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CHOLECALCIFEROL (VITAMIN D3) IN THE PREVENTION
AND TREATMENT OF PROSTATE CANCER: EFFECTS
AND MECHANISMS OF ACTION

presented by

ERIK J. TOKAR

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**CHOLECALCIFEROL (VITAMIN D₃) IN THE PREVENTION AND
TREATMENT OF PROSTATE CANCER: EFFECTS AND MECHANISMS OF
ACTION**

VOLUME I

By

Erik J. Tokar

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

2005

CHOLECALCEROL TREATMENT

Prostate cancer
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ABSTRACT

CHOLECALCIFEROL (VITAMIN D₃) IN THE PREVENTION AND TREATMENT OF PROSTATE CANCER: EFFECTS AND MECHANISMS OF ACTION

By

Erik J. Tokar

Prostate carcinogenesis, a multi-step process, which occurs over a period of 20-30 years, provides the opportunity for cancer prevention and treatment. Epidemiological evidence suggests an inverse relationship between prostate cancer incidence and serum vitamin D levels. Vitamin D plays an important role in cell proliferation and differentiation. The objectives of this study were to examine: i) the potential of cholecalciferol (vitamin D₃) as an agent for treatment by inhibiting or reversing malignant characteristics of prostate cancer cells; ii) its ability as a chemoprevention agent by reversing cellular changes associated with malignant transformation, and iii) its mechanisms of action. The malignant RWPE2-W99, human prostate epithelial cell line, which forms slow-growing tumors in nude mice and mimics behavior of primary human prostate cancers, was used. Results show that cholecalciferol, at physiological levels: i) inhibited anchorage-dependent and -independent growth in agar; ii) induced differentiation by decreasing vimentin but increasing PSA expression; iii) decreased MMP-9 and MMP-2 activity with concomitant decrease in motility and invasion; and iv) exerted its effects, by up-regulating vitamin D receptor (VDR), retinoid-X receptors (RXRs), and androgen receptor (AR) in a dose-dependent manner. Cholecalciferol inhibited changes associated with a malignant phenotype. I also report, for the first time, that human prostate epithelial cells constitutively express 25-hydroxylase protein

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(CYP27A1), the enzyme that converts cholecalciferol to 25OHD₃ and that CYP27A1 is up-regulated by cholecalciferol. Cholecalciferol has effects similar to those of calcitriol on growth, MMP activity, and VDR. Because cholecalciferol can inhibit cellular changes associated with malignant transformation and invasion, I propose that cholecalciferol may be an effective agent for treatment, and for secondary and tertiary prevention of prostate cancer.

Chemoprevention is the ultimate answer to reducing the high incidence of prostate cancer. The ability of cholecalciferol, to block or reverse changes associated with malignant transformation, was examined. Since immortalization is an early step in carcinogenesis, the immortalized but non-tumorigenic RWPE-1 human prostate epithelial cell line was used. RWPE-1 cells do not grow in agar and are not invasive. Physiological levels of cholecalciferol: i) inhibited anchorage-dependent growth; ii) decreased MMP-9 and MMP-2 activity; iii) induced differentiation by decreasing vimentin but increasing PSA expression, and iv) up-regulated VDR, RXRs, and AR, suggesting that its effects, in part, are receptor-mediated. Based on these *in vitro* studies, cholecalciferol appears to be an effective chemopreventive agent. I further show that RWPE-1 cells also express CYP27A1, and its expression is up-regulated by cholecalciferol. Since the presence of 1 α -hydroxylase has previously been shown, my results demonstrate that human prostate epithelial cells, target cells for vitamin D, have the necessary enzymes and the rare ability to locally produce the biologically active calcitriol from cholecalciferol. The mechanisms of action of cholecalciferol appear to involve the up-regulation of nuclear receptors and CYP27A1. Taken together, my results show that cholecalciferol may be an effective agent for chemoprevention and treatment of prostate cancer.

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To my family and my mentor
Thank you, for everything

First and foremost

for support, patience, w

for believing in me and

inspiration, none of

for Dr. Stephen C. B

Dr. Daniel E. Williams

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SOHD:

HPR

DHT

FS RA

ADAM

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ANOVA

AR

ATP

ATRA

BM

BMP

BPE

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T

LA

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D-PBS

DR-3

DNA

ECM

EDTA

ELISA

EGF

ER

FBS

FGF

FLP

FN

FRAP

GDP

GFAP

HSPIN

HEV

HN

IGF

IGFBP

LIST OF ABBREVIATIONS

1,25(OH) ₂ D ₃	1,25-dihydroxyvitamin D ₃ , calcitriol
25OHD ₃	25-hydroxyvitamin D ₃
4-HPR	<i>N</i> -(4-hydroxyphenyl)retinamide
DHT	5- α dihydrotestosterone
9-cis RA	9-cis-retinoic acid
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AR	Androgen receptor
ATP	Adenosine triphosphate
ATRA	all-trans retinoic acid
BM	Basement membrane
BMP	Bone morphogenetic protein
BPE	Bovine pituitary extract
BPH	Benign prostatic hyperplasia
bp	Base pairs
cdk	cyclin-dependent kinase
cdki	cyclin-dependent kinase inhibitor
CFE	Colony forming efficiency
cK	cytokeratin
D-box	Distal box
DBD	DNA binding domain
DBP	Vitamin D binding protein
D-PBS	Dulbecco's phosphate buffered saline
DR-3	Direct repeat with three nucleotide spacer
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
ER	Estrogen receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FLIP	Fluorescence loss in photobleaching
FN	Fibronectin
FRAP	Fluorescence recovery after photobleaching
GDP	Guanine diphosphate
GFAP	Glial fibrillary acidic protein
HGPIN	High-grade prostatic intraepithelial neoplasia
HPV	Human papilloma virus
IEN	Intraepithelial neoplasia
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein

