Cells

Because we were interested in developing a culture condition in which we could get large differential effects of added steroids or other growth factors, ("unmasking serum factors"), LNCaP/A-dep cells were adapted to grow over a 3 month period in reduced serum concentrations. They were grown in 10% charcoal-stripped (2 x) fetal-calf serum in a 50:50 media of DMEM:Ham's F12 supplemented with 5 µg/ml insulin, 5 µg/ml transferrin and 10⁻⁸M selenium plus T (Testosterone 10⁻⁷M). This media is referred to as 2 x CS/FCS/DFITS plus T. Every 2-3 weeks, the LNCaP/A-dep cells were trypsinized and replated in DFITS with progressively lower 2 x CS/FCS plus T (5% 2% 1% 0.2%). The LNCaP/A-dep cells are now routinely grown in 0.2% 2x CS/FCS plus or minus T at 10⁻⁸M and other growth factors. The growth rate in the presence of 10 ng/ml EGF and/or 10 ng/ml HBGF-I plus 10⁻⁹ to 10⁻⁸M testosterone is equivalent to growth in 10% FCS. What can be shown in this low serum media, is a 7-10 fold growth response to T in the presence of aFGF/HBGF-I or EGF. Testosterone, HBGF-I and EGF are the major mitogenic factors which can replace the serum requirements.

The Effect of T, aFGF/HBGF-I, EGF, TGF-β1, Cyproterone Acetate, (CY) Hydroxylutamide (HOFLU) and Estradiol 17β (E2) on Growth of LNCaP/A-dep Cells.

Using the low serum media (0.2% 2x CS/FCS/DFITS), the affect of T, HBGF-I, EGF, TGF-β1, CY, HOFLU and E2 on LNCaP/A-dep growth was documented (Figure 1, Table 1). Figure 1A shows the affect of T (10⁻⁷M) on growth as observed at 14 days. The plates have been stained with 0.1% crystal violet which gives an approximate estimate of cell number (7). T stimulates growth 3-5 x. T plus HBGF-I or EGF gives a further increase in cell number of 2 x. Both EGF and HBGF-I at 10 ng/ml added together give no further increase. aFGF/HBGF-I or EGF by itself can stimulate growth 3-4 x (3,8).

With the low serum media, we could thus demonstrate a growth requirement for aFGF/HBGF-I. In the presence of T (10⁻⁷M) there is an additive effect of aFGF/HBGF-I or EGF with T. In Figure 1A, the effect of TGF-β1 at 0.1 ng/ml was also tested. TGF-β1 slightly inhibited growth 20% either in the presence or absence of 10⁻⁷M T. TGF-β1 also inhibits EGF stimulated growth, mainly in absence of T and as previously reported (15). TGF-β1 (0.1 to 3 ng/ml) does not significantly block the androgen induced growth of LNCaP/A-dep cells (20% inhibition). This is consistent with the low levels of TGF-β receptors on these LNCaP cells (Wilding, this symposium). Figure 1B and Figure 1C demonstrate the effect of cyproterone acetate (CY) and estradiol 17β (E2) on growth of LNCaP/A-dep cells in the low serum media. CY at 10⁻⁷M stimulated growth 3 x and, as with testosterone, there is an additive affect of either EGF or aFGF/HBGF-I with the CY (Figure 1B).

Likewise, we tested estradiol 17β (E2) in this system and found similar results. E2 stimulates 5 x and E2 plus aFGF/HBGF-I and/or EGF stimulates growth 8 x (Figure 1C). TGF-β1 at 0.1 ng/ml shows little affect on growth either in presence or absence of 10⁻⁷M E2 (Figure 1C).

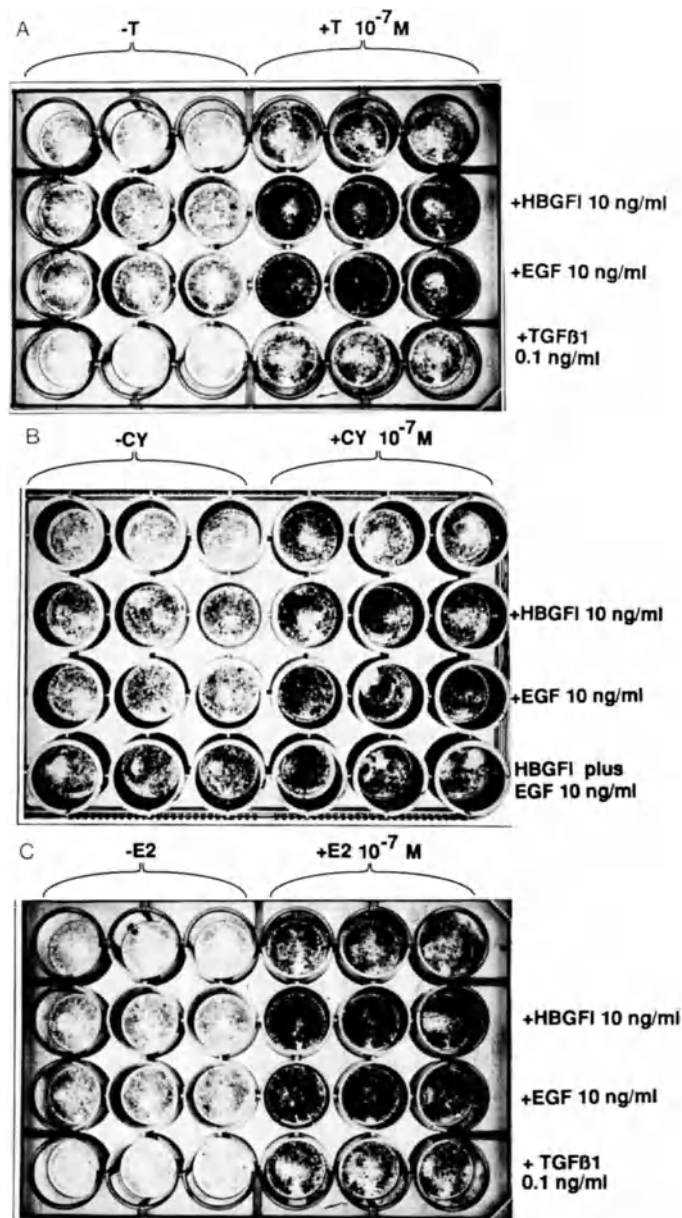


Figure 1: Growth assay of LNCAP/A-dep cells. 24 well plates were fixed and stained with 0.1% crystal violet after growth for 14 days in 0.2% 2 x CS-DFITS media. (a) Effect of testosterone (T) at 10^{-7} M, EGF, aFGF/HBGF-I and TGF- β 1. (b) Effect of cyproterone acetate at 10^{-7} M, aFGF/HBGF-I and EGF. (c) Effect of estradiol 17β (E2) at (10^{-7} M) aFGF/HBGF-I, EGF and TGF- β 1.

Table 1: LNCaP/A-dep Cell Growth in Monolayer in 0.2% 2 x CS/FCS-DFITS

Treatment	Fold increase over -T at 14 days	
Control (-T)	1.0	
T (10M)	5.0	
T + aFGF/HBGF-I		8.2
T + EGF (10 ng/ml)	8.2	
CY (10M)		3.6
CY + aFGF/HBGF-I (10 ng/ml)	5.0	
CY + EGF (10 ng/ml)	4.8	
E2 (10M)		6.5
E2 + aFGF/HBGF-I (10 ng/ml)	8.7	
E2 + EGF (10 ng/ml)	8.7	
HOFLU (10M)		5.8
HOFLU + aFGF/HBGF-I (10 ng/ml)	11.0	
HOFLU + EGF (10 ng/ml)		14.0

EGF = epidermal growth factor

HOFLU = hydroxyflutamide

T = testosterone

CY = cyproterone acetate

E2 = estradiol 17 β

aFGF/HBGF-I = acidic fibroblast growth factor or heparin-binding growth factor 1 2 x CS/FCS - DFITS = twice charcoal-stripped fetal calf serum -

DMEM:F12 (1:1) with insulin (5 μ g/ml), transferrin (5 μ g/ml) and selenium (10M)

Experiments were also performed with hydroxyflutamide (HOFLU), a pure anti-androgens with growth stimulator properties on LNCaP cells (11). Again, we found an additive affect aFGF/HBGF-I and EGF with HOFLU addition.

The fold increase in growth at 14 days in this assay over the -T levels for all compounds and steroids tested are summarized in Table 1.

Figure 2 illustrates a typical growth curve of LNCaP/A-dep cells, initially plated at 5,000 cells/well (1.0 ml). The media was changed 2 x (6th and 10th days) during the 14 day growth assay. The estimated doubling time in 0.2% 2 x CS/FCS DFITS is 8 days. When T is added to the media at 10^{-9} to 10^{-7} M, the doubling time is approximately 4 days, while the addition of aFGF/HBGF-I or EGF to the T containing media decreases the doubling time to about 3 days. There is thus, an additive effect of T and aFGF/HBGF-I or EGF on growth of LNCaP/A-dep cells.

Figure 3 illustrates a dose response curve to aFGF/HBGF-I and EGF in the presence and absence of 10^{-7} M testosterone (T). The optimum response for aFGF/HBGF-I and EGF in the absence or presence of T is 1-10 ng/ml. There is however, a much larger differential affect of aFGF/HBGF-I or EGF on growth of LNCaP/A-dep in the absence of T. This is presumably in part due to testosterone's ability to stimulate TGF- α and aFGF/HBGF-I production and secretion in these prostate cancer cells (see below).

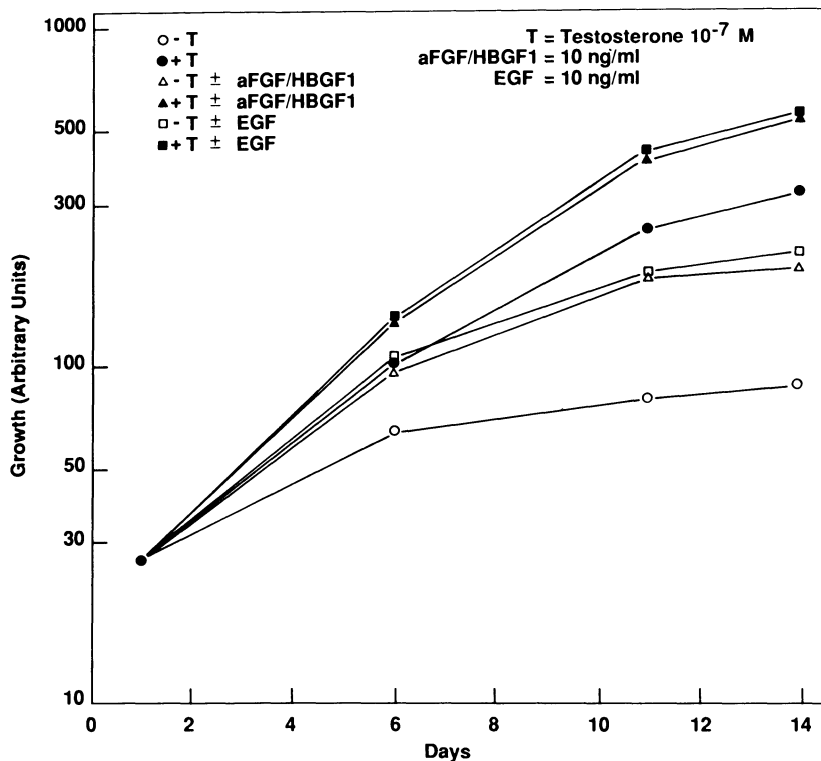


Figure 2: Growth curve of LNCaP/A-dep cells in 0.2% 2 x CS/FCS DFITS media. $\circ$, -T; $\bullet$, +T (10^{-7} M); $\triangle$, -T plus aFGF/HBGF-I at 10 ng/ml; $\blacktriangle$, +T plus aFGF/HBGF-I; $\square$, -T plus EGF 10 ng/ml; $\blacksquare$, +T plus EGF. Each data point represents the average of triplicate determinations with a standard error of less than 10%. Similar results have been obtained in three independent experiments.

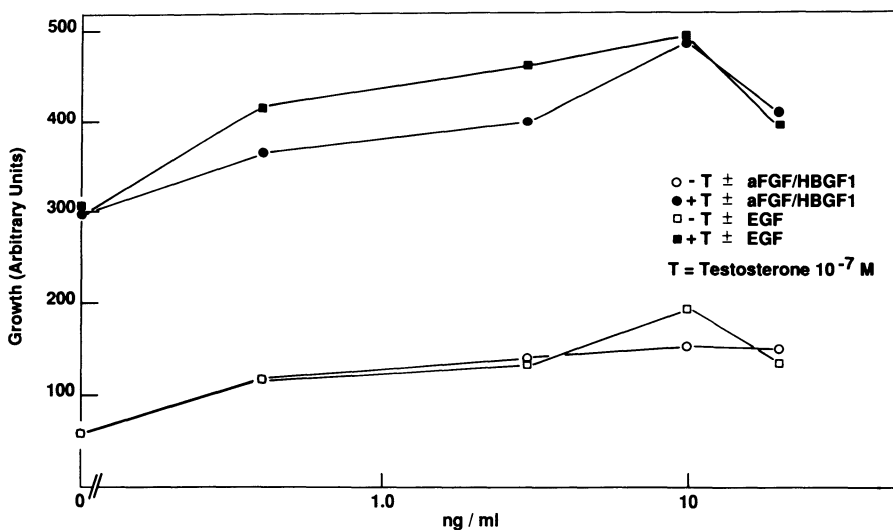


Figure 3: Dose-response (growth) of LNCaP/A-dep to aFGF/HBGF-I and EGF in the presence and absence of 10^{-7} M testosterone (T) $\circ$, -T plus aFGF/HBGF-I; $\bullet$, +T plus aFGF/HBGF-I; $\triangle$, -T plus EGF; $\blacktriangle$, +T plus EGF.

Figure 4 illustrates a dose response curve to testosterone (T) and estradiol 17 β (E2) in the presence and absence of aFGF/HBGF-I. The optimum T concentration is 10⁻⁹M (ED₅₀ = 5 x 10⁻¹⁰M) while the optimum E2 concentration is 10⁻⁸M (ED₅₀ = 3 x 10⁻⁹M). The ED₅₀ of T on growth is approximately the same in either the presence or absence of 10 ng/ml aFGF/HBGF-I. Likewise, the ED₅₀ of E2 on growth is approximately the same in the presence or absence of 10 ng/ml aFGF/HBGF-I. In summary, it takes 10 x more E2 to get the same growth as T whether growth factors are present or not. The ED₅₀ of T on growth (ED₅₀ = 5 x 10⁻¹⁰M) is similar to the estimated Kd (Kd = 3 x 10⁻¹⁰M) for the androgen receptor using ³H-R1881 as ligand (11).

In Figure 5 are photographs of LNCaP/A-dep cells grown in the presence and absence of T and/or CY. Without T in the low-serum media cells grow very slowly over the 14 day culture period. They form flat "webs" of cells which interconnect. However, cells grown in T or T+ growth factors are more rounded and pile up on top of each other. They are much more ruffled on their surface. LNCaP/A-dep cells grown in CY or CY+ growth factor show this behavior of "rounded aggregates" and ruffling even in a more pronounced way than cells grown with T.

Androgen and Estrogen Stimulates aFGF/HBGF-I mRNA Accumulation in LNCaP/A-dep Cells

Figure 6 is a Northern blot of 2 x Oligo dT cellulose purified poly (A⁺)-RNA probed with ³²P-cRNA to human aFGF/HBGF-I. The 4.8, 2.8, 1.8 Kb, and 0.9 Kb aFGF/HBGF-I mRNA hybridizing bands are 10 x more intense in the poly (A⁺) RNA from LNCaP/A-dep cells treated with T and EGF. The RNA from E2 and EGF treated cells also indicated even larger accumulation of the main aFGF/HBGF-I mRNA transcripts. These different sizes of aFGF/HBGF-I mRNA are presumably due to alternate poly (A⁺) addition sites in the long 3'-non-coding region of aFGF/HBGF-I mRNA alternate spliced 5'-non-coding regions, or degradation intermediates (16).