NT
LPAC
NWM
DA
NOF
KSEM
LBD
MMP
ME
-ENA
MT-MMP
MTT
NH
NLS
NR
PDA
PA
PAP
PIC
PIN
PSA
PTH
RAR
RE
RER
SD
SDS-PAGE
TGF
TMP
PA
TR
PA
TV
TOR
TORE

INT	<i>p</i> -iodonitrotetrazolium
IUPAC	International Union of Pure and Applied Chemists
IVVM	Intravital video microscopy
kDa	Kilodalton
KGF	Keratinocyte growth factor
KSFM	Keratinocyte serum free medium
LBD	Ligand binding domain
MMP	Matrix metalloproteinase
Mr	Molecular weight
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane-type matrix metalloproteinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NIH	National institutes of health
NLS	Nuclear localization signal
NR	Nuclear receptor
P-box	Proximal box
PA	Plasminogen activator
PAP	Prostate acid phosphatase
PIC	Pre-initiation complex
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTH	Parathyroid hormone
RAR	Retinoic acid receptor
Rb	Retinoblastoma
RXR	Retinoid X receptor
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
tPA	Tissue-type plasminogen activator
TR	Thyroid hormone receptor
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	Vitamin D response element

Hypotheses

The main hypothesis is that all-trans-retinoic acid (ATRA) treatment of acute promyelocytic leukemia (APL) cells is associated with morphological and functional changes in these cells, and that these changes are associated with the induction of differentiation. The following hypotheses will be used to test the main hypothesis: ATRA treatment of APL cells will induce differentiation. The following hypotheses are that:

1. ATRA inhibits the growth of APL cells.

2. ATRA induces cell differentiation.

a. down-regulation of

b. induction of

differentiation

3. ATRA inhibits invasion.

a. vimentin

b. matrix metalloproteinase

4. ATRA alters its biological activity.

(VDR), retinoic acid receptor (RAR),

(CYP27A1)

5. ATRA will prove to be effective in the treatment of APL.

treatment through the induction of differentiation.

Hypotheses

The main objectives of this study are: i) to examine the potential of cholecalciferol (vitamin D₃) as a chemoprevention agent by reversing cellular changes associated with malignant transformation; ii) to examine its potential as an agent for cancer treatment by inhibiting or reversing malignant characteristics of prostate cancer cells; and iii) to explore its mechanisms of action. The non-tumorigenic RWPE-1 cell line will be used to test the chemopreventive ability of cholecalciferol and the malignant RWPE2-W99 cell line will be used for prostate cancer treatment studies. The proposed hypotheses are that cholecalciferol:

- 1) inhibits the growth of pre-malignant and malignant human prostate epithelial cells
- 2) induces cell differentiation, indicated by changes in protein expression, by:
 - a. down-regulating vimentin, a marker of loss of differentiation
 - b. inducing prostate specific antigen (PSA) expression, a marker for cell differentiation
- 3) inhibits invasion by decreasing:
 - a. vimentin-associated motility
 - b. matrix metalloproteinase (MMP-9 and MMP-2) activity
- 4) exerts its biological effects by modulating the expression of vitamin D receptor (VDR), retinoid X receptors (RXRs), androgen receptor (AR), and 25-hydroxylase (CYP27A1)
- 5) will prove to be an effective potential agent for prostate cancer prevention and treatment through its multiple effects



Part I

Literature Review

The

Chapter 1

The Prostate: Structure, Function, and Neoplastic Diseases

Abstract

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Abstract

In order to understand the organ under investigation, one must have a basic knowledge of its anatomy, structure, histology, and function. This is especially true in the case of the prostate gland because different diseases of the prostate can be associated with its different anatomical regions. The prostate is a small accessory male sex gland with an increasing tendency to develop both benign and malignant tumors with increasing age. This chapter provides information about the zonal anatomy, structure and function of the prostate gland and the role of the components of the prostatic secretions, including growth factors, prostasomes, proteins, lipids, and enzymes. This chapter also describes the morphology and histology of the different cell types that make up the prostate gland. Prostatic diseases, including benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and prostate cancer will also be discussed.

Anatomy

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Anatomy

The prostate is a gland found in all orders of male mammals (Krongrad, 2000). The prostate is considered to be a gland because it makes secretions, thus, meeting the definition of a gland. The human prostate is considered an exocrine accessory sex gland because of its indirect involvement in procreation (**Figure 1-1**). Regardless of its small size, it is the largest of the accessory sex glands. Its shape and size resemble that of a chestnut and it weighs approximately 20 grams, or a little less than an ounce. The prostate is about the size of a pea at birth and gradually increases in size until puberty (Rous, 1988). After puberty there is a rapid period of growth until the prostate reaches its normal adult size sometime between the third and fourth decade of life.