By *in situ* hybridization, we also demonstrate androgen induction of aFGF/HBGF-I mRNA. LNCaP/A-dep cells were plated at low density (5,000 cells/cm²) on Lab-Tek slide/chamber in 10% Nu-Serum/DMEM. This media was removed and replaced with DMEM plus or minus R1881 at 10⁻⁸M for 72 hrs. The cells were fixed and processed for *in situ* hybridization (13). Figure 7 shows the results from one such experiment where we can observe 10 x increases in hybridization with the anti-sense ³²P-cRNA probe to

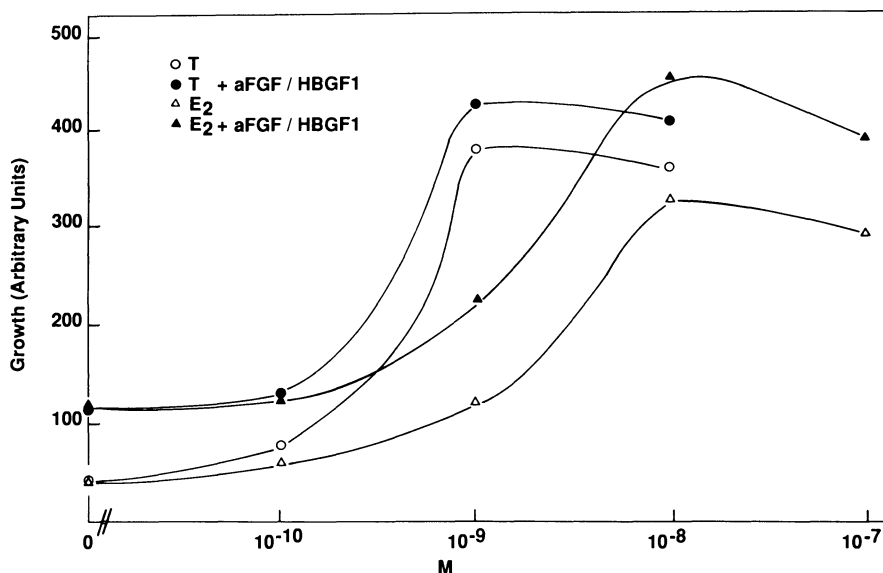


Figure 4: Dose-response of LNCaP/A-dep cells to testosterone (T) or estradiol 17 (E2) in the presence and absence of 10 ng/ml aFGF/HBGF-I. ○, +T; ●, +T + aFGF/HBGF-I; △, +E2; ▲, +E2 + aFGF/HBGF-I.

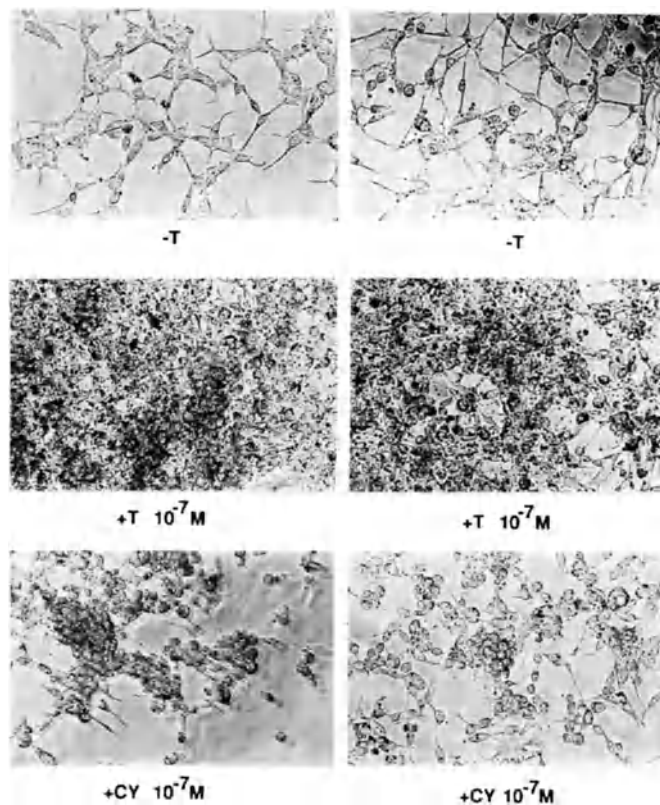


Figure 5: Phase-contrast photomicrograph of LNCaP/A-dep cells grown for 12 days in the absence (-T) or presence of 10^{-7} M testosterone (+T) or 10^{-7} M cyproterone acetate (CY).

human aFGF/HBGF-I cDNA by 72 hrs. By similar *in situ* hybridization experiments, we established that accumulation of androgen induced aFGF/HBGF-I mRNA could not be observed at 16 hrs, but could be observed by 40-72 hrs. Thus aFGF/HBGF-I appears to be a key androgen-induced growth factor in the human prostate carcinoma LNCaP system. Analysis at the protein level is now underway.

Cloning of the LNCaP/A-dep aFGF/HBGF-I cDNA

Eighteen positive signals were isolated using a human HBGF-I cDNA probe (518 bp Eco R1/Bgl II fragment [17]). All clones had short 500 bp inserts. The inserts from two clones were sequenced and found to be identical to the normal human aFGF/HBGF-I cDNA (17). The DNA sequence is shown in Figure 8. It lacks 2 amino acids at the N-terminal and stops two amino acids before the termination signal.

Identification of a Single-Base Pair Mutation in the C-Terminal Region of the Steroid Binding Domain of the LNCaP Androgen Receptor.

From our LNCaP cDNA library, we isolated three independent AR cDNAs (different sizes) and sequenced the DNA binding, hinge and steroid binding domains (Figure 9). In all three AR cDNA clones we found one mutation of an A to G which results in amino acid 877 in the normal AR, a threonine, being converted to an alanine in the LNCaP AR (14). We confirmed the mutation in exon H of the AR gene using LNCaP/A-dep DNA, and normal liver DNA as a control, by PCR reaction as outlined in Figure 10. Figure 11 shows the DNA sequence pattern of LNCaP and normal liver DNA PCR products in the region of interest. Thus with three cDNA clones (no PCR involved) and PCR of genomic DNA, we have confirmed the Thr to Ala mutation in the LNCaP-AR.

DISCUSSION

We have developed a low serum media for analyzing the interacting affect of androgens, anti-androgens, and estrogens with the growth factors EGF and aFGF/HBGF-I on growth of LNCaP/A-dep cells. EGF and/or aFGF/HBGF-1 can essentially replace testosterone (T) if the media is changed sufficient times and fresh growth factor added at appropriate intervals during the growth assay. By growing the LNCaP/A-dep cells in this low serum media (0.2% CS/FCS-DFITS), we revealed a requirement for aFGF/HBGF-I and an additive affect of T plus aFGF/HBGF-I or EGF on growth.

Testosterone increases EGF receptor levels and stimulates secretion of TGF- α from LNCaP cells (8,3 Wilding, this symposium). This is consistent with our results on the additive affect of T and EGF on LNCaP/A-dep cell growth. EGF by itself stimulates growth 4 x (50 to 200 Artec units). However, EGF only stimulates 1.7 x in the presence of T (300 to 500 Artec units). The EGF we add to the media presumably adds to the TGF- α /EGF pool in interacting with higher levels of EGF-R in the T stimulated LNCaP/A-dep cells.

Likewise, similar phenomena are observed with T $\pm$ aFGF/HBGF-I. Again we interpret these results to suggest that T stimulates endogenous aFGF/HBGF-I production by LNCaP/A-dep cells and this endogenously produced aFGF/HBGF-I would be in a "better position" to interact with higher levels of FGF/HBGF-E receptor levels due to endogenously produced aFGF's association with the LNCaP/A-dep extracellular matrix. The levels of aFGF/HBGF-I peptide and levels aFGF/HBGF-I receptor are now being investigated.

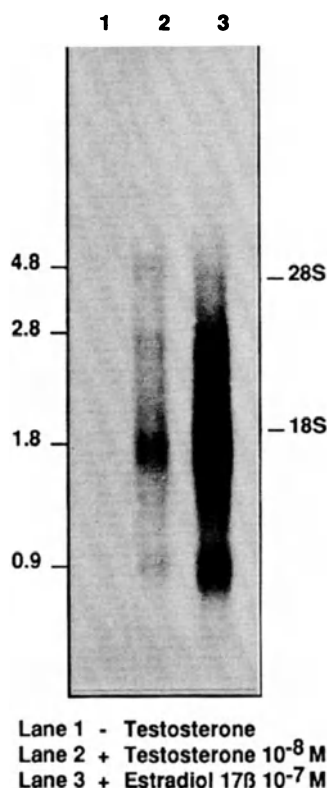


Figure 6: Northern analysis of 2 x Oligo dT- cellulose purified poly (A+)-RNA from LNCaP/A-dep cells grown for 5 days in 0.2% 2 x CS/FCS DFITS plus 5 ng/ml EGF media. Lane 1, -T; lane 2, plus T at 10^{-8} M; and lane 3, plus estradiol 17β at 10^{-7} M. ^{32} P cRNA (antisense) to human aFGF/HBGF-I coding region (518 bp ECOR1-Bgl II fragment) was used as the probe with 10^6 cpm/ml and a specific activity of 8×10^8 cpm/ μ g.

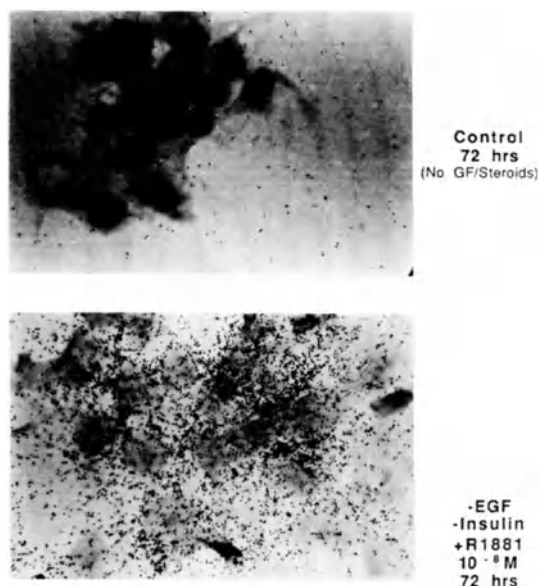


Figure 7: *In situ* hybridization of LNCaP/A-dep cells in DMEM media. ^{32}P -cRNA probe to human aFGF/HBGF-I was used as described in MATERIALS AND METHODS and reference 13. LNCaP/A-dep cells were exposed to 10^{-8}M R1881 for 72 hrs (bottom panel) or no steroid, (top panel). No growth factors were present in the media during the 72 hrs.

We thus began analysis of steady-state aFGF/HBGF-I mRNA levels in LNCaP/A-dep cells grown in low serum media plus and minus testosterone and estradiol 17β . As noted in RESULTS, we observed a large increase in aFGF/HBGF-I mRNAs (4.8 Kb, 2.8 Kb, 1.8 Kb and 0.9 Kb) from cells treated with T + EGF or E2 + EGF vs. EGF alone. The different size bands are possibly due to utilization of alternate poly (A) addition sites and/or intracellular degradation intermediates (16). B-Actin mRNA signals from the same filter blot indicates the 2 x oligo dT poly (A +) RNA in these experiments is undergraded as isolated from the cells.

By *in situ* hybridization experiments we confirmed the androgen induction of aFGF/HBGF-I mRNA using R1881 at 10^{-8}M . By 72 hrs, we observe at 5-10 x induction of hybridizing grains in cells treated with R1881. EGF with androgens enhances aFGF/HBGF-I mRNA expression. EGF by itself does little or nothing to aFGF/HBGF-I mRNA expression as assayed by *in situ* hybridization.

As stated in the RESULTS we isolated several aFGF/HBGF-I cDNA clones from a LNCaP/A-dep cDNA library. The sequence of the presumed coding region of LNCaP/A-dep aFGF/HBGF-I is identical to the normal human aFGF/HBGF-I (17). By *in situ* hybridization little or no bFGF/HBGF-II expression has been found in LNCaP/A-dep cells (data not shown). No unusual oncogenic form of the FGF/HBGF family so far has been found in LNCaP/A-dep cells, but other FGF/HBGF members may be expressed and should be investigated. bFGF/HBGF-II expression seems to be found more often in mesenchymal elements of BPH and slow growing tumors (6,9) and co-expressed with aFGF/HBGF-I in androgen independent rapidly growing, metastatic prostate carcinoma cells, such as rat Dunning tumor AT-3 cells (9).

In summary steroid regulation of aFGF/HBGF-I synthesis and secretion/receptors etc., seems worthy of further investigation as a possible targets for interfering with prostate carcinoma growth, both *in vivo* and *in vitro*.

GGG GAA ATC ACC ACC TTC ACA GCC CTG ACC GAG AAG TTT AAT CTG	57
Gly Glu Ile Thr Thr Phe Thr Ala Leu Thr Glu Lys Phe Asn Leu	
CCT CCA GGG AAT TAC AAG AAG CCC AAA CTC CTC TAC TGT AGC AAC	102
Pro Pro Gly Asn Tyr Lys Lys Pro Lys Leu Leu Tyr Cys Ser Asn	
GGG GGC CAC TTC CTG AGG ATC CTT CCG GAT GGC ACA GTG GAT GGG	147
Gly Gly His Phe Leu Arg Ile Leu Pro Asp Gly Thr Val Asp Gly	
ACA AGG GAC AGG AGC GAC CAG CAC ATT CAG CTG CAG CTC AGT GCG	192
Thr Arg Asp Arg Ser Asp Gln His Ile Gln Leu Gln Leu Ser Ala	
GAA AGC GTG GGG GAG GTG TAT ATA AAG AGT ACC GAG ACT GGC CAG	237
Glu Ser Val Gly Glu Val Tyr Ile Lys Ser Thr Glu Thr Gly Gln	
TAC TTG GCC ATG GAC ACC GAC GGG CTT TTA TAC GGC TCA CAG ACA	282
Tyr Leu Ala Met Asp Thr Asp Gly Leu Leu Tyr Gly Ser Gln Thr	
CCA AAT GAG GAA TGT TTG TTC CTG GAA AGG CTG GAG GAG AAC CAT	327
Pro Asn Glu Glu Cys Leu Phe Leu Glu Arg Leu Glu Glu Asn His	
TAC AAC ACC TAT ATA TCC AAG AAG CAT GCA GAG AAG AAT TGG TTT	372
Tyr Asn Thr Tyr Ile Ser Lys Lys His Ala Glu Lys Asn Trp Phe	
GTT GGC CTC AAG AAG AAT GGG AGC TGC AAA CGC GGT CCT CGG ACT	417
Val Gly Leu Lys Lys Asn Gly Ser Cys Lys Arg Gly Pro Arg Thr	
CAC TAT GGC CAG AAA GCA ATC TTG TTT CTC CCC CTG CCA GTC TCT	462
His Tyr Gly Gln Lys Ala Ile Leu Phe Leu Pro Leu Pro Val Ser	
TCT	
Ser	

Figure 8: DNA sequence of LNCaP/A-dep aFGF/HBGF-I cDNA clone. The aFGF/HBGF-I DNA insert was 465 bp and contained information for 151 of 155 amino acid of the coding region of normal human aFGF/HBGF-I. The first glycine (Gly) in the sequence corresponds to amino acid 3 in human aFGF/HBGF-I.