The normal prostate gland in the adult male lies caudal to the urinary bladder and cranial to the external urethral sphincter (**Figure 1-1**) and consists of a large series of branching ducts that enter the prostatic urethra (Isaacs, 1983). The prostate is divided into two distinct segments, distal and proximal, by the 35 degree angle of deviation the urethra takes as it passes through the prostate (McNeal, 1989). The urethra also divides the prostate gland into a ventral fibromuscular portion and a dorsal glandular portion, both of which are encapsulated by a thin layer of connective tissue. The prostatic urethra is the portion of the urethra which is within the prostate and has the same anatomical relationship to the gland as the core of an apple has to the apple itself. It is a channel through the prostate gland through which urine flows as it leaves the bladder.

The prostate gland consists of approximately 30-50 tubuloalveolar glands contained in the fibroelastic stroma. The stroma is composed of smooth muscle and completely shields the three zones from view. The fibromuscular stroma portion

vas deferens —

prostatic
urethra —

penis —

scrotum —

Figure 1-1: The location of the male reproductive system relative to the prostate gland.

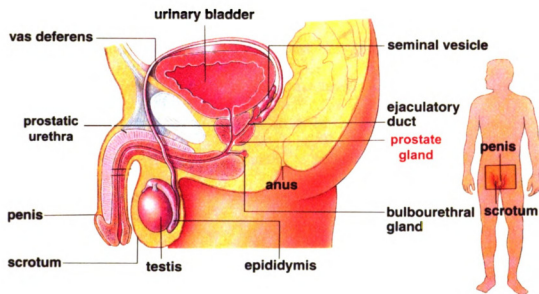


Figure 1-1: The location of the prostate gland with respect to the remainder of the male reproductive and urinary anatomy. The prostate gland lies caudal to the urinary bladder and is divided into two segments by the prostatic urethra (modified from Starr and McMillian, 1997).

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occupies the anterior surface of the prostate and makes up roughly one third of the total tissue of the prostate capsule. The fibroelastic capsule surrounds the prostate gland and also contains small bundles of smooth muscle. The capsule and fibromuscular stroma portions of the prostate are of less importance than the glandular portion from a pathological and histological standpoint.

The glandular portion of the prostate is composed of three zones that differ biologically and histologically: the peripheral, central, and transitional zones (**Figure 1-2**). These concentric zones, first established by McNeal (McNeal, 1981), divide the prostate based on morphological, functional, and pathological significance (Coffey, 1992). The peripheral zone in the normal prostate comprises the majority (approximately 70%) of the prostatic volume. As its name implies, it extends around the posterolateral peripheral aspects of the gland from its apex to its base (Kirby, et al., 1996). The ducts of the peripheral zone, which predominantly consist of the main prostatic glands, open into the distal prostatic urethra. The histological appearance of this zone is identified by small, acinar spaces lined by tall columnar secretory epithelial cells. The majority (approximately 70%) of prostatic adenocarcinomas arise in this zone (Ahmed et al., 1997).

The central zone is the second largest zone of the normal prostate and it comprises about 25% of the glandular volume. The majority of glands in this zone are mucosal glands. This zone is cone-shaped and surrounds the ejaculatory ducts with its apex at the verumontanum and its base against the bladder neck (Dixon et al., 1999). The central zone is surrounded by the peripheral zone and makes up the majority of the prostatic base. The central zone is characterized by relatively large acini with irregular contours,



Figure 1-2. Zonal structure of fibronectin.

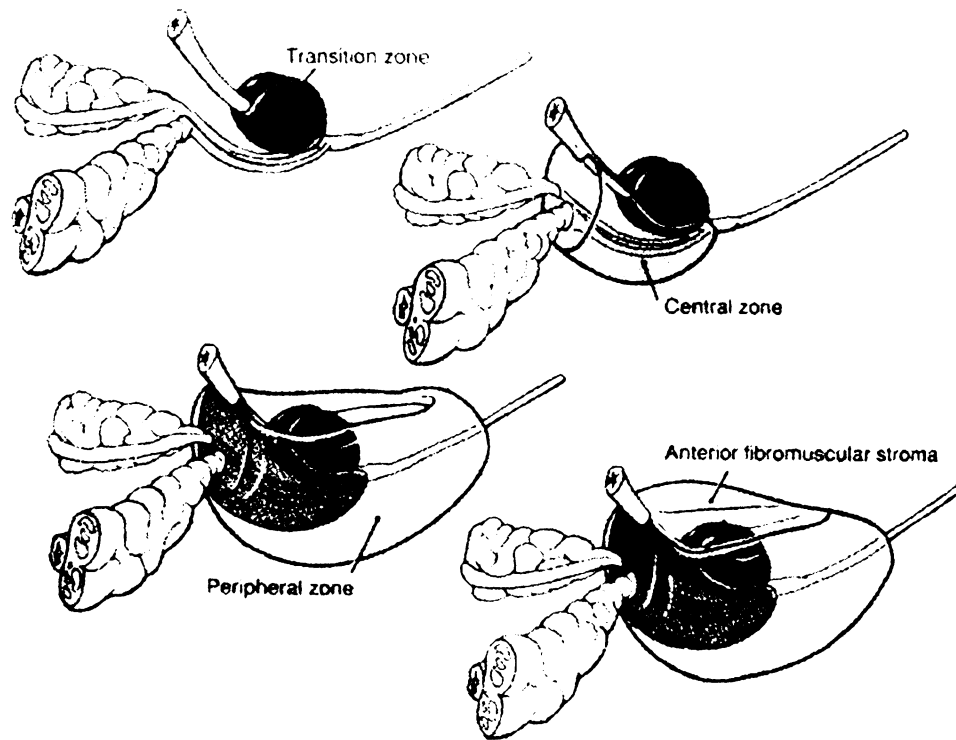


Figure 1-2: Zonal anatomy of the prostate, showing the three glandular zones and the fibromuscular stroma (Ahmed et al., 1997).