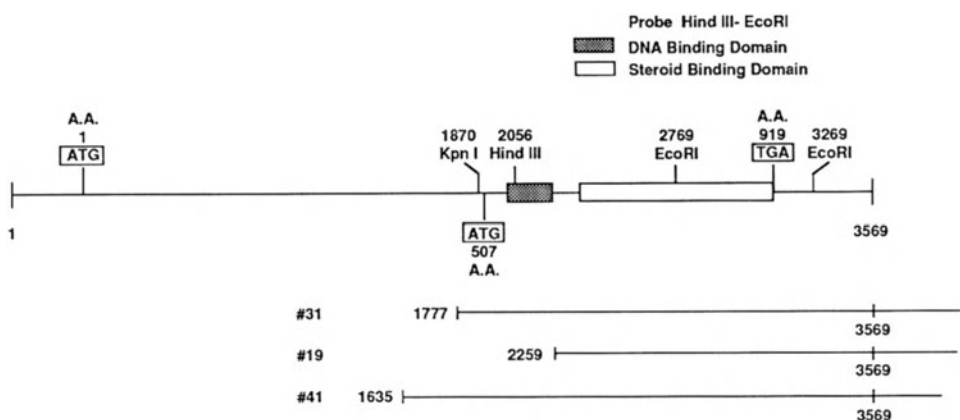


Figure 9: LNCaP-Adep Androgen Receptor cDNA Clones. Restriction map of the human androgen receptor cDNA (14) and diagram of the three independent AR cDNA clones isolated from the LNCaP/A-dep cDNA library.

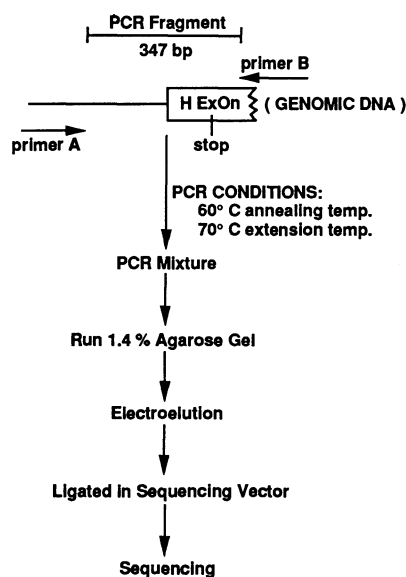


Figure 10: PCR of LNCaP and Normal Liver Genomic DNA. Outline of PCR protocol used to isolate exon H (8) DNA of the androgen receptor gene from normal and LNCaP total genomic DNA.

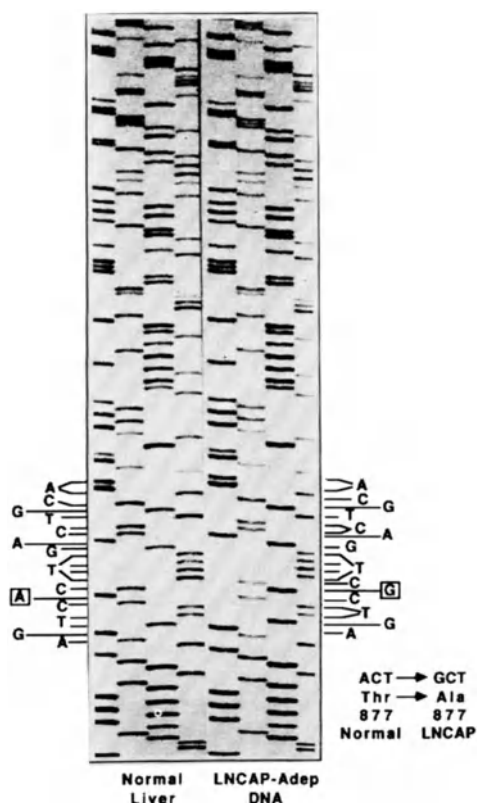


Figure 11: DNA sequence of the region in the AR gene (exon H) in which there is a single-base pair mutation between the normal AR gene (liver) and the LNCaP/A-dep AR gene. This mutation (A to G) converts Thr 877 to Ala 877, using the number system in reference 14.

The peculiar positive growth response of LNCaP/A-dep and induction of aFGF/HBGF-I mRNA cells by cyproterone acetate (CY), hydroxyflutamide (HOFLU) and estrogen is most intriguing (10,11). We have repeated some of the growth experiments in our low serum media and have found similar results (Fig. 1, Table 1) as previously reported (10,11). The data so far suggests that CY, HOFLU and estradiol 17 β are all stimulating the growth "circuits" through the androgen receptor (AR); that is, whatever genes are being activated by testosterone's ability to bind AR and alter the AR such that it acquires the proper *positive* steroid-dependent transactivation functions, can also be carried out by HOFLU, CY and E2. These compounds may be *positive* activators of the androgen receptors steroid-dependent transactivation functions. Although estradiol 17 β binds the "normal" AR at 100 x higher concentrations, it does not usually result in *positive* gene expression of androgen responsive genes. These results imply the LNCaP/A-dep AR may be

In the light of the previous discussion, we undertook an analysis of the DNA sequence of the LNCaP/A-dep androgen receptor. Our AR cDNA clones so far analyzed, encompass amino acids 434 to 919 and 526 bp of 3'-non-coding region. We found only one mutation (A to G) which converts Thr 877 to alanine (Ala 877) (14). The position of this mutation is interesting. Eleven amino acids upstream (Val 866) there is a mutation in the AR of some individuals with androgen insensitive syndrome (Wilson, this symposium). Recently, Parker et al., (18) has mapped an area in the estrogen receptor which plays a role in steroid-dependent (estrogen) transactivation. The mutation in the LNCaP (ala 877) AR is in the same homologous region of the ER that is involved in steroid-dependent transactivation (-47 to -61 amino acids from the c-terminal) (18). We have prepared AR expression vectors with the normal human AR and the LNCaP/A-dep AR and will test the ability of T, CY, HOFLU and E2 to *positively* transactivate the normal and LNCaP/A-dep AR using MMTV-CAT and rat SVS IV-CAT reporter genes as a first step in analyzing this androgen receptor mutation in LNCaP/A-dep cells.

Future studies will involve studying how the androgen receptor is involved in increasing both aFGF/HBGF-I and its mRNA both at the transcription and mRNA turnover levels. These studies should lead to new insights into understanding steroid-regulated growth in general and possibly to new insights in understanding the development of androgenindependent prostate carcinoma.

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DISCUSSION

Dr. Smith:

This mutation in the androgen receptor could have profound clinical significance, since prostate cancer cell growth would be stimulated by estrogen treatment. It is very important to find out how common this mutation is, do you have plans to pursue that?

Dr. Harris:

Yes. The idea that I had was to actually talk to Jim Karr and see if we could find the siblings of this patient and to see if that mutation was actually in the brothers or the sisters, or if there was any of that tissue around. We don't know if this is a tissue culture phenomena. The cells have been in culture since 1982. If there was any normal tissue around, then potentially we could PCR family members to see if the LNCaP AR mutation can be found in their genome. This LNCaP mutation is 11 amino acids from the mutation that Dr. Wilson talked about yesterday, where a valine to methionine seemed to cause the androgen insensitive syndrome. I think that area of the steroid binding domain is going to turn out to be extremely interesting.

Dr. Tindall:

If the C-terminal mutation is responsible for the differential functional activity of these steroids, you would expect that if you simply looked at steroid binding by competition analysis that you would see some unusual differences. Have you done these types of studies to show that there is actually a difference in the steroid binding specificity of the LNCaP receptor?

Dr. Harris:

No, I haven't, but Carlo Sonnenschein and others have studied the steroid binding properties of the LNCaP AR. He has labelled LNCaP androgen receptor with R1881 and then he can compete that off with 17-beta estradiol, but it does not compete with DES. That's very important. The androgen receptor binds estradiol but does not bind DES. He has done the reciprocal experiment of binding tritiated estradiol and can compete that off with various androgens and anti-androgens, but I have myself not done that. One of the critical things about the estrogen binding to the androgen receptor is that it is 17-beta specific and DES will not work.

Dr. Tindall:

That's right, but the normal androgen receptor at reasonably high concentrations of estradiol will be competed, so that's the same as you normally see with the endogenous receptor.

Dr. Harris:

I think what we are talking about is a factor of ten or less. For example, for the normal androgen receptor, it takes on the order of 10^{-7} molar E_2 to begin to see binding. You begin to see competition even in your DNA binding domain experiments and previously published materials. What seems to be the LNCaP case in terms of the ED_{50} of growth response, for example, by estrogens, it is in the 10^{-8} molar range. If you look at testosterone response it is in the 10^{-10} to 10^{-9} molar range. So the mutation may have shifted the estrogen binding to the LNCaP androgen receptor less than 10x.

From the Sonnenschein data, the RBA for estradiol-17-beta is 1 where R1881 binding is set at 100 in these LNCaP cells. We may also be distinguishing binding from biological activity with this LNCaP AR mutation that is altered transactivation functions.

Dr. Tindall:

Maybe you ought to check the effect of diethylstilbestrol on the LNCaP cells.

Dr. Harris:

Well I even have a better idea. Dr. Liao mentioned that he had two phenanthrene ring designed anti-androgens. It's another possibility. I have tried flutamide and it doesn't work, so LNCaP cells do not have the ability to hydroxylate the flutamide.

Dr. French:

I believe Dr. Eppo Mulder and co-workers in Rotterdam have done competition binding studies with the LNCaP androgen receptor and found that the affinity for progesterone was higher than for testosterone. Since cyproterone acetate is a progestin, it might explain or at least correlate with the activity of cyproterone in this tumor cell.

Dr. Smith:

We did some studies a long time ago with LNCaP's and showed their growth was stimulated with progesterone. However, we could not detect the presence of progesterone receptors so we did not publish the data since we had no explanation for the phenomenon.

DNA METHYLATION, DIFFERENTIATION AND CANCER

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INTRODUCTION

The methylation of cytosine residues in DNA is potentially dangerous for living cells for two reasons. Firstly, the application of a methyl group to the 5 position of a cytosine residue increases the rate of spontaneous deamination of the pyrimidine and the product of this deamination cannot be recognized easily by repair enzymes. Thus, 5-methylcytosine is inherently mutagenic and evidence has accumulated to suggest that this mutagenic activity is responsible for a high proportion of spontaneous mutations within the human genome. Secondly, methylation of cytosine residues in DNA is a powerful mechanism for the silencing of vertebrate genes. The inappropriate methylation of controlling regions of genes can result in heritable epigenetic changes in phenotype which are potentially capable of giving rise to cellular immortality and transformation. This review will focus on these two biological properties of DNA methylation as they relate to human cancer.

Biochemistry of DNA Methylation

Approximately 3-4% of cytosine residues in Vertebrate DNA become modified post-synthetically by the application of a methyl group from S-adenosylmethionine specifically to the 5 position of the pyrimidine ring. Almost all of the methylation which occurs in vertebrate DNA is confined to the simple dinucleotide CpG. This palindromic sequence is either completely methylated at both cytosine residues or neither is methylated, with a very small percentage of methylation sites existing in the half-methylated configuration (1). This is most probably due to the fact that the half-methylated state, which is generated after DNA synthesis, serves as the preferred substrate for the DNA methyltransferase enzyme (2-3). The function of this enzyme seems to be the modification of half methylated CpG dinucleotides into the fully methylated configuration thus ensuring the clonal heritability of methylation patterns (4).

The mouse DNA methyltransferase has recently been cloned and codes for a 175 kilodalton protein which has similarities, in the carboxyl terminal of the protein to the catalytic site of bacterial type II methyltransferases (5). Unlike the bacterial DNA methyltransferases, the mammalian enzyme appears to be non-specific for the sequence surrounding the CpG acceptor site and applies the methyl group with the highest efficiency to hemimethylated sites. The function of this enzyme therefore seems to be the copying of existing methylation patterns so that they may be transmitted from cell to cell.

Distribution of Methylation Sites in the Vertebrate Genome

The CpG dinucleotide is unusual in that it is strongly underrepresented in the vertebrate genome occurring approximately one fifth as frequently as would be predicted on a random statistical basis (6). However there are regions of DNA called "CpG islands" in which the dinucleotide occurs at a frequency

close to its expected value (7-9). These islands, which constitute approximately 1% of vertebrate DNA, are often associated with genes and appear to have survived in the genome because they have never been methylated (10). There is little evidence that CpG islands are methylated in the germ line of vertebrates and Bird (8,10) has suggested that they have survived because they have never been modified. The remaining CpG sites are scattered approximately 20 times less frequently through the genome and these sites are often methylated.

The vertebrate genome can therefore be separated into two compartments based on CpG distribution-islands constituting 1% of DNA with the remaining 99% of DNA containing regions highly suppressed in CpG frequencies. It has been proposed that the deficiency of CpG is due to the fact that sites within bulk DNA were previously methylated, but have been subsequently lost during genome evolution due to increased mutability of 5-methylcytosine residues (10).

The increased mutability of 5-methylcytosine relative to cytosine is due to an increased rate of spontaneous pyrimidine deamination caused by the methyl moiety. The product of the reaction, thymine rather than uracil is more difficult for the cell to recognize and repair. Although there is evidence that mammalian cells can repair the resulting T-G mismatch in the correct direction (11), the possibility of mutation is increased and CpGs are well known hot spots for mutation. It has been shown that the CpG deficiency is compensated for by an excess of the mutation products TpG and CpA deficiency of CpG might therefore be regarded as an evolutionary consequence of cytosine methylation. Evidence for CpG mutational hot spots is that the highest frequencies of restriction fragment length polymorphisms in the human genome are revealed by enzymes which contain CpGs within their recognition sequences (12). This increased mutation frequency is still inducing new mutations into the human genome. For example, direct genomic sequencing has identified CpG as being a hot spot for mutation in the factor VIII and IX antigens hemophiliacs (13,14).

Role of DNA Methylation in Gene Expression

The role of DNA methylation in controlling the activities of eukaryotic genes remains controversial (8). Numerous studies have shown that active genes are less methylated than their inactive counterparts (15) but, it has been difficult to establish a cause and effect relationship between these two phenomena. For example, it has not been clearly demonstrated that undermethylation of a gene is a prerequisite for its expression during normal development. It remains possible that active expression results in undermethylation rather than the other way around.

On the other hand, the evidence that the methylation of CpG islands can silence gene activity is strong. For example, genes located on inactive X chromosomes have been shown to be more methylated in their islands than their counterparts on the active X chromosome (16). Many experiments have shown that the methylation of islands can silence genes (e.g., 17) by a mechanism probably requiring the assembly of a chromatin configuration (18). The methylation of islands associated with X chromosome inactivation appears to occur subsequent to gene inactivity so that methylation may serve to stabilize transcriptional incompetency (19).

There is also evidence from studies in tissue culture systems, that island-containing genes located on autosomes can become silenced by a mechanism involving DNA methylation. Many housekeeping genes appear to become silenced in culture by sporadic DNA methylation events (10) and can subsequently be easily activated by exposure to 5-azacytidine (20,21) which induces massive demethylation of treated cells (22). These relationships between methylation and expression are well documented but it has not been shown that methylation of islands on autosomal genes occurs during normal organismal development. This, of course, does not preclude the possibility that this methylation is important in abnormal growth situations which might occur during cellular immortalization during carcinogenesis.

The relationship between methylation and expression of non-island containing genes remains unclear although a correlation between expression and undermethylation has been firmly established. On the other hand the evidence that methylation of CpG islands can silence genes and is involved in transcriptional incompetency has strengthened considerably. There is no evidence, as yet, that specific demethylation events of islands are important during normal development.

DNA Methylation in Cancer Cells

Overall levels of DNA methylation are generally decreased within cancer cells (23,24). Importantly, these decreases seem to appear early in the transformation process and are one of the first genomic changes that can be detected in colon adenomas (25). There is also evidence that chemical carcinogens can interfere with enzymatic DNA methylation (see 23). These two findings have led to the hypothesis that undermethylation might be associated with the activation of genes in tumor cells resulting in abnormal growth and oncogene activation.

Many of these earlier studies on tumor cells did not distinguish clearly between island-containing genes and those genes which are unlikely to be subject to methylation inhibition. Also, little attention has been paid to the overall patterns of methylation which might be established in tumor cells relative to their normal counterparts. Recently Baylin's group has shown that specific chromosomal regions might be prone to hypermethylation rather than hypomethylation (26-28). These experiments have focused predominantly on chromosome 11p, a region known to harbor tumor suppressor genes and it has been proposed that this region might be a "hot spot" for hypermethylation (28). The concept is therefore developing that methylation of these regions might result in the extinction of activity of negative regulators of cell growth rather than activation or positively acting factors. These ideas are particularly interesting in view of the current interest in tumor suppressor genes, where gene inactivity seems to be more related to transformation than activation.

DNA Methylation of an Island-Containing Gene During the Establishment of Mouse Cell Lines

We have recently completed a study on the methylation of the MyoD1 CpG island in mouse cells as a function of immortalization (29). The result showed that all mouse cell lines examined have aberrant methylation of the CpG island when compared to normal mouse tissues. The methylation, which is normally present in the non-island regions of the gene has begun to move into the island-containing regions where presumably it can begin to exert an influence on cellular growth. Interestingly we have also observed that cells which have become transformed following exposure to chemical carcinogens show even further increases in methylation of CpG islands (Marziasz and Jones, unpublished). These experiments suggest that it is not the overall level of methylation which might be important, but rather the relative distribution of methyl groups in island versus non-island regions of genomic DNA. The biochemical reasons for this remain completely unknown but it is perhaps significant that transformed cells contain higher levels of DNA methyltransferase than their nontransformed counterparts (30). This apparent lack of control of DNA methylation may be linked to the aberrant methylation of islands seen during the transformation process.

SUMMARY

DNA methylation has the potential to introduce new mutations into the vertebrate genome and to lead to gene paralysis when it spreads into CpG islands. We have evidence that the methylation of islands, which only occurs on X-linked genes in normal tissues, is frequently induced during the establishment of cell lines and may be linked to cellular immortality. Treatment with carcinogens causes even further disturbances in DNA methylation patterns. The silencing of genes important in cellular growth control may be more important for the development of tumors than hypomethylation of sequences in non-island containing regions of DNA.

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DISCUSSION

Dr. Thompson:

Are you starting to look yet at the effects of *myc* genes on methylation of other genes involved in differentiation?

Dr. Jones:

Is the question, are we looking at *myc* genes?

Dr. Thompson:

No. Are you looking at the effects of *myc* genes on other genes which are markers for differentiation?

Dr. Jones:

It's very exciting now to have this first gene which is really responsible for differentiation and so that's the only one we have been able to study so far. We would like to do that. We don't know whether this kind of methylation is really important in tumorigenesis for example. I think what's very interesting is that something which is never seen in a normal tissue is seen in every single cell line without exception.

Dr. Buttyan:

Are you suggesting that DNA methylation is a routine means of eucaryotic transcriptional regulation? I had thought that that was a long-abandoned idea and in fact that gene methylation was a consequence of transcriptional inactivity rather than the cause.

Dr. Jones:

What I've shown you is that if you take that gene at the top, and you methylate the *cpa* island in an *in vitro* experiment and put it into a cell, it will be inactive. So methylation could clearly turn a gene off. This occurs in normal genes when they are located on the inactive X chromosome and results in their inactivity. What I am now showing you is that it occurs also during the formation of cell lines. I don't know which comes first, but all I can say is that the methylation may stabilize transcriptional inactivity and that once it's occurred, it's heritable for the rest of the life of that cell. So it's a mechanism probably for stabilizing transcriptional inactivity.

Dr. Buttyan:

So, in other words, you wouldn't specify that methylation is the first step towards transcriptional inactivation? Can a gene be transcribed *in vitro* in the presence of appropriate transcription factors, even if it is methylated?

Dr. Jones:

If it isn't too heavily methylated then you can turn it on by throwing in a transcription factor which activates the gene. In 3T3 cells where I showed you a very high level of methylation, the answer is no, you can't turn it on. These kinds of experiments really explain why azacytidine is so good at turning on suppressed information in cultured cells. All kinds of selectable genes such as thymidine kinase have these islands, and azacytidine is terrific at turning those kinds of genes on.

Dr. Coffey:

What do you think directs the localization of what becomes hypomethylated and hypermethylated in various genes in the tumor?

Dr. Jones:

I think that what we have to do is to separate the genome into island and non-islands and then everything fits together. What Dr. Baylin is seeing is hypermethylation of the islands, and what Dr. Vogelstein saw was hypomethylation of the coding sequences. I don't know what's causing the thing to spread except that we have evidence that the DNA methyl transferase level is different in transformed cells relative to normal cells.

Dr. Coffey:

But that won't explain the distribution of it between various genes. In the same cell you have 5 genes hypomethylated and 5 others that are not affected. There has to be some specificity within there to know what to hit.

Dr. Jones:

It depends what you mean by the gene. What I am trying to display here is one gene which has an island and that it's a general shift in methylation from the non-island to the island.

Dr. Coffey:

What I am saying is that if you had 10 of those genes in one cell, some would shift, some would not shift and I am trying to get at what causes that specificity. The enzyme level won't explain that.

Dr. Jones:

I don't know what causes the specificity, but I can see that even if it is a totally random event, then the strong Darwinian selection occurring within the animal could select for an inactive gene. If this is a gene which can cause a cell to differentiate, for example, and you now start turning it off by this mechanism, then after a series of cell divisions you would constantly select cells which have become more and more methylated within the islands of those kinds of growth controlling genes. I have no idea about the mechanism. I'm really struck at the commonality.

Dr. Tindall:

Have you identified any specific *cis* acting elements that are methylated in the 5 prime flanking region?

Dr. Jones:

No, not yet, but we have a new PCR based genomic sequencing method which will allow us to look at those and we have somebody doing those experiments, hopefully right now.

Dr. Miller:

With respect to the degree of methylation, if you take your methylcholanthrene transformed cells and put them into a nude mouse and grow them up as a solid tumor and then take that back out and look at it, are they still as hypermethylated as they were in the tissue culture state? In other words, is this just a phenomenon of the culture state or does it maintain if you grow the same cells as a solid tumor?

Dr. Jones:

We haven't done that experiment yet. I would suspect that they would remain methylated based upon Dr. Baylin's experiments, but I really don't know the answer to that yet.

EVIDENCE FOR THE INVOLVEMENT OF GENETIC DIFFERENCES AND MESENCHYMAL FACTORS IN THE PROGRESSION OF ONCOGENE-INDUCED PROSTATE CANCER IN RECONSTITUTED MOUSE PROSTATE

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INTRODUCTION

Carcinogenesis is a multi-step process involving the accumulation of alterations within the cellular genome (reviewed in 1,2). These alterations can occur in protooncogenes converting them into activated oncogenes, and it has been suggested that the multi-step nature of carcinogenesis may partly reflect the consecutive activation of several dominantly acting oncogenes (3-7). It seems reasonable to presume that prostatic cancer follows this pattern. In support of this notion, oncogene activities have been reported to be present in human prostatic adenocarcinoma (8-10), in cell lines derived from cancerous prostatic tissues (11), and in Dunning R3327 rat prostatic adenocarcinomas (12,13). Recently, utilizing the mouse prostate reconstitution model system, it was demonstrated that activated *ras* or *myc* oncogenes singly were able to induce distinct dysplastic and hyperplastic lesions, respectively (14). The two oncogenes in combination induced rapidly growing, poorly differentiated malignant adenocarcinomas. Thus, the activation of the *ras* and *myc* oncogenes can induce a step-like progression of carcinogenesis in this experimental animal model.

Although still a matter of controversy, variations in histological patterns of human prostate glands, which have been collectively termed dysplasias and cytological atypias, are considered by many investigators to represent early step premalignant conditions (15-18). These lesions together with small focal lesions of frank adenocarcinoma are frequently detected in prostatic tissues obtained during transurethral resections and in radical prostatectomy specimens (19,20). Focal, small sized carcinoma of the prostate is exceedingly frequent and has been observed in autopsy series with a reported incidence many times greater than that of clinical carcinoma in studies conducted in seven different countries (21-23). Although these incidental prostate cancers are common worldwide, there are profound geographical differences with regard to the development of more extensive clinical adenocarcinoma of the prostate (21,24). Specifically, the incidence of large sized, clinically detectable carcinomas is significantly increased in the Western countries when compared to Oriental countries. The reasons for such geographical differences in the rate of progression remain unclear and may include environmental factors. However, it remains a distinct possibility that heritable genetic resistance/susceptibility is involved in the progression to malignancy.

It has been known for several decades that heritable predisposition is a significant factor for many forms of cancer (reviewed in 25). Despite the fact that most observers postulate that well-established heritable predisposition accounts for less than 5% of the total cancer burden, it has become increasingly clear that the actual impact of heredity on cancer incidence may far exceed the cases where clear-cut Mendelian patterns of inheritance have been demonstrated. Animal studies have clearly shown that susceptibility to exogenous carcinogens is inherited (reviewed in 26,27). Although the general conclusions

of these studies are valid, it is often difficult to disentangle specific target organ responses to carcinogens versus carcinogen-induced systemic effects which could affect target organ responses indirectly. In addition, the potential of carcinogens to induce multiple genetic mutations and alterations can further limit the interpretation of results. We have utilized the mouse prostate reconstitution system to ask whether genetic differences in the carcinogenic response to a specific pair of cooperating oncogenes exist. Our preliminary results suggest that the response to the *ras* + *myc* oncogene combination is strain specific. Interestingly, the differential response resides in the mechanism(s) which converts benign hyperplastic glands to malignant ones. Furthermore, the differential response appears to be in part intrinsic to the prostatic mesenchymal cells.

Mouse Prostate Reconstitution

Recently, technologies have been combined and extended toward the goal of understanding oncogene function within the context of the prostate *in vivo* (14). Anlagen of the mouse prostate urogenital sinuses (UGS) are isolated from day 17 mouse fetuses and dissociated enzymatically to single cell suspensions. Oncogenes are then introduced into these cells, which include cells derived from the urogenital sinus epithelium (UGE) and cells derived from the urogenital sinus mesenchyme (UGM), utilizing recombinant retroviruses produced in genetically engineered packaging cells which are free from helper-virus contamination (28,29). After infection, the fetal organ is "reconstituted" by resuspending the cells in a collagen matrix and grafting this cell suspension under the renal capsule of a syngeneic male host (Figure 1A). The entire *in vitro* manipulation takes only a few hours.

It has been shown in control infections, using retroviral vectors without oncogenic sequences, that the fetal cells reorganize and undergo normal growth and morphogenesis, resulting in a differentiated mouse prostate after four to six weeks. Thus far, the effects of the *ras* and *myc* oncogenes singly and in combination on prostatic growth and differentiation have been studied (14). The results showed that v-Ha-*ras* induces a grossly dysplastic phenotype with a strong angiogenesis component. The v-*gagmyc* oncogene induces epithelial hyperplasia. Since the cells which contain the oncogenes can be identified either by using specific antisera to the gene product or by including the bacterial β -galactosidase gene in the retroviral vector sequences and staining the tissue for β -galactosidase activity, it has been demonstrated in *ras*-induced dysplasia and *myc*-induced hyperplasia that both direct and indirect effects of the oncogene contribute to the phenotype suggesting that paracrine mechanisms are involved in both cases. The *ras* and *myc* oncogenes together induce tumors which express a specific cytokeratin normally seen in the prostatic luminal epithelial cells. Analysis of tumor-derived cell line DNA as well as tumor DNA by Southern blotting demonstrated that the carcinomas were mono- or oligoclonal, suggesting that the *ras* + *myc*-induced carcinomas had acquired a subsequent genetic alteration(s) during the four-week growth period. These initial studies have shown that the effects of *ras* and *myc* singly on prostatic growth and development

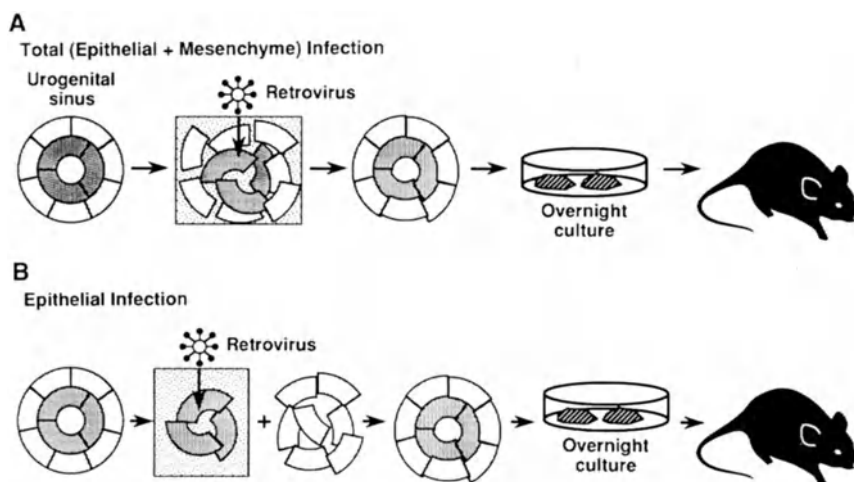


Figure 1: Mouse prostate reconstitution protocol: A. Total (UGE + UGM) infection. B. Epithelial (UGE-specific) infection variation.

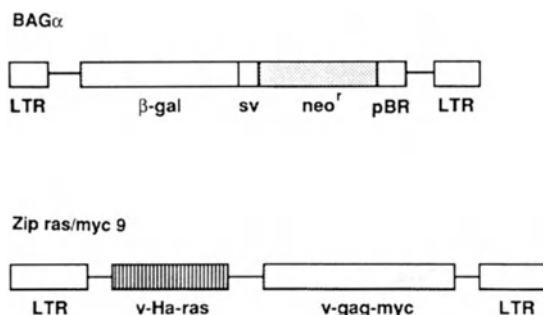


Figure 2: Schematic representation of recombinant retroviruses used in mouse prostate reconstitutions: BAG-alpha (reference 34) and Zipras/myc 9 (reference 14).

are oncogene- and cell-specific in nature, and in combination the two cooperating oncogenes induce carcinomas, but additional genetic alterations were involved in the progression of the tumors.

We have continued the development of this model system to provide more specific information. At the time of dissection it is possible to mechanically separate the epithelial and mesenchymal components of the urogenital sinus (30-32). We have used recombinant retroviruses to examine the effects of the *ras* and *myc* oncogenes on the growth and differentiation of the mouse prostate when they are introduced selectively into the isolated UGE (Figure 1B).

The results of control experiments using non-oncogenic retroviral vectors were similar to those in which total UGSs were used in that differentiated mouse prostate tissue was recovered after a four-week growth period as a renal subcapsular graft. The expanded mouse prostate reconstitution model system offers unique experimental advantages. The possibility of introducing genes specifically into the epithelial compartment while leaving the adjacent mesenchyme unaltered presents an opportunity to study the direct effects of transforming genes on the epithelium under conditions where the mesenchymal cells are either normal or also transformed. It is also possible to manipulate the ratio of transformed to normal cells by using retroviral supernatants of various titers. These parameters are critical to creating *in vivo* conditions which mimic the rare initiating events of tumorigenesis such as somatic mutations. This model system also permits the analysis of multiple experiments sufficient for statistical analysis. This potential, combined with the biological property of retroviruses to integrate "at random" into the genome of target cells, validates the use of this model to study discrete oncogene-specific phenotypic alterations superimposed onto various genetic backgrounds.

Application of Mouse Prostate Reconstitution to the Study of Genetic Differences in Oncogene-Induced Carcinogenesis

The initial studies which reported *ras* + *myc*-induced carcinogenesis using the mouse prostate reconstitution model system were carried out with tissues obtained from inbred C57BL/tan mice (14). This strain is a coat color variant of C57BL/6 which was isolated in London in 1958 and subsequently maintained as an inbred mouse strain (33). Using two Moloney Murine leukemia virus-based retroviral vectors (Figure 2), BAG-alpha (34) as a non-oncogenic control virus and Zipras/myc 9 (14), which carries both the *v-Ha-ras* as well as the *v-gagmyc* oncogenes, we have explored the possibility that the C57BL/6 mouse and the BALB/c respond differentially to the *ras* + *myc* tumor induction protocol. We first employed the basic total infection protocol (Figure 1A) using high titer ($> 10^5$ colony or focus-forming units/ml) virus supernatants. These supernatants were tested for the presence of helper-virus using a highly sensitive assay (35) prior to use.