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The transitional zone comprises the remaining 5 to 10% of the prostatic volume. This zone is composed of two bilaterally symmetrical lobules that surround the prostatic urethra. The main component of the transitional zone is a cylindrical smooth muscle sphincter whose main function is to prevent the reflux of seminal fluid into the bladder at the time of ejaculation (Coffey, 1992). The submucosal glands of this zone have ducts that empty into the urethra at the base of the verumontanum. Histologically, the transition zone acini resemble those of the peripheral zone; however, the surrounding stroma is more compact and resembles that of the central zone. The transition zone develops 25% of prostate cancers and it is the exclusive site of benign prostatic hyperplasia (Coffey, 1992). The histology of the prostate gland will be discussed next.

Histology

The normal prostate is a highly complex tissue. The glands in the normal prostate are lined with pseudostratified epithelium (**Figure 1-3**) and are surrounded by stroma that contains nerve fibers, smooth muscle, fibroblasts, blood vessels, and extracellular matrix. In the normal prostate, the pseudostratified epithelium is attached to the basement membrane (**Figure 1-4**). But when the prostate becomes cancerous the epithelial cells become disorganized and form a cluster of cells (**Figure 1-5**). Constant interaction between epithelial cells and stromal cells is crucial for maintaining the structure and

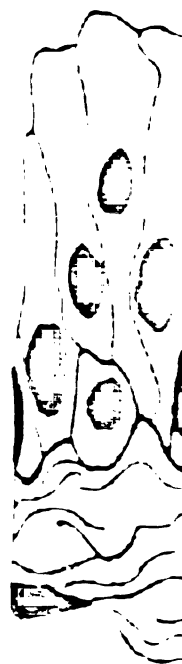


Figure 1-3: Pseud.
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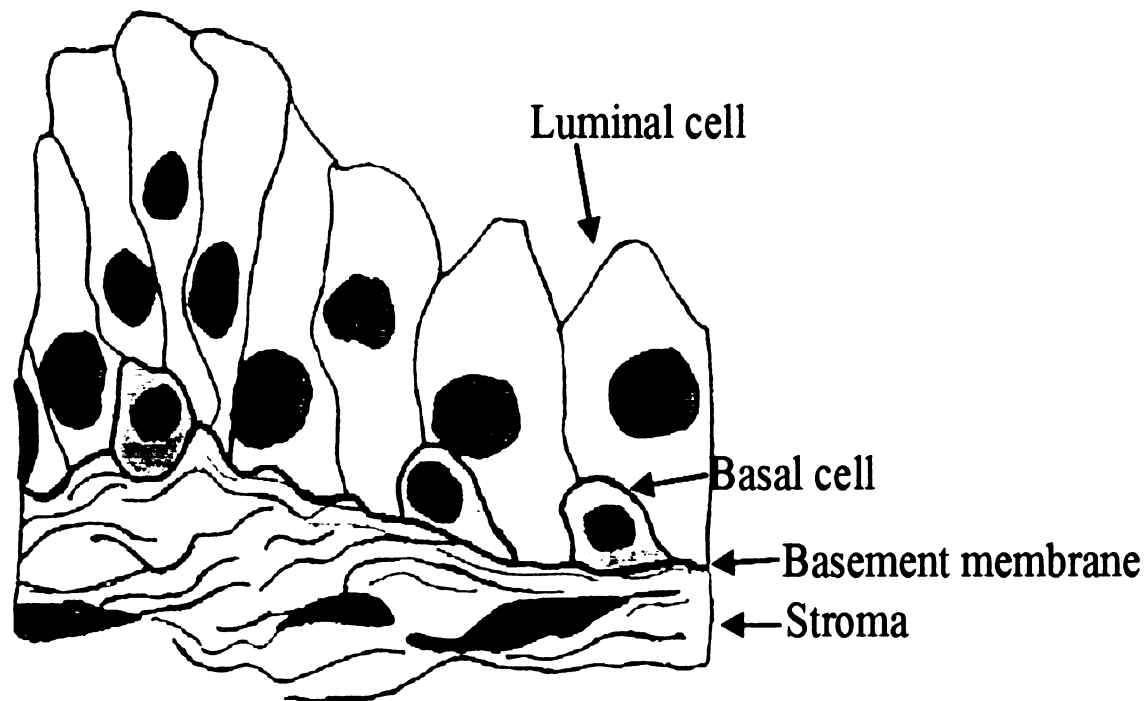


Figure 1-3: Pseudostratified epithelial cell layer of the normal prostate. The basal cells (gray) and the luminal cells (white) are shown attached to the extracellular matrix (ECM). Fibroblasts (black) are located in the ECM (modified from Bello, 1996).

Figure 1-4: Histology
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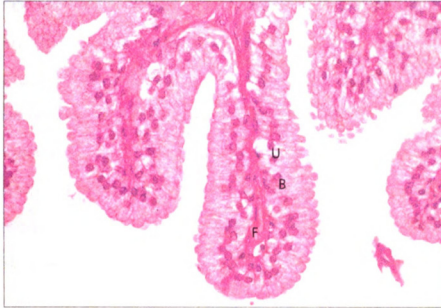


Figure 1-4: Histological section of the benign prostate, showing the uniform columnar cells (U), the basal cell (B), and fibrovascular stalk (F) (Kirby et al., 1996).