The results of the control BAG-alpha infections using both BALB/c as well as C57BL/6 mice were similar in that differentiated mouse prostate tissue was recovered after the four-week growth period (Figure 3A). In contrast, Zipras/myc 9-induced BALB/c versus C57BL/6 glands demonstrated two distinct phenotypes representing different stages of tumor progression. The BALB/c reconstitutions were

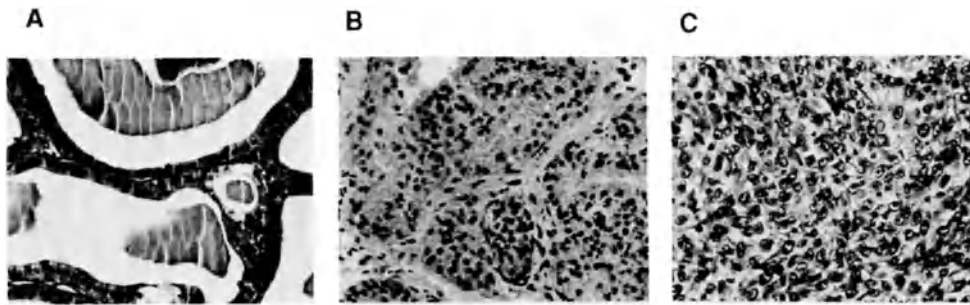


Figure 3: Hematoxylin and eosin stained tissue sections of the results of: A. BAG-alpha total infection of C57BL/6 UGS, B. *ras/myc 9* total infection of BALB/c, UGS, and C. *ras/myc 9* total infection of C57BL/6 UGS.

consistently hyperplastic with individual lumina being replaced completely with abnormally, growing epithelial cells (Figure 3B). However, in all cases examined thus far, the hyperplastic glands were surrounded by relatively normal appearing mesenchymal, presumably smooth muscle, cells. Thus, the BALB/c urogenital sinus cells formed hyperplastic glands when induced with *ras* and *myc* under conditions where both epithelial as well as mesenchymal cells were infected. It was felt that one possible explanation for the obvious difference between these BALB/c results and those obtained previously using the C57BL/tan strain might be a minor variation in the response time to *ras + myc* induction. This point was addressed in experiments which included an extended eight-week growth period (Table 1). Despite a substantial increase in the time over which a conversion to malignancy could occur, the hyperplastic phenotype persisted and no carcinomas were seen.

Inbred C57BL/6 urogenital sinus tissues were then used in identical *ras + myc* induction protocols. Poorly differentiated malignant adenocarcinomas were recovered with high frequency after four weeks' growth (Figure 3C and Table 1). Although the tumors in some cases demonstrated a more differentiated phenotype in small localized areas, they were very similar to those induced in C57BL/tan UGS previously (14). At this time two explanations for the differential response to the *ras + myc* induction protocol are possible. Either *ras + myc*-containing cells in both mouse strains respond differentially to comparable levels of the *ras* and *myc* oncoproteins, or the levels of expression of the two proteins differ between the two strains eliciting different responses. Although the answer to this question must await direct analysis of viral gene expression in both strains, it is of interest that a previous study showed that Zipras/*myc 9* is capable of producing tumors in both BALB/c as well as C57BL/6 mice when the virus is introduced into midgestation embryos (36).

Normal Mesenchyme is Non-Permissive for High Frequency Malignant Conversion

In order to probe the system further, isolated UGEs were recovered from BALB/c as well as C57BL/6 fetuses and used as a specific target for Zipras/*myc 9* infections. These infected cells were then reconstituted with normal uninfected UGMs from the same strain, grafted and allowed to grow for four weeks. Control BAG-alpha infections of both strains produced normal appearing differentiated prostatic tissues (Table 2). *Ras + myc*-induced BALB/c UGE when reconstituted with uninfected BALB/c UGM produced hyperplastic glands which in most cases were indistinguishable from those produced when both compartments were infected (see Figure 3B). Interestingly, the C57BL/6 *ras + myc*-induced UGE which was reconstituted with normal uninfected UGM did not form malignant tumors, as was the case with the total infection protocol, but progressed only to the hyperplastic stage (Table 2). In general, the C57BL/6 hyperplastic glands recovered thus far were similar to those produced in BALB/c tissues (see Figure 3B). Thus, the genetic differences in response to the total (UGE + UGM) *ras* induction protocol were reconciled by excluding the oncogenes from the mesenchyme.

These results indicate that an activity within the mesenchyme of the C57BL/6 mouse is either induced or altered by *ras + myc* induction, and that this activity modulation is either permissive or contributory to malignant conversion of the *ras + myc*-induced epithelium. Furthermore, these data suggest that this mesenchymal response is not present in the BALB/c-derived UGM.

Table 1: Phenotypic alterations in BALB/c versus C57BL/6 mice resulting from control BAG-alpha (Figure 2 and reference 34) and Zipras/*myc* 9 (Figure 2 and reference 14) total UGS (UGM + UGE) infections.

BAG α					
BALB/c			C57/BL6		
<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>	<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>
2	2 normal	42	7	7 normal	32 $\pm$ 6

Zipras/ <i>myc</i> 9					
BALB/c			C57/BL6		
<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>	<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>
6	6 hyperplastic	63 $\pm$ 14	20	1 normal	15
5*	5 hyperplastic	166 $\pm$ 39	19	carcinomas	427 $\pm$ 99

* extended 8 wk. growth period

SUMMARY

We have used the mouse prostate reconstitution model system to study the carcinogenic response to the *ras* and *myc* oncogenes. Our preliminary data suggest that there are genetic differences in the response to this tumor induction under conditions where the two oncogenes are introduced into both the epithelial and mesenchymal components of the urogenital sinus. Whereas BALB/c mice respond to this protocol by producing epithelial hyperplasia, C57BL/6 mice produce a more progressed malignant prostate adenocarcinoma. Interestingly, these genetic differences can be eliminated by altering the experimental conditions such that the two oncogenes are selectively introduced into only the urogenital sinus epithelium. Thus far under these conditions, both strains produce epithelial hyperplasia.

These preliminary experimental results are consistent with the observation that geographical differences and thus potential genetic differences in the rate of progression of human prostate cancer exist (21).

We intend to confirm these findings and further investigate these phenomena, with the hope of revealing useful information concerning the heritable predisposition to prostate cancer and the mesenchymal-epithelial interactions associated with its progression.

Table 2: Phenotypic alterations in BALB/c versus C57BL/6 mice resulting from control BAG-alpha (Figure 2 and reference 34) and Zipras/*myc* 9 (Figure 2 and reference 14) UGE-specific infections.

BAG α					
BALB/c			C57/BL6		
<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>	<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>
4	4 normal	27 $\pm$ 12	5	5 normal	35 $\pm$ 6

Zipras/ <i>myc</i> 9					
BALB/c			C57/BL6		
<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>	<u>n</u>	<u>Phenotype</u>	<u>W.W. (mg)</u>
9	9 hyperplastic	58 $\pm$ 13	6	6 hyperplastic	61 $\pm$ 8

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DISCUSSION

Dr. Buttyan:

It's hard for me to understand how the mesenchyme from one strain of mouse can be different from the mesenchyme of another. Is there a possibility that there is some retroviral difference between these two strains? Say for example, endogenous retrovirus in one strain that your retrovirus may interact with and cause these changes that you see?

Dr. Thompson:

Well that's possible, but we checked the serum of every animal for reverse transcriptase. It's very important to our experiments that there not be any helper virus in the animal or anywhere in the system and we completely rule that out and we don't consider helper virus - positive experiments. I must admit that some of these experiments haven't been checked but we only find that about 2 to 4% of host animals, this is the London data, had any kind of activity in the serum so we very carefully control that.

Question from the floor:

Do you think there is a latent retrovirus that your virus induces or activates?

Dr. Thompson:

Why would it induce only in mesenchyme? I suppose it's possible. I think there are well documented cell specific effects to oncogenes, I think that's very clear and I would prefer to look to that for an explanation.

Dr. Tindall:

Have you looked at androgenic effects, male vs. female or Tfm mice?

Dr. Thompson:

Not yet, we have some experiments like that planned. You mean just in tumor promotion? I can tell you one thing about these tumors. We've let them go out to 8 weeks and transplanted them, made single cell suspensions and they are basically androgen insensitive by that time. I think there is probably a window where there is some sensitivity, but it may be tricky to find. We're sort of concentrating on that question.

Dr. Isaacs:

That was beautiful work and exciting and there is obviously a precedent in many systems for a genetic background within the strains of animals to get various differences in tumor susceptibility. I hate to point out an obvious thing but you are planning on doing mixing experiments where you use the stroma from one strain and the epithelium from the other. Do you have any preliminary data on that?

Dr. Thompson:

No, we don't.

Dr. Isaacs:

In those experiments it may also be very interesting to see if you add both types of stroma or again some type of suppressor function.

DIFFERENTIAL HYBRIDIZATION ANALYSIS AS A TOOL TO STUDY PROSTATIC CANCER METASTASIS

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INTRODUCTION

The progression of prostate cancer can be described in terms of growth rate, hormone responsiveness, histology and metastatic ability. Some of these parameters can be interdependent. Clearly, metastatic ability of tumor cells is clinically most significant. It is this process in which primary tumor cells spread through the body and grow out at secondary sites that leads to much of lethality due to cancer. For prostate cancer the clinical consequences of established metastatic disease are profound, since no curative therapy is available (1). Systemic palliative methods, based on androgen ablation, are usually successful, but of limited duration. The outgrowth of androgen insensitive cells is inevitable, and will eventually result in the patients death (2).

The process of metastasis is a complex one, in which at least four stages can be distinguished, i.e. - invasion; - dissemination of tumor cells, either lymphatic or hematogenic; - extravasation and - secondary outgrowth (3). Both molecular characteristics of tumor cells, as well as, host mediated factors are likely to play a role in the metastatic cascade. In this chapter the characterization and isolation of tumor cell specific molecular factors implicated in prostate cancer metastasis will be discussed. As model system, the serially transplantable Dunning R-3327 tumor and therefrom derived tumor lines were used. The methods used are all based on the determination and comparison of steady state mRNA levels during the progression of prostate cancer. Moreover, the rationale and application of so called differential/subtraction hybridization analyses are discussed and illustrated.

Identification of Molecular Characteristics of Metastatic Prostate Cancer Cells; The Rationale for Differential/Subtraction Hybridization Analysis

The molecular basis of tumor progression can be studied at three levels, i.e. -DNA, -RNA and - Protein. The protein expression pattern determines the growth potential, biochemical profile and structural organization of a cell. Aberrant protein expression patterns, leading to cellular transformation and progression, however, find their origin in genomic alterations. Therefore, studies on the involvement of specific genetic changes, such as chromosomal abnormalities, can serve as a lead to identify genes that are implicated in the onset and progression of cancer (4). A less complicated technique that is more widely applicable is the so called allelotyping; DNA probes that map on known chromosomal locations and detect Restriction Fragment Length Polymorphisms (RFLPs) can be used to identify consistent deletions of (parts of) chromosomes. Likewise, allelotyping colorectal carcinomas revealed a frequent deletion of the long arm of chromosome 17, which led to the identification of p53 as being a tumor suppressor gene (5, 6). The few karyotypes determined from human prostate cancers indicated that the long arm of chromosome 10

(10q23-ter) was deleted in a number of cases (7). Clearly, the above mentioned technique can be used to investigate this putative involvement of chromosome 10 in an extensive group of patients with prostate tumors of varying histological grade and clinical stage (See also, W. Isaacs). The chromosomal analyses and allelotyping do not enable us to determine subtle changes in the genome, such as point mutations and/or small deletions. These studies require the use of Southern analyses and/or differential hybridization with oligonucleotides. Such approaches are "self limiting", in that respect that they are confined to known genes and/or previously described aberrations. Genes that should be considered relevant are, - oncogenes, - tumor suppressor genes, and genes encoding - growth factors, growth factor receptors, - transcription factors, extracellular matrix proteins, - cell adhesion molecules. The term "self limiting", therefore, seems to be paradoxical, since taken all this into account, at least 300 known genes should be studied. Studies at the RNA level by Northern blot assays have similar "constraints"; only previously characterized genes can be studied. It should be noted, however, that an overwhelming amount of circumstantial evidence indicates that the above mentioned genes can actually be involved in the onset and progression of cancer. Therefore, the study of these genes and their relation to the etiology and progression of prostate cancer are of major importance.

Just as chromosomal analysis and allelotyping can roughly indicate the genomic location of relevant genes, similar direct approaches are necessary to identify proteins that are differentially expressed during the progression of prostate cancer. Unfortunately, no such methods are available yet. Comparison of the protein expression profile by 2D-gel electrograms are feasible. Differentially expressed proteins that are thus identified, however, cannot be routinely characterized. Further purification and identification is usually difficult, requiring sophisticated protein technology.

A direct approach to identify genes that are differentially expressed is based on comparison of steady state mRNA populations, such as differential and subtraction hybridization analyses. These techniques, however, do not enable the identification of genes that are aberrantly expressed (i.e. different size of transcripts). Unless the level of steady state mRNA has changed, no aberrant expression patterns can be identified. Another constraint of these techniques is, that a number of genes can be regulated post-transcriptionally (mRNA-stability), or post-translationally. Such subtle differences cannot be identified using this approach.

The advantages of differential/subtraction hybridization analyses are, 1) that yet unknown, though relevant genes can be isolated. Moreover, both genes that are upregulated, as well as, down modulated can be identified. Hence, dominant acting genes (e.g. oncogenes, growth factor -, and growth factor receptor encoding genes), as well as recessive genetic elements (e.g. tumor suppressor genes) can thus be characterized 2) that the reagents that become available, cDNA-clones, can be routinely characterized by DNA sequence analysis and comparison of the resulting DNA sequence with nucleic acid/protein data bases 3) that regardless whether the gene is etiologically related to the disease or (merely) a marker for its progression, the cDNA can be immediately evaluated for its use in diagnosis by RNA *in situ* hybridization analysis.

Therefore, differential/subtraction hybridization analysis can be a powerful approach to study tumorigenesis and cancer progression. Due to its limitations, however, the technique should be considered complementary to other molecular approaches.

Differential/Subtraction Hybridization Analysis: The Approach

There are different methods to compare mRNA populations. When comparing mRNA populations two aspects are of great importance.

First, the complexity of the mRNA population. This is usually expressed in nucleotides/cell, and can range from 6×10^8 n/cell for a complex population like brain, to 1×10^8 n/cell for spleen (8). Secondly, the relative abundance of the mRNA population should be considered. In eukaryotic cells, only few mRNAs are present at high abundance ($> 1\%$); most mRNAs are present at low abundance (1% to $0,01\%$) or very low abundance ($< 0,01\%$). Essentially, these two factors determine which approach to use. When one is interested in high- and low abundance transcripts ($>0,01\%$) the differential screening of cDNA libraries is a valid approach ("differential hybridization analysis"), for a review, see ref. 9. The identification of very low abundance ($< 0.01\%$), differentially expressed genes requires a more sophisticated approach, i.e. subtraction hybridization analysis.

Without elaborating on technical details, the principles of these techniques will be discussed, referring to the most recent/state-of-the-art papers regarding molecular biological details (protocols).

The "flow scheme" for a differential hybridization assay is depicted in Fig. 1. A cDNA library is constructed from a given mRNA population (e.g. "A"). The resulting cDNA library "A" represents all transcripts from population "A". The library is plated and replicate filters are screened differentially; Number 1 (A-1) with a DNA probe representing mRNA population "A" (poly(A)⁺ mRNA of "A" is used as a template for a first strand cDNA reaction; second strand cDNA is then labeled to high specific activity, using the random prime labeling method, according to Feinberg and Vogelstein, (10)).

Thus cDNAs can be detected, if the corresponding transcript is present at a relative abundance greater than 0,01% in the template mRNA population that was used to synthesize the probe, i.e. sensitivity is approximately 0.01%. Likewise, replica number two (A-2) is screened with a probe representing mRNA population "B". The differences in the resulting autoradiographs represent cDNA clones that detect transcripts that are down modulated in "B" (vs. "A"). Usually, transcripts with an expression level below 0,01% are not detectable using this assay.

To improve the sensitivity of the technique, the method of liquid subtraction hybridization analysis is used (Fig. 2). In this approach first strand cDNA from "A" is hybridized in solution to excess "B" mRNA (mRNA "driven", subtraction hybridization). When the amount of the transcript of interest in the "driver" mRNA population is constant, then simple first order kinetics can be applied (11,12). Under these conditions the time of hybridization, to obtain an effective enrichment for the mRNAs of interest, can be approximately calculated (Rot value). Subsequently, the ssDNA molecules can be separated from dsDNAs; a cDNA library is then constructed using the subtracted first strand DNA population. This library represents genes that are differentially expressed at very low abundance (<0,001%).

Applications of Differential/Subtraction Hybridization Analysis to Cancer Metastasis

The above mentioned techniques have been successfully used in the identification of genes induced by growth factors (13-15). Also genes expressed during the induction of differentiation have thus been identified (16, 17). Furthermore, comparisons of normal vs. malignant tissues using differential hybridization analysis have been successful (18-20).

For metastasis, however, few reports are available on the use of this technique, to identify genes that are upregulated or down modulated during this process. Interestingly, the publications on this topic all report on down regulation of genes during progression.

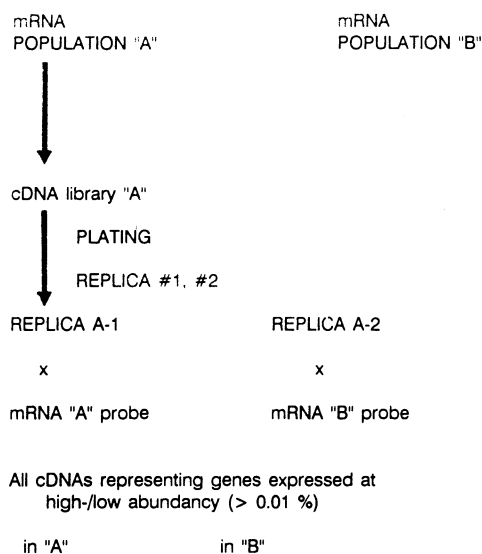


Figure 1: Flow scheme for differential hybridization analysis.

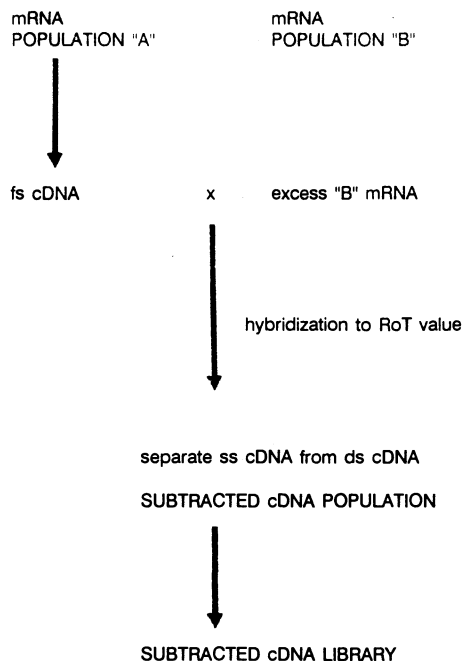


Figure 2: Flow scheme for the synthesis of a subtracted cDNA library (fs cDNA = first strand cDNA; ss-DNA = single stranded DNA; ds DNA = double stranded DNA).

Our group identified fibronectin as being down modulated in prostatic cancer cells (21). Yet another gene, NM23, was down regulated in melanoma cells with low metastatic capacity (22). WDNM1 is a gene down regulated in metastatic mammary adenocarcinoma (23). These results indicate that like in tumorigenesis, also in metastasis, suppressor genes might be involved ("metastasis suppressor genes").

The Dunning R 3327 Rat Prostatic Cancer Model System: The Model System of Choice for Differential/Subtraction Hybridization Analysis?

It should be stressed that the approach of differential hybridization analysis is a powerful one, that must be applied to metastasis more frequent in the near future. For such studies, however, a good model system is an essential prerequisite. None of the human *in vivo* tumor lines available to date, exhibit metastatic potential *in vivo* (after s.c. transplantation). For studies on the molecular basis of prostate cancer the Dunning-R-3327 rat model system seems to meet the demands for subtraction/differential hybridization analyses.

The parental tumor from which all the rat prostatic tumor sublines were derived, is the original R-3327 tumor described by Dunning (24). To denote differences in the history of the original R-3327 tumor passaged at several institutes, the tumor line serially passaged at Johns Hopkins Hospital (Baltimore, Md) was termed R-3327-H. The tumor line passaged at the Papanicolaou Cancer Research Institute (Miami, Fl) was termed R-3327-P (25). The phylogeny of the Dunning sublines that were used for this study is described in Fig. 3.

The P and the H tumor are well differentiated tumors that grow in response to androgens. The sublines AT-1, AT-2 arose from the Dunning R-3327-H and PAT-1 from the R-3327-P line passaged at Johns Hopkins Hospital (25). The CUB line was derived from the H line at Dr. Shimazaki's laboratory (Cheba University, Japan, 26). All these lines arose within one passage and are anaplastic, hormone-independently growing tumors with a low metastatic ability (25), i.e. less than 10% of the animals inoculated with tumor sublines develop distant metastases. MAT-LyLu (27) and MAT-Lu (28) both arose from the AT-1 tumor. PAT-2 and AT-3 arose from the P- and H-line, respectively. In rats, these last four sublines grow as anaplastic tumors with a high metastatic ability, i.e. more than 90% of the animals inoculated with these tumor sublines develop distant metastases (25). The characteristics of the Dunning sublines are summarized in Table 1.

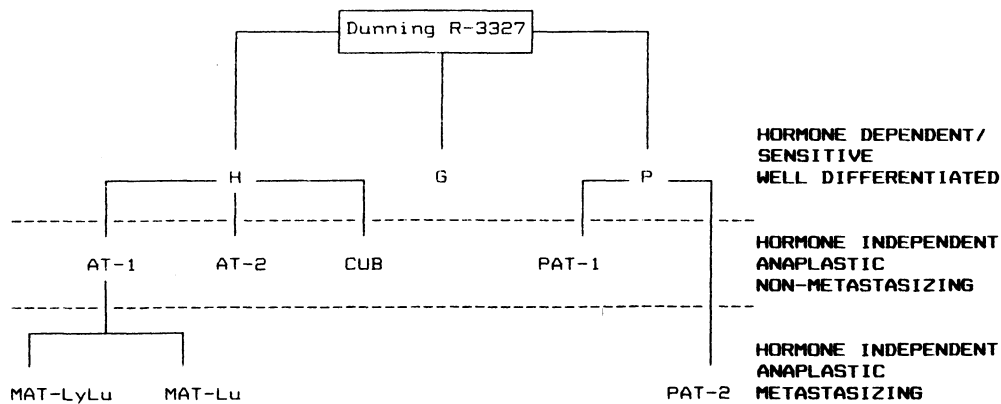


Figure 3: Phylogeny of the Dunning R-3327 sublines.

Table 1: *In vivo* biological characteristics of Dunning R-3327 rat prostatic cancer sublines

Subline	Histology	Growth rate Doubling time in days)	Androgen responsive	Metastatic ability*
H	Well-differentiated	22 ± 5	Yes	Low
P	Well-differentiated	10.5 ± 2	Yes	Low
G	Poorly-differentiated	4.0 ± 0.2	Yes	Low
CUB	Anaplastic	3.4 ± 0.6	No	Low
PAT-1	Anaplastic	2.8 ± 0.7	No	Low
MAT-Lu	Anaplastic	2.7 ± 0.2	No	High (lungs)**
AT-1	Anaplastic	2.5 ± 0.2	No	Low
AT-2	Anaplastic	2.5 ± 0.2	No	Low to moderate (lungs)**
AT-3	Anaplastic	1.8 ± 0.2	No	High (lymph nodes & lungs)**
PAT-2	Anaplastic	1.8 ± 0.2	No	High (lymph nodes & lungs)**
MATLyLu	Anaplastic	1.5 ± 0.1	No	High (lymph nodes & lungs)**

* Low metastatic ability, <5% of s.c. inoculated rats develop distant metastases; moderate ability, >5%, <20%; high ability, >75% develop distant metastases.

** Organs in the parentheses are the site of the distant metastases for the individual sublines.

The Dunning R-3327 system is a compilation of well characterized sublines that represent the full spectrum of parameters in which prostate tumor progression can be described (androgen responsiveness, growth rate, histology, metastatic capacity). Hence, we have chosen this system as model for our differential hybridization analyses.

Identification of Differentially Expressed Genes During the Progression of Prostatic Cancer Cells; A Study on the Dunning R-3327 Sublines

To identify genes whose expression is down-modulated in the process of metastasis, a cDNA library from the anaplastic non-metastasizing Dunning R-3327 subline, AT-1, was used for a differential hybridization assay, using probes derived from AT-1 and MAT-LyLu mRNAs. Mat-LyLu is a Dunning R-3327 subline that differs from AT-1 in its metastatic capacity, i.e. MAT-LyLu is highly metastatic. The resulting cDNA clones isolated according to the criteria used in a typical differential hybridization experiment, were further investigated by using these clones as probes for Northern- and dot blots, containing mRNAs of the AT-1 and MAT-LyLu *in vitro* cell lines. The cDNA clones that detected a differentially expressed mRNA were, subsequently, used as probe on a Northern blot, containing mRNAs of a panel of Dunning tumors that differed widely in their characteristics. The differentially expressed mRNA detected with recombinant clone pSE-1, correlated well with the anaplastic non-metastasizing phenotype (Table 2). Nucleotide sequence analysis revealed that recombinant clone pSE-1, whose expression was high in all tested anaplastic non-metastasizing sublines, was identical to fibronectin. To further investigate the down-modulation of this gene, we studied its expression in AT-2.1 and therefrom derived lines that were induced to metastasize by transfection with the v-Ha-ras oncogene (29). Also during this experimentally induced metastasis, fibronectin mRNA levels were approximately 8 fold lowered.

Fibronectin being specifically down modulated in metastatic rat prostatic cancer cells is in good agreement with earlier findings, that describe the loss of this extracellular matrix protein upon morphological transformation (30,31). The loss of fibronectin, a protein that is important in adhesive interactions, was thought to be one of the large number of factors that play a role during metastasis. The loss of cell-adhesion points in metastasizing BSp73 rat adenocarcinoma cells (32) and the decrease of fibronectin in K-1375 melanoma cell variants having low metastasizing capacity (32-34) sustained this hypothesis. Our studies indicate that also in metastasizing prostatic cancer cells, a down-modulation of fibronectin is evident. Unlike the previous studies, we present evidence that fibronectin expression is decreased at the mRNA level. The Dunning R-3327 AT-2.1 subline could be genetically manipulated, resulting in experimentally induced metastasizing lines, that appeared to exhibit a down-modulation of fibronectin. This seems to support the specificity of this phenomenon.

Table 2: Expression levels measured with pSE-1 as a molecular probe

SUBLINE	DIFFERENTIATION	ANDROGEN SENSITIVITY	METASTATIC CAPACITY**	pSE-1 EXPRESSION*
G	POOR	SENSITIVE	LOW	0
H	GOOD	DEPENDENT	LOW	0
AT-1	NONE	INDEPENDENT	LOW	++++
AT-2	NONE	INDEPENDENT	LOW	+++++
CUB	NONE	INDEPENDENT	LOW	++++
PAT-1	NONE	INDEPENDENT	LOW	++++
MAT-LyLu	NONE	INDEPENDENT	HIGH	+
MAT-Lu	NONE	INDEPENDENT	HIGH	++
AT-3	NONE	INDEPENDENT	HIGH	+
PAT-2	NONE	INDEPENDENT	HIGH	+

*Quantification by densitometric scanning of autoradiographs; 0 = control; level, + = 2 x control level, +++++ = 10 x control level.

**LOW, i.e. less than 10 % of the animals develop distant metastases; HIGH, i.e. more than 90 % of the animals develop distant metastases.

One of the questions that could not be answered, using this approach, is whether the processing of the fibronectin mRNA is different. Alternative splicing of the fibronectin transcript, in transformed cells, putatively resulting in modified proteins, has been reported (35-37) and might be an important effect in addition to the down-modulation of fibronectin in such cells. Further analysis, possibly by doing S₁ and per analyses, might result in more insight in that phenomenon. The relevance for diagnosis remains to be established.

The comparison of AT-1 vs. MAT-LyLu, revealed relatively few differences. Under experimental conditions used (21), the threshold level for abundance of mRNA detected was estimated to be 0.1-0.05%. Furthermore, all the cDNAs found, recognized transcripts that were down modulated in the metastatic cancer cells. The few reports to date, that use similar techniques all describe down modulation of genes in metastasizing cancer cells (21-23). At the given sensitivity this might indicate that upon metastasis the loss of molecular characteristics is the more pronounced effect. It should be noted that this is highly speculative in light of the few reports available to date.

Further improvements in the technique enabled us to increase the sensitivity and the specificity (38). cDNAs representing 0.01% of the mRNA population can now be detected. Furthermore, the number of clones that appeared to be "false positive" in the secondary screening was decreased (10-20%). Thus, the differential screening became more sensitive and efficient.

In a next series of experiments in which we screened for mRNAs that were overexpressed/induced in metastatic prostatic cancer cells, such cDNA clones could be found. A cDNA library of the highly metastatic MAT-LyLu (Table 1), was differentially screened with probes representing MAT-LyLu and H (well differentiated, non-metastasizing) mRNAs. Upon differential screening of 10.000 recombinant (cDNA insert containing) clones, >50 differences were found. Of the 24 clones that have been evaluated to day, in a secondary screening on a panel of three Dunning sublines (i.e. H, AT-2, MAT-LyLu), 20 revealed differential expression patterns. Six clones recognized transcripts whose expression level was at least ten-fold lower in the H-tumor when compared to MAT-LyLu. Those were used as probes to screen ten Dunning R-3327 sublines. It appeared that two clones, pBUS1 and -19, could specifically discriminate the metastatic tumor lines from the non-metastatic variants. pBUS-19, however, was not elevated in MAT-Lu, a tumor line that metastasizes exclusively to the lungs.

Thus, two new genes were identified that might be of relevance to the study of prostate cancer etiology and/or progression. Further studies are under way to establish the relevance and usefulness of these genes.

DISCUSSION

This chapter describes the use of differential hybridization analysis to isolate and characterize genes that are upregulated or down modulated in metastatic prostatic cancer cells. Whether the mRNAs detected with these cDNAs represent genes, that are etiologically related to prostate cancer and the progression of the disease, or (merely) a marker for progression/metastasis, remains to be established.

The obvious next steps are the cloning of the genes and transfection on recipient cells. This could be under its own promotor or heterologous (inducible) promotor. Evaluation of transfectants can give a clue to the causal relation between the overexpression/down-modulation of the gene and the metastatic phenotype.

If the thus identified genes are only a marker of progression of the disease, clearly, they could still be of great value. Diagnosis of prostate cancer is still hampered by the lack of such markers, that predict the relative aggressiveness of an individual tumor. The need for such "progression markers" is further indicated by the fact that most likely more localized cancers (i.e. stage A-2, B-1,2) will be clinically diagnosed in the near future, due to the improvement of diagnostic tools (e.g. Transrectal Ultrasound) and the screening of population groups at risk (male > 50 years old). Taken all this into account, the primary clinical application of cDNAs identified by differential hybridization analyses might very well be, to determine the individual aggressiveness of a tumor on basis of its expression pattern.

The mRNA abundance threshold of the differential hybridization assay, > 0.01% (i.e. only cDNAs detecting mRNAs that are expressed at more than 0.01% of the total mRNA population will be identified), indicates that the reagents can be used immediately in diagnosis. Either in RNA *in situ* hybridization or by Northern blot analysis of mRNA extracted from pathologically defined tissue.

We think that the results obtained to date confirm the applicability of differential/subtraction hybridization analysis to cancer research and many new aspects of tumorigenesis and cancer progression are expected to be defined at the gene expression level using this approach.