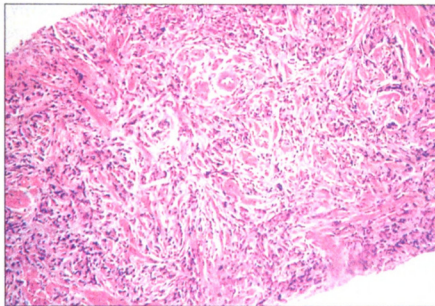


Figure 1-5: Histological section of the cancerous prostate (Kirby et al., 1996).

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The luminal cells are the most common cell type in the prostate gland. They are tall columnar, terminally differentiated secretory epithelial cells. They are attached to the basement membrane by integrin receptors. These cells can be distinguished by their shape, enzymes and secretory granules that stain for prostate specific antigen (PSA), prostate acid phosphatase (PAP), and other proteins. These secretory granules and enzymes are located in the luminal end of the cells. The nuclei are located towards the base of the cells below a zone of abundant Golgi apparatus. With androgen ablation, these cells decrease in number by up to 90%, become cuboidal, and shrink by as much as 80% and 60% in volume and height, respectively.

Some of the basal cells are considered to be the stem cells from which the luminal cells arise (Verhagen et al., 1992; Tokar et al, 2005). These cells are less abundant than the luminal cells, comprising only 10% of the secretory epithelial component. They are far smaller in size than the luminal cells and may appear absent from some of the ducts and acini. Basal cells have a round morphology, possess large nuclei, and are most abundant in the central zone of the prostate gland. Basal cell proliferation has been directly related to the development of benign prostatic hyperplasia (Partin, 2000).

Stem cells are elongated cells that contain little cytoplasm. They are located in the basal cell layer (Foster and Ke, 1997). Stem cells within the basal layer give rise to the basal cells, luminal cells, and neuroendocrine cells. The neuroendocrine cells rest on the basal cell layer between the secretory cells and often have dendritic processes. They are randomly scattered throughout the prostate and exist in all three zones. The

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The principal function of the seminal fluid. The spermatozoa can travel through the fluid is to provide the vaginal canal with a favorable environment.

The prostate gland (1900). Its major secretory product is the prostatic component of the seminal fluid. The soluble components of the fluid include hormones, lipids, and proteins. These are taken up from the blood and secreted into the prostatic ducts (PSA) and prostatic secretions. PSA is expressed in both the prostatic and seminal vesicles. It appears as prostatic secretions. ATP, cholesterol, and other components of the fluid and other seminal

preceding sections have provided background information on the anatomy, structure, and histology of the human prostate necessary for an understanding of prostate function.

Function

The principal function of the prostate gland is to contribute its secretion to the seminal fluid. The main purpose of this fluid is to act as a vehicle in which the spermatozoa can travel to the outside at time of ejaculation. The secondary purpose of the fluid is to provide nourishment to the sperm. The fluid is slightly alkaline, making the vaginal canal less acidic, which allows the sperm to survive longer in that environment.

The prostatic component makes up 18-20% of the total ejaculate volume (Dixon, 1999). Its major secretory products such as acid phosphatase, citrate, and zinc identify the prostatic component in semen. The secretion is in the form of soluble and particulate matter. The soluble fraction of the semen includes proteins, carbohydrates, electrolytes, hormones, lipids, and growth factors. Molecules such as IgA, transferrin, and albumin are taken up from the blood. Prostatic acid phosphatase (PAP), prostate specific antigen (PSA), and prostate binding protein, all major proteins specific to the prostate, are expressed in both pubertal and adult man. In the seminal plasma, particulate matter appears as prostasomes, which are membrane-bound organelles rich in Ca^{2+} , GDP, ADP, ATP, cholesterol, and membrane-bound enzymes and proteins. In humans, different components of the prostatic secretions may interact as well as act upon ejaculated sperm and other seminal elements.

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It is believed that seminal plasma has an effect on sperm function, such as, motility, viability, fructose and oxygen consumption, along with its primary purpose of sperm transport. Prostatic fluid can enhance the motility of epididymal, ejaculated sperm (Lindholmer, 1973). In clinical studies, it was found that men with disturbed prostatic function show decreased motility and viability (Eliasson, 1970). At ejaculation, the sperm take up zinc from the prostatic fluid, which temporarily inhibits sperm chromatin decondensation. This is important in fertility since nuclear zinc content of ejaculated sperm correlates with fertility status in men (Dixon et al., 1999). Antibacterial activities of seminal plasma have been attributed to low molecular weight products of the prostate such as zinc, spermine, and a heat-stable fraction (Stamey et al., 1968).

Prostasomes, part of the particulate matter in seminal plasma, may play a complementary role in prolonging the life of sperm by preventing hypersensitization of the female to antigenic proteins present on the surface of sperm. Lipids from the prostatic fluid in the seminal plasma play a role in reducing certain macrophage and neutrophil functions that are normally unfavorable to sperm.

A major activity of prostatic secretion is proteolysis. The prostate contains a vast array of enzymes that are involved in tissue remodeling during growth. The most studied of these enzymes is prostate specific antigen (PSA), a serine protease (Webber et al., 1995). PSA is used as a serum marker for diagnosis of prostate cancer and for monitoring efficacy of treatment for prostate cancer. PSA has many activities in the prostate including the breakdown and modulation of insulin-like growth factor-binding protein (IGFBP) and degradation of secretory leukocyte protease inhibitor (SLPI), which protects tissues against degradation by proteases released during inflammation (Dixon, 1999).

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PSA is also involved in the dissolution of semen coagulum. This involves degradation of fibronectin contributed to the seminal plasma by the seminal vesicles (Webber et al., 1995). Prostatin, a serine protease isolated from seminal plasma, may have a general proteolytic function. PSP94, one of the most abundant proteins in human prostatic fluid, may function in modifying properties of mucus in the female and male reproductive tracts (Dixon, 1999). Kinins, tissue hormones known to promote sperm motility, may also be regulated by the enzymatic activities in the prostate.

Prostatic acid phosphatase (PAP), a glycoprotein, is used as a serum marker in prostate cancer diagnosis and therapy. Although its exact biological function is not known, PAP may play an important role in prostate function. PAP may contribute to the formation of bone around neoplastic prostatic metastasis sites. PAP may also contribute to creating a balance between phosphorylation and dephosphorylation of proteins necessary for proper growth of cells (Lee et al., 1991).

Other accessory sex gland products are affected by prostatic proteases and metalloproteinases, such as seminal plasma motility inhibitor. Other degradation processes may produce amino acids that aid in energy metabolism of sperm. Prostatic secretion may assist in the conversion of fructose to glucose as well as being a major contributor to the inositol content in human seminal fluid. Calcium is required for the motility and fertilization of sperm and the citrate from the prostatic fluid is one of the most important factors in regulating the calcium levels in the semen.

Presently, growth factors are one of the most researched areas in the study of the functions of the prostate (Dixon, 1999). Growth factors are needed for normal cellular growth and development and they work by binding to cell-surface receptors in order to

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stimulate a variety of cellular responses including proliferation, secretion, and communication. Transforming growth factor (TGF) β 1 and β 2 are secreted by the stroma and epithelium, respectively. TGF- β is known as a suppressor of epithelial cell proliferation. Insulin-like growth factors (IGF) are potent mitogens of prostatic epithelial cells but not stromal cells. Stromal cells express type I IGF receptors and epithelial cells express IGF, IGF receptors and their binding proteins (IGFBP). IGFs, IGFBP, and IGFBP proteases are implicated in the regulation of prostatic growth, with an emphasis on keeping IGF effects to a minimum in the normal gland. These growth factors, along with the other components of the prostatic secretions, are important for the maintenance of tissues that make up the prostate. There remains a lot to be learned about prostate cancer but the better our understanding of the anatomy, structure, function, and histology of the normal prostate the more we will be prepared to diagnose, treat, and prevent prostate cancer.

Prostatic Diseases

The human prostate is composed of several glandular and nonglandular elements each with a characteristic and unique predisposition to disease. The following section will discuss the anatomical considerations that are implicated in the development of these diseases.

Benign Prostatic Hyperplasia

The transition zone is the exclusive site of benign prostatic hyperplasia (BPH) (Coffey, 1992). This exclusivity of BPH origin may be due to delayed prepubertal

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development, continued growth into adult life, and intermingling of different stroma types, which are all features unique to the transition zone (McNeal, 1990). BPH can be described as being microscopic, macroscopic, or symptomatic. Microscopic evidence can be seen as early as the fourth decade of life (McNeal, 1990) and can be found in approximately 50% of men by the age of 60, and over 90% by the age of 80 (Ahmed et al., 1997). Macroscopic disease, which is evident on physical examination, is seen in only half of these men and is not necessarily symptomatic.

It is not known what causes BPH to develop but three theories have been proposed. The first hypothesis is that the changes in the levels or ratios of androgens and/or estrogens with age stimulate prostate cell growth. The second hypothesis is that alterations in epithelial-mesenchymal interactions, through re-awakening of the embryonic inductive potential of the stroma, lead to increased proliferation. And the third hypothesis is that there is an expansion of an epithelial stem cell population (Hudson et al., 2001).

The tissues (muscle, glands, and fibrous tissue) that comprise BPH when it arises are the same that compose the normal prostate. The only difference is that BPH tissue generally has more glands and less muscle and fibrous tissue than the normal prostate. Histologically, BPH is extremely variable, ranging from a highly epithelial type to a fibromuscular type that is largely devoid of epithelial components (**Figure 1-6**) (Kirby et al., 2001).

The early stages of BPH in the transition zone produce microscopic nodules, but do not lead to clinical symptoms. As BPH continues to progress, the microscopic nodules undergo further hyperplastic changes with a substantial increase in size. The

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B

Figure 1-6: (A)

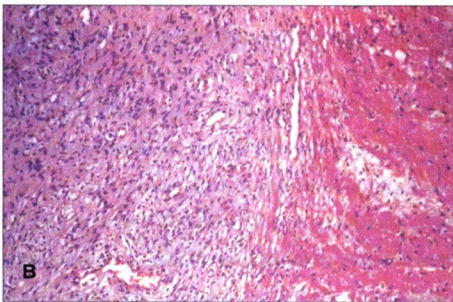
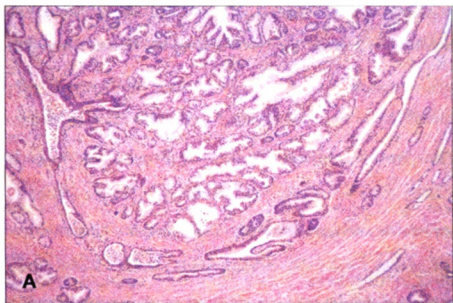


Figure 1-6: (A) BPH hyperplastic nodule of the highly epithelial type. (B) BPH stromal nodule of the fibromuscular type (Kirby et al, 2001).

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stimulation of the prostatic ducts next to these nodules leads to the formation of macroscopic nodules. The macroscopic nodules displace prostatic tissue both caudally and posteriorly, leading to the formation of the surgical capsule. There is considerable growth of BPH tissue during this stage of development. The final stage of histogenesis of BPH is the development of clinical symptoms.

The precise causes of BPH remain enigmatic but numerous risk factors have been proposed, and there is undoubtedly interplay of a number of factors. The increasing incidence of microscopic BPH with age is clear evidence that age is possibly the most important risk factor of this disease. Normally functioning testes are also an extremely important prerequisite for disease development as this plays a role in the hormonal (androgens) activity levels in the body. While these are the only two risk factors that have been definitively linked to BPH, there is increasing evidence to suggest several others could possibly play a role in its development.

There is evidence to suggest that hereditary predisposition of BPH is a risk factor. Recent molecular observations have suggested that DNA abnormalities may contribute the BPH pathogenesis. These studies demonstrated abnormalities such as: 1) a high frequency of genomic alterations in BPH compared with normal tissue, 2) molecular abnormalities in the DNA sequence of p53, a tumor suppressor gene, in up to 40% of BPH tissues, and 3) increases or decreases in the methylation patterns of nucleotides in BPH tissue when compared to normal tissue (Partin, 2000). Further investigations have shown that the observed familial aggregation of BPH follows a Mendelian pattern of transmission. Factors controlling prostatic growth in hereditary BPH may be androgen-independent, and larger prostates in young men with hereditary BPH are characterized by

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Race and ethnicity have also been suggested as risk factors for BPH. Studies suggest that there is a higher incidence rate in African-American men when compared to age-matched white American men. Men of Oriental races have been shown to have lower incidence rates for BPH than any other race. It is also suggested that these racial differences may be attributed to dietary differences. While men of Asian descent have the lowest incidence of BPH, studies show that descendants of men born and raised in China and Japan who migrated to the United States demonstrate microscopic and clinical evidence of BPH similar to that seen in the indigenous American population (Partin, 2000). This change in incidence may be due to the change in diet. The Asian diet is rich in yellow vegetables and soy products, all of which may inhibit BPH development. Conversely, the Western diet of the United States is high in fats, which could possibly promote the development of BPH. There are other miscellaneous risk factors that have been implicated with the development of BPH. These include smoking, alcohol intake, obesity, certain medications, diabetes mellitus, cirrhosis of the liver, and hypertension (Kirby and Christmas, 1993; (Partin, 2000).

Prostatic Intraepithelial Neoplasia (PIN)

Precancer or intraepithelial neoplasia (IEN) is a noninvasive lesion that has genetic abnormalities, loss of cellular growth control functions, cellular heterogeneity, and some phenotypic characteristics of invasive cancer and that predicts a substantial likelihood of developing invasive cancer (O'Shaughnessy et al., 2002). IEN occurs in most epithelial tissue as a moderate to severe dysplasia, is on the causal pathway leading

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from normal tissue to cancer, and is close in stage of progression to cancer (invasive neoplasia) (O'Shaughnessy et al., 2002). In aggressive cancers this progression may happen within months to a few years, but generally these changes appear to occur over a long period of time. Progression is mapped in target tissues by the appearance of specific molecular and genotypic damage associated with increasingly severe dysplasia and is influenced by factors specific to the environment of the host tissue.

Subjects with IEN are at significantly higher risk than unaffected populations for developing invasive cancer in the same tissue (O'Shaughnessy et al., 2002). A notable example to illustrate the invasive cancer risk associated with IEN is the prostate. Prostatic intraepithelial neoplasia (PIN) is a risk marker for prostate cancer and shares many characteristics with prostate adenocarcinoma (**Table 1-1**).

Apart from the characteristics mentioned in Table 1, PIN lesions also share the following characteristics with malignancy: loss of basal layer continuity, production of acid mucin, altered lectin binding, evidence of loss of differentiation as evidenced by decreased prostate specific antigen (PSA) immunostaining, and abnormalities of ploidy (Kirby et al, 2001).

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Table 1-1: Evidence that PIN is premalignant (modified from Kirby et al., 2001)

- Morphological similarity to invasive cancer – cellular atypia in high-grade PIN is virtually indistinguishable from invasive cancer, except no invasion has occurred in high-grade PIN
- Phenotypic similarity to cancer – cytoskeletal proteins, secreted proteins, and degree of glycosylation are shared by PIN and cancer but not BPH or normal prostatic epithelium
- Incidence is greater in organs harboring invasive cancer
- Severity is increased with cancer
- Extent is greater with concomitant cancer
- Spatial relationship to invasive cancer – both being found primarily in the peripheral zone, found less frequently in the transition zone
- Microinvasion present from the premalignant change

PIN is divided into two grades, low-grade PIN and high-grade PIN. Low-grade PIN (**Figure 1-7A**) architecture shows the epithelial crowding and stratification, with irregular spacing. The nuclei are enlarged with a marked size increase, the chromatin appears normal, and nucleoli are rarely prominent. In low-grade PIN the basal cell layer and the basement membrane both remain intact. Some pathologists prefer not to report low-grade PIN due to the difficulty in separating this lesion from benign epithelium and reactive atypia (Bostwick and Sakr, 1998).