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DISCUSSION

Dr. Isaacs:

As kind of a corollary to the studies that you're doing, Dr. Tomaischikowa has taken non-metastatic AT2 tumor cells, a clone variant which has basically very low metastatic ability, and he has hybridized it with the AT3, which is a tumor line that is highly metastatic. In these he has been able to characterize both the chromosomes of both parents. When he makes a hybrid that has all of the parental chromosomes, the phenotype of the hybrid is non-metastatic. When he passages both in culture and in animals he gets segregants that begin to start losing chromosomes. Some of these still are non-metastatic. However some of them also re-express their metastatic ability. So, I think it's a very interesting system because as we have already heard about suppressor genes, it looks like there are going to be genes that also can suppress the metastatic ability. Now it's interesting, in the hybrids that are not metastatic, there is an effect on growth rate, so it hasn't suppressed tumorigenicity *per se*, but in all of the hybrids so far there has been a fairly substantial change also in growth rate. So we have to be a little bit careful, because it is possible that some of the genes that are so called metastatic suppressor genes may do that by an indirect mechanism of simply changing growth rates. I think it is very interesting because in your screens you are going to need to do it both ways, i.e. look at genes that are on and metastatic and also genes that are off and metastatic.

Dr. Schalken:

I forgot to mention that the first screen that I mentioned was the other way around, but at that time I think that the threshold mRNA abundance that we could detect was about .1%. So perhaps the real

important genes would not be picked up. Moreover, this method still tends to pick up genes which are > .01% (relatively high abundance), so I think the next step is to do the real subtractive libraries in which you throw away all of the relatively high abundantly expressed genes and add that to the analysis. But I agree with you, the model system you are describing is very interesting to screen those genes on too, and of course you should do it both ways.

Dr. Smith:

You've demonstrated that you have increases in mRNA levels. Have you done Southern blot analysis to see whether you have any gene amplification or rearrangement?

Dr. Schalken:

Not yet. We are now really at the point of sequencing them all. From this screening came 48 clones and 10 of them were interesting. So we are running them all through the sequence analysis and comparison with the EMBO data base, and certainly the Southern blots have to be done too.

MOLECULAR BIOLOGY OF ANDROGEN RECEPTORS IN PROSTATE CANCER CELLS

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INTRODUCTION

Androgenic hormones regulate the development and growth of many normal and tumor cells. It is clear that the androgen receptor (AR) plays a key role in the mechanism of androgen action. Activation of the androgen receptor by binding of the male hormone (either testosterone or dihydrotestosterone) results in the differential regulation of both mRNA and protein synthesis in target cells (1-5). Cloning of the androgen receptor cDNA, (the last of the known steroid receptor genes) has been achieved in several laboratories, including our own (6-10). Sequence comparison with the other steroid hormone receptor genes has revealed that the androgen receptor gene contains two highly conserved regions. Functional analysis of the estrogen- (11), progesterone- (12), and glucocorticoid receptor cDNA's (13) has demonstrated that these two discrete regions correspond to DNA-binding and steroid-binding, respectively.

The low abundance of the androgen receptor within tissues (0.001% of total cellular protein) and its relative instability have complicated studies of the receptor's physical and functional properties. Moreover, these same factors have contributed significant difficulties in achieving purification of the receptor in large quantities. Although the androgen receptor cDNA has been expressed in androgen receptor deficient eukaryotic cells (7,8) or *in vitro* translation system (6,10), the quantity of the expressed protein still remained at a similar level as that found in androgen target tissues.

Expression of the androgen receptor in a suitable heterologous system could circumvent previous difficulties of obtaining sufficient quantities of the receptor for analyzing the molecular properties of the protein. The advantage of using *E. coli* as an expression host is that foreign genes can be easily manipulated into a suitable vector for producing large quantities of the gene products as proteins. Recently a number of mammalian proteins have been expressed in *E. coli* in their biologically active forms (14,16). Moreover, the rat glucocorticoid receptor- (17) and the chicken progesterone receptor cDNA's (18) were successfully expressed in *E. coli* and shown to exhibit specific binding to their cognate steroids and/or steroid response elements in the mouse mammary tumor virus long terminal repeat region. Thus, these bacterially produced receptor derivatives exhibited biological activities similar to their *in vivo* counterparts.

In this manuscript we report the overexpression of the human androgen receptor cDNA as a fusion protein in *E. coli*. Utility of this fusion protein should facilitate the elucidation of the structure and function of the DNA- and steroid-binding domains of the androgen receptor and the further characterization of other androgen-regulated genes.

CLONING AND CHARACTERIZATION OF cDNA CODED FOR THE ANDROGEN RECEPTOR

In order to obtain an androgen receptor cDNA, a 32 bp synthetic oligonucleotide DNA binding consensus probe was used to screen both an LNCaP cDNA library and other commercially-available cDNA libraries which were derived from human prostate and testis. A total of 30 positive clones were isolated. Twenty-four of these clones were excluded on the basis of hybridization with probes, which were specific for the estrogen, progesterone or glucocorticoid receptor cDNAs. The inserts of the remaining recombinants were subcloned into the DNA sequencing vectors, pGEM4, pSP72 and M13 phage. Sequence analysis revealed that six of these clones not only shared the same sequence in their overlapping regions (Figure 1) but also contained a characteristic DNA binding consensus sequence, which was slightly different from that of the other steroid receptors.

To examine whether any of these clones contained the sequence of the androgen receptor transcript, clone HART52 in pGEM4 was converted to RNA by Sp6 RNA polymerase and translated in a nuclease-treated rabbit reticulocyte lysate system. The translation product from the *in vitro* transcribed RNA was able to bind both [³H]DHT and [³H]Mibolerone specifically (data not shown). Next, the [³⁵S]labeled translation product, derived from clone HART39 in pGEM4 was recognized by an anti-AR monoclonal antibody, CB54 (19) (Figure 2).

In addition to the cDNA clones isolated above, a 1157 bp Eco47 III fragment was isolated from an X-chromosome library (ATCC 57750) using the HART39 cDNA insert as a probe. This insert (HARGX3) contained sequences which spanned 165 bp upstream from the putative initiation codon and about 1 kbp of the 5' end of the coding region (Figure 1), when compared with published data (6-8). This fragment, together with the other hAR cDNA inserts, allowed us to construct a cDNA containing the full-length coding sequence for the hAR protein with 926 amino acid residues.

STUDY OF EXPRESSION OF HUMAN ANDROGEN RECEPTOR mRNA IN HUMAN CELLS

Although our composite hAR cDNA (measuring 5.5 kb) is much longer than the published size of the hAR cDNA (6-8), it does not represent the full length sequence for the hAR mRNA, since at least two species of hAR mRNA (measuring 7.5 and 9.5 kb in length) have been detected in both LNCaP and normal human foreskin fibroblast cells by Northern blot analysis (Figure 3; Lanes 1 and 2). The hAR mRNA (at least the 9.5 kb species) could not be detected in human foreskin fibroblast cells from a patient with the

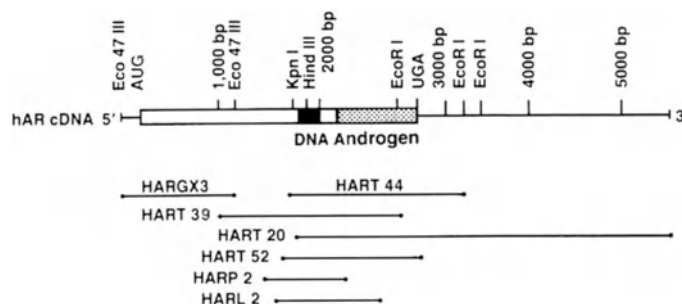


Figure 1: Partial structure of the hAR transcript deduced by overlapping hAR cDNA and genomic clones. Representative fragments of overlapping human androgen receptor (hAR) cDNA clones are denoted: HART 20, 39, 44, and 52 from a human testis lambda gt11 library, HARP 2 from a human prostate lambda gt11 library, HARL 2 from a human LNCaP cell line lambda gt11 library. Ends of the cDNA clones are denoted by dots. The Eco 47 III fragment, HARGX3, was obtained from a genomic clone isolated from a human X chromosome library (ATCC S7750) and by sequence analysis, corresponds to the 5' region of hAR mRNA (6-8). The total available cDNA is about 5.5 kbp. The region corresponding to the predicted coding sequence for the hAR protein is indicated by the outlined box. The predicted initiation codon, the in-frame stop codon, the DNA binding domain (DNA), and the androgen binding domain (Androgen) are indicated.

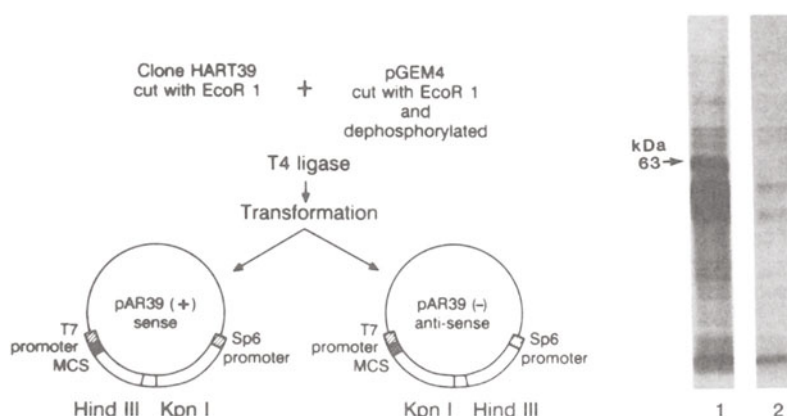


Figure 2: SDS-polyacrylamide gel Analysis of *In Vitro* Expressed Human Androgen Receptor Precipitated by Monoclonal Anti-AR Antibodies. An EcoR1 fragment (1.9 kb) from cDNA clone HART39 was ligated to pGEM4, which had been digested by EcoR1 and dephosphorylated by bovine alkaline phosphatase. Two orientations (sense and antisense) of the hAR cDNA insert in pGEM4 were selected and designated as pAR39 (+) (Lane 1) and pAR39 (-) (Lane 2) with respect to the Sp6 promoter. The pGEM4 transcription vector containing 1.9 kb of the human androgen receptor cDNA, was linearized with the restriction endonuclease, Xba I. The linearized plasmid (0.5 ug) was transcribed in the Promega Riboprobe Gemini system using Sp6 RNA polymerase. After treatment with RNase-free DNase, transcripts were extracted with phenol-chloroform, precipitated by ethanol and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of 50 uCi of [³⁵S]methionine (1465 mCi/mmol; Amersham). The lysate was incubated with human anti-AR monoclonal antibody (CB54) at 4° C overnight and incubated with Pansorbin for an additional 4 h at 4° C. The immunoprecipitate was washed extensively with PBS, resuspended in SDS-page sample buffer and heated at 100° C for 4 min. The supernate was separated from Pansorbin by centrifugation and applied to a 10% polyacrylamide gel. Standard proteins were used as molecular weight markers: myosin (200 kDa); B-galactosidase (116 kDa); phosphorylase b (97 kDa); BSA (66 kDa); and ovalbumin (45 kDa).

complete androgen resistance syndrome or from patients with the incomplete androgen resistance syndrome (Figure 3; Lanes 3 -5).

EXPRESSION OF HUMAN ANDROGEN RECEPTOR IN *E. COLI*

Elucidation of the structure and function of the androgen receptor protein has always been hampered by its scarcity and lability. Overexpression of AR in a heterologous system, such as *E. coli*, would provide a good source for obtaining large quantities of AR. In order to accomplish this, the hAR cDNA from hAR52 was subcloned into pSp72. An Asp 718-Cla 1 fragment, which codes for 412 amino acid residues and contains both the putative DNA binding and the steroid binding domains, was generated from that vector, blunt-ended with the Klenow fragment of *E. coli* DNA polymerase and fused to the C-terminus of the *E. coli* β -galactosidase gene in the plasmid, pSS20*a (20). The bacterially expressed androgen receptor could be obtained in a soluble active form. More importantly, the bacterially expressed hAR hybrid protein had an affinity and specificity for androgen binding similar to the native AR.

Previously we have shown (21,22) that the synthetic androgen, R1881, can be used for covalently labeling the androgen receptor. Therefore, photoaffinity labeling with R1881 was used to demonstrate specificity and selectivity of the androgen binding to the bacterially expressed hAR. A band with an Mr of 150,000 of the resulting product (Figure 4, right lane) confirmed the identity of the inducible fusion protein observed SDS PAGE gel (data not shown). This band was displaced with a 200-fold excess of either nonradioactive R1881 (Figure 4, left lane) or dihydrotestosterone (data not shown).

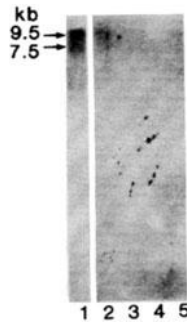


Figure 3: Expression of Androgen Receptor mRNA Analyzed by a Northern Blot. Total RNA (20 μ g) extracted from human cells was fractionated on a formaldehyde-agarose (1.5%) gel and blotttransferred onto nitrocellulose paper. The paper was probed with a [^{32} P]labeled Hind III-EcoRI fragment from clone HART39 to identify the androgen receptor mRNA. An RNA ladder (BRL) was used for molecular size standards. The autoradiograph was obtained after 3 days' exposure using double intensifying screens. Lane 1: LNCaP cells; Lane 2: Normal foreskin fibroblasts; Lane 3: Foreskin fibroblasts from patient 1 with the incomplete androgen insensitive syndrome; Lane 4: Foreskin fibroblasts from patient 2 with incomplete androgen insensitive syndrome; Lane 5: Foreskin fibroblasts from a patient with the complete androgen insensitive syndrome.



Figure 4: Photoaffinity labeling of the hybrid hAR with R1881. Whole bacterial cells were labeled with 5nM [^{3} H]R1881 in the absence or presence of 1000nM nonradioactive steroid for 18 h, 4 $^{\circ}$ C. The cells were washed with buffer to remove unbound steroids. Harvested cells were resuspended in buffer, sonicated and centrifuged at 9000 rpm 15 min at 4 $^{\circ}$ C. Aliquots (10 ml) of the supernate were placed in a plastic petri dish set on the top of a UV light box, which had been precooled at 4 $^{\circ}$ C. The [^{3} H]R1881(labeled hAR was irradiated with UV light (330 nm) for 15 min at 4 $^{\circ}$ C, warmed for 30 min at 55 $^{\circ}$ C and precipitated with 50% ammonium sulfate at 4 $^{\circ}$ C overnight. The ammonium sulfate precipitate (0.5 ml) was dialyzed against 2 liters of double distilled water overnight and subjected to SDS-polyacrylamide gel electrophoresis. Fluorograph was accomplished after a two-week exposure using EN ^{3} HANCE.

REMARKS

In this work we have isolated about 5.5 kbp of overlapping cDNA clones for the hAR gene, which consisted of 165 bp of the 5' - upstream region from the putative initiation codon, the entire coding region for the receptor polypeptide and 2.5 kbp of the 3' -untranslated region. The isolated cDNA was transcribed *in vitro*, and the resultant mRNA was translated in a rabbit reticulocyte lysate system. The authenticity of the cDNA for the hAR gene was demonstrated by sequence analysis, anti-AR antibody precipitation and steroid binding analysis of the *in vitro* translation product. Northern blot analysis of the hAR mRNA in LNCaP cells and human normal skin fibroblasts, and the isolation of long stretches of cDNA at the 3' end, suggested that the hAR mRNA is comprised of a heterogenous species with long untranslated tails. This may be of significance since the 3' - untranslated region of many mRNA species appear to serve important regulatory roles in gene expression, as well as maturation and transportation into cytoplasm (23). Although long tails have been found in all of the steroid hormone receptor gene transcripts (24-26), its significance in expression of these genes is unknown.

Moreover, our genetically engineered androgen receptor could readily be detected by assaying the steroid-binding activity in whole bacterial cells. Interestingly, whether the steroid was added to whole bacterial cells or to sonicated bacterial extracts, the amounts of the androgen binding activity recovered in a soluble form were very similar.