The architecture of high-grade PIN (**Figure 1-7B**) is similar to low-grade PIN but with more epithelial crowding and stratification. High-grade PIN can be divided into four architectural patterns: tufting, microcapillary, cribriform, and flat (Bostwick, 1994). Although most cases of PIN have multiple patterns, tufting is the most common and is



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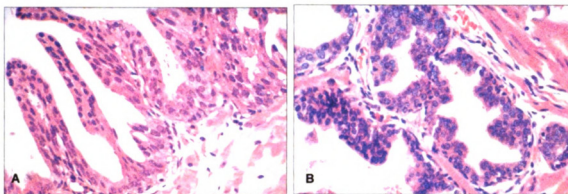


Figure 1-7: (A) Low-grade prostatic intraepithelial neoplasia. (B) High-grade prostatic intraepithelial neoplasia. Both panels show H and E staining (Kirby et al., 2001).

found in at least 97% of radical prostatectomy specimens with PIN and cancer. High-grade PIN also shows enlarged nuclei with some size and shape differentiation. The chromatin is of increased density with clumping and the nucleoli are frequently large and prominent, which is similar to invasive carcinoma. High-grade PIN is associated with disruption of the basal cell layer and basement membrane, as identified by high-molecular-weight cytokeratin immunostaining (Kirby et al., 2001). This has led to the development of an increasingly accepted model for prostate carcinogenesis (**Figure 1-8**). The significance in recognizing PIN is based on its strong association with prostatic carcinoma. PIN has a high predictive rate of prostate adenocarcinoma, which makes it a novel intermediate marker to indicate likelihood of cancer.

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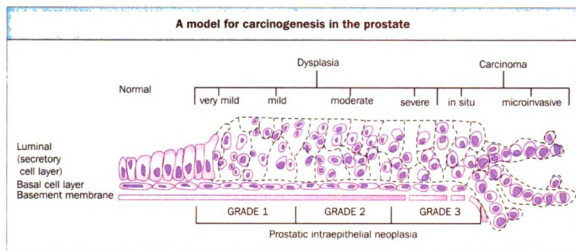


Figure 1-8: Model for PIN – carcinogenesis in the prostate showing characteristics associated with progression from normal prostatic cells to the cancerous phenotype (Kirby et al., 2001).

Prostate Cancer

Prostate cancer is the most common non-skin cancer in American men. Its incidence increases sharply with age from about the age of 60 onwards and approximately 1 out of every 7 men over the age of 60 will develop this disease. More than 80% of prostate cancer is found in men over the age of 65. It is estimated that prostate cancer will have accounted for 33% of all new cancer cases in men in 2003, with 220,900 new cases expected to be diagnosed (Jemal et al. 2003). Second only to lung and bronchus cancer, prostate cancer will be responsible for approximately 10% of all cancer deaths among men in 2003, accounting for an estimated 28,900 deaths (Jemal et al., 2003). While the exact cause of prostate cancer remains obscure, several factors have

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Table 1-2:

Kirby et al.

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been implicated in giving rise to this disease. Old age, race, environment, hormones, and diet are the most noticeable of these factors but genetic predisposition, sexual history, and infection are other factors that may be associated with risk for prostate cancer (Table 1-2).

Table 1-2: Factors implicated in the development of prostate cancer (modified from Kirby et al., 2001).

Genetic – Familial autosomal dominant in some

Racial – More common in blacks, Scandinavians, US whites, and Western Europeans

Age – More common with increasing age

Diet – Animal fats and low intakes of yellow/green vegetables

UV light – Higher incidence in areas with lower exposure

Pollution – Urban dwelling, cadmium exposure, exposure to radioactive agents

Sexual History – More common in men with early experience and multiple partners

Infection* – Gonococcol infection, Herpes simplex type 2, human papillomaviruses 16 and 18

* = possible risk factors

Most carcinomas of the prostate are classical adenocarcinomas, being made up of epithelial cells with varying degrees of glandular architecture. The basal cell layer is absent causing a departure from the normal two layers of cells. The definitive site of origin is difficult to identify due to infiltrative growth pattern resulting in difficulty in defining the actual edge of the tumor. Approximately 70% of prostate carcinomas originate in the peripheral zone, 20% originate in the transition zone, and about 5-10% originate in the central zone. The frequency of carcinomas in the transition zone is

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disproportionately high given that this zone comprises only 5-10% of the volume of the normal prostate but accounts for 25% of the carcinomas. Conversely, the central zone accounts for about 5% of carcinomas, a disproportionately low frequency considering its large volume.

The problem with prostate cancer is very complicated because of what is called autopsy cancer. Even though only about 10% of men over the age of 50 will have clinically diagnosed prostate cancer, approximately 30% of them will have histologic evidence of adenocarcinoma at autopsy (Lin and Lange, 2000). Since it is often diagnosed at an advanced stage, the majority of men die with their disease.

In recent years there has been a reduction in the incidence and mortality rates of prostate cancer. The reduction in mortality is due to either 1) reduction of incidence, 2) improvements in therapy, and/or 3) early detection. Reduction of incidence is due in part to the education of the public about prostate cancer and the methods for possibly preventing the development of this disease. Improvements in therapy are a result of the many investigations conducted on men and animal models with prostate cancer. Early detection, or finding men with prostate cancer at a time when it is curable, is due mainly in part to the yearly