Production of large amounts of hAR fusion protein should facilitate the purification and characterization of the androgen receptor. Purification could be achieved by a combination of anti- β -galactosidase antibody-immunosorbent and SRE-DNA affinity chromatography. The β -galactosidase portion of the hybrid hAR could be removed by cleavage at the collagenase recognition site of the protein. The availability of purified protein should allow studies of some important biological functions of the receptor such as zinc-, DNA-, and steroid binding. Furthermore, the purified protein could be utilized as a probe to isolate and characterize other androgen regulated genes.

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DISCUSSION

Dr. Smith:

I have a question but I would like to make a general comment first. We have heard a lot of papers presented at this meeting and posters on the LNCaP cell line compared to this meeting 4 years ago. We've also seen karyotype analysis of the LNCaP's. One in which there were two X chromosomes and the other in which there was only one X chromosome, and we saw the marker chromosomes in one case. One thing that I would like to urge is that as research continues with the LNCaP cell lines, investigators should actually karyotype their cell lines so that we can see which LNCaP they are dealing with. I use this comment really to preface my question. We've seen now from three different laboratories that hydroxyflutamide stimulates growth in LNCaP cells and you demonstrate here that hydroxyflutamide can antagonize the effect of androgens on PSA expression. Is that because PSA expression can be dissociated from the effects of hydroxyflutamide on growth or is it perhaps a different subclone of LNCaP's? Have you looked at the effects of hydroxyflutamide on growth of your LNCaP's?

Dr. Young:

That is a very intriguing question. We haven't really looked at the growth effect of the hydroxyflutamide but I think that we have to go back now to look at this. For now, we just would like to first know whether it is due to the androgen effect.

Dr. Isaacs:

In many laboratories, the LNCaP cells which are very nice to work with *in vitro* have not been able to produce tumors *in vivo*. I would strongly urge that if anyone is working with these cells, that they test the tumorigenicity of these cells in nude mice, and if they should happen to have a sub-line which comes up fairly reproducibly it would be very, very useful. As far as I'm aware, there is not a situation where you can inject, for example, a million cells or even 10^7 of these cells and get any type of reproducible frequency of producing a tumor no matter how much antigen you give or even if you give anti-lymphocyte serum to nude mice. So I think that it is very important for people working with this as a culture model to know that in many systems it is not tumorigenic.

Dr. Matusik:

Have you done nuclear run off experiments to check if androgens are inducing the rate of transcription of the PSA gene?

Dr. Young:

Not yet, that is the next step.

Dr. Harris:

What was the concentration of hydroxyflutamide that you used in your experiments?

Dr. Young:

It was 5 micro molar.

Dr. Harris:

It's possible that at that concentration you might begin to see inhibition of growth.

Dr. Young:

Right now we are just looking at the hypertrophy phenomena, but during that period of time we don't really see that the cell number changes because the time is too short, not longer than 3 or 4 days. Since the period of time of our experiments was within 48 hours, it was too short to see the changing cell numbers.

PANEL DISCUSSION

DNA Structure and Gene Expression

PANELISTS--David Bilhartz, Aaron Katz, Dolores Lamb, Ming-Fong Lin and Roy Smith

Dr. Bilhartz:

Dr. Oefner, concerning the PCR technique for measuring gene expression, I don't understand how you can quantitate the relative levels of gene expression in your model. How do you decide what level would be biologically active? There should be a cut off. With PCR we may be measuring the presence of RNA that's not biologically significant. How did you decide to stop after 25 reactions?

Dr. Oefner:

Regarding your second question, 25 cycles turned out to be optimum because afterwards we ran out of reagents. And you have to have more enzyme to do more cycles. As far as the quantitation of the PCR reaction is concerned, at the beginning we were having tremendous difficulty doing it and that is why we are starting to analyze the PCR reaction mixtures by means of capillary electrophoresis which is very sensitive. But as Dr. Schalken has pointed out and has shown, there are tremendous differences between the different Dunning tumor lines in the expression of GAPDH dehydrophosphate dehydrogenase, so as soon as we are able to quantitate our PCR reactions we have to look at that problem. It is definitely there. If we want to answer some of the questions related to prostate cancer, we have to look at the cells in the patient and we do the PCR reaction not only for this quantitative purpose but also for subsequent sequencing. I think it is a powerful technique in combination with the findings of aspiration biopsy. I don't see another way for repeated analysis of gene expression in the patient.

Dr. Bilhartz:

Early on in your talk you mentioned Viola's work with prostate cancer and said that you agree with that completely. I was wondering, have you actually repeated some of the immunohistochemical staining with the same antibody they used? Because I know that I have examined many different prostate cancer specimens with that particular antibody and have had trouble reproducing the strong correlation with tumor grade.

Dr. Schalken:

We haven't worked with it at that level. I just mentioned that we used the Dunning system to test the hypothesis at the RNA level, so the answer is simply, no.

Dr. Bilhartz:

I don't feel that it is a problem with the *ras*-oncogene expression. I feel that this antibody may lead to some false positive results.

Dr. Katz:

With regard to the methylation of the *ras* oncogene, I'm interested to know if you have determined the specific sequences which are methylated and the specific structures or function that they code for and if you've looked at the specific codons of 12 and 61 which seem to be involved in the generation of the T24 bladder cell carcinoma line?

Dr. Jones:

We haven't done much work on the methylation of the *ras* gene. That work has been done by a group in Italy who have shown that if you methylate the promoter you turn the gene off. We, of course, realizing that methylation was a mutational hot spot, did look at those particular codons to see if it was C-

G site at that region which could have become mutated as a result of cytosine methylation. But there isn't, so those two mutations are not due to cytosine methylation. Scanning the literature for hemophilia mutations, both with factor VIII and factor IX, there is a very strong prevalence of those kinds of mutations and if you think about it, if you're doing RFLP analysis, the enzymes which are most useful in the human genome are those enzymes which contain C-G's in their recognition sites. So you will often see people using Taq1 and Msp1 because those are the enzymes that reveal the largest number of RFLP's because they have C-G's which have become mutated. So I think it's in the random sort of RFLP stuff and also in very specific sites within the genome. It certainly looked to me like it's in that androgen receptor.

Mr. Fabian:

My question is to Dr. Timothy Thompson. In addition to considering the mixing experiment suggested by Dr. John Isaacs, I was wondering if you perhaps were considering infecting cells from F-1 hybrids, to examine the genetic basis of the tumor phenotype seen in C57BL/6 as compared to the hyperplastic phenotype seen in BALB/C.

Dr. Thompson:

Well, I think the power of genetics is that you can get to the answer, you can get to the genes very slowly and right now we are involved in molecular characterization of the system and confirming some results. I've already bred F-1's, we're going to go into F-1's as a recipient host to rule out environmental possibilities here. Then you go F-2's then you do the back. All of this is completely doable with this system. You can do genetics with this system and eventually people get to the gene in this manner.

Mr. Fabian:

And is this specific to any BALB/C subline or do you see this in different sublines?

Dr. Thompson:

We've checked two sources, two distributors.

Mr. Fabian:

There are genetic differences in BALB/C sublines.

Dr. Thompson:

Yes, but a lot of those letters at the end are the source and we've checked two, Harlan-Sprague-Dawley and Sasco and the response is the same. We've also checked two distributors of Black 6's and the response is the same. I'm new to genetics as well, so it's a new thing for me.

Dr. Lamb:

Dr. Schalken, my question concerns the problem of doing differential hybridization. Mostly you are going to pick up very high abundance message differences, and if you are looking at growth factors or something like that, these are very low abundance messages (that are causing really very dramatic differences in growth). I am wondering whether you will end up picking out the things that have to do with cells dividing or metastasis, which reflect the end result rather than the causative factor? Do you understand the question?

Dr. Schalken:

Yes, I get the point. With this technique, people are really down at .001%'s (very low abundant genes). The groups working on differential gene expression in different parts of the brain pick up genes with an expression level of .001 to .003%. PCR has helped quite a lot. So what you do essentially is, the first step, take messenger population B and use it as a driver in solution subtractive hybridization and then you lose a lot of the things that you're now picking up, so at that approach, secondary to this one, it is certainly possible albeit pretty difficult. But the technique is powerful enough to pick up low abundant genes.

Dr. Lamb:

So the answer then is that the potential is there, but it hasn't been fully reached.

Dr. Schalken:

It's the next step that is coming from this point on.

Dr. Lamb:

When you showed us the initial pictures (that were out of your Cell paper) of the cells that were

expressing the beta galactosidase, is it possible that all of those cells are actually infected but not with a high enough copy number? Because you talked about the possibility of a paracrine action.

Dr. Thompson:

The copy number at low multiplicity of infection is almost always one. In a few cases in the tumors I saw two or three integrations, but with the multiplicities that we're using, there should be very little variation in terms of copy number.

Dr. Lamb:

So you feel that the fact that you see the beta galactosidase indicates that there would have to be one?

Dr. Thompson:

The strongest evidence for that is on northern blots where the genomic RNA is roughly at a 20 to 6 ratio to the sub-genomic message. So that indicates very strongly that where you do see beta-gal mRNA you are going to see *ras* RNA.

Dr. Lin:

Could you tell me what you propose regarding the function of your protein?

Dr. Matusik:

I don't really know what the function of probasin is or what the ligand is. The fact that it is in the secretions and gets into the nucleus seems contradictory. It seems quite illogical and the only way I can rationalize it is to say that the target in the secretions is the sperm which is all nucleus and the target that we see in the cell again is the nucleus. So it is carrying some transcription factor ligand, I believe, to those two different targets. It may be carrying a ligand in the semen to the oocyte. Probasin may be bound to sperm and carried to the oocyte. The structure of the probasin protein and its homology to ligand suggests that it does bind a ligand. This ligand remains to be identified.

Dr. Lin:

Do they synthesize both forms at an equal or different rate?

Dr. Matusik:

At different rates. I didn't show the data, but if you do the *in situ* hybridization and look at the level of the mRNA in different lobes of the prostate, you find out that the total messenger content in the lateral lobe for this message is probably close to 10%. If you do the immunohistochemistry, it looks like the protein is fairly equally distributed between secretory product and nuclear product. If you look in the dorsal lobe, the messenger content is probably about 3% yet we don't see any secretory product, we only see nuclear product. If you look in the ventral lobe the level of the PB mRNA is probably about .1% or less, we still see only the nuclear product and no secretory product. By S₁ protection and primary extension, we know that all three lobes have the intact mRNA and we've actually done hybrid selection where we isolated and purified the mRNA from the ventral prostate and have *in vitro* translation. It makes both proteins. So the message is there, but there is preferential translation at different start sites. I don't know how that works.

Dr. Lin:

You have determined that the mRNA level of PSA goes down in carcinoma tissue. How about the PSA protein level? Does the protein level decrease? Since the secretion of PSA goes up, can you propose any possible mechanism to explain the increase of PSA in the patient's serum?

Dr. Young:

In prostatic carcinoma tissues, PSA protein was also decreased. One of the possible explanations for the increase in PSA in prostate cancer patients' sera is that, while tumor cells lose PSA synthesis ability, the PSA synthesizing cells in cancerous tissues still remain in sufficient number to give rise to high levels of PSA in sera.

Dr. Smith:

This is a technical question because I am fascinated by your results. After you disperse the cells and you infect with the retrovirus, the provirus will probably only integrate into the genome when you have subsequent proliferation of those cells. You said that you let them sit overnight. Do they proliferate, and was the copy number the same in the two sets of experiments that you showed, that is, where you had opposing results?

Dr. Thompson:

With respect to the copy number question, different copy numbers I think can be ruled out. Identical titers of virus were used and at this multiplicity, which is less than one, it's been shown in other papers that integration events rarely exceed one.

Dr. Smith:

But it is difficult to get integration unless you have DNA replication, irrespective of how many retroviral particles you put in.

Dr. Thompson:

It is generally thought that you need proliferation for the provirus to integrate. So you're suggesting that there are different initial proliferative rates of the two strains.

Dr. Smith:

I'm asking, is that a possible explanation of your results?

Dr. Thompson:

I don't think that's a possibility. What we do to measure the percent infectivity is to do a two-day graft and take the tissue out, section it, stain it, or use antibodies and then look at the number of cells which show signal. With a virus of about 10 to the 5th CFU's per ml it's about 1 in 1000, i.e. 0.1%. I think I can probably exceed that by using concentrated virus, but that's about the upper limit right now. You can also co-culture. One thing that we are not really interested in yet is maximizing that number, but you can co-culture these grafts with psi-2-producers so that they could sit, for example overnight, as a collagen clot on top or on a pedestal or something and have constant exposure to viral particles overnight, and you would definitely increase titer at least one order of magnitude, possibly two under those conditions.

Dr. Lee:

Your interesting observation of probasin reminds me, years back, when John Grayhack and I worked on rat prostate lateral and dorsal lobe stimulation by prolactin. It's interesting that prolactin will only stimulate prostate growth in the dorsal and lateral lobe but it has very little effect on the ventral lobe. We also observed that castration will lead to prostatic regression, but if you treat these castrated rats with prolactin the dorsal and the lateral lobe will regress very slowly. We have looked at various mechanisms. There is no increase in DNA synthesis as compared to the control. There is one interesting observation which we have not reported. If we look at the prolactin treated rats' lateral prostate, almost every time we can find on two dimensional electrophoresis gels a protein spot at about 20 kilodalton at the very, very basic end. Sometimes it will run off the gel. It's more in the prolactin treated prostate than in the control and I was wondering if there is some way you could help us to identify if that is indeed probasin?

Dr. Matusik:

The answer is yes, that is probasin. Probasin has a pI probably between 10 to 11.5 and when you run it onto the gel, it smears in exactly like that. The other members of this ligand carrier family are all acidic proteins and if you line up the amino acid sequence, you find that in probasin where there are basic amino acids, the other members of the family have acidic amino acids, with one exception and that's in the helical structure where it is highly composed of acidic amino acids. In terms of prolactin, this is some work we have done with Gail Prins that Don Coffey had asked about earlier. When we have pituitary grafts in the animals and looked at the prolactin effect on the dorsolateral prostate, we found a couple of events. One was that the ventral prostate gene, prostatic binding protein was now induced in the dorsolateral prostate. Also, we found that the probasin mRNA went up about 2-fold. Now a 2-fold rise doesn't sound like a lot, but when you think that the mRNA is probably close to 10% to begin with and we've got a 2-fold increase because of prolactin, we're going to 20%. So I think that it is a very impressive change.

Dr. Matuo:

When I first found probasin, I did not know its function. This is the reason why I changed my strategy from probasin to growth factors. I can guess two functions. 1) Probasin can bind non-HBGF and heparin binding growth factor and probasin also had a high affinity for heparin. 2) Probasin has a high, strong affinity for the head of rat sperm, not the whole sperm body. So, maybe including growth factor, probasin has some functional role in fertilization. But we don't know anything as shown by Dr. Matusik.

Dr. Coffey:

I think this is a very exciting thing Dr. Jones is proposing here and Peter, if I remember correctly, several years back you showed that in aging fibroblasts there was an overall level of hypomethylation that

occurred with senescence. Is the immortalization and senescence the same? Have you had a chance to go back and look at the aging fibroblasts to see if in the islands it hypermethylates now but the overall gene is a hypo, so that the gross is down?

Dr. Jones:

Your memory is far too good! I did actually have those old slides to show today but I ran out of time. What you see when you put a mouse cell in culture is a precipitous drop in DNA methylation as a function of passage during crisis, so the methylation goes right down as the cells are dying. Then as you see the establishment of a cell line the methylation goes up again. You are erasing the methylation and then you are re-establishing a new pattern which is selected for because you're extinguishing genes which prevent cellular growth.

Dr. Coffey:

What is the role of hypomethylation and what you're seeing here in relation to chromosome fuzziness and condensation?

Dr. Jones:

I really don't know. A lot of those experiments utilize 5 azacytidine to show changes in chromosome structure and I'm not sure whether you can be certain that the fuzziness you see is due to hypomethylation *per se* or to the presence of azacytidine in the DNA. So I think that it remains unclear. I know that Bert Vogelstein often starts out his talk with the idea that this hypomethylation might cause chromosomal instability.

Question from the floor:

Do you think this causes chromosomal instability?

Dr. Jones:

I don't know.

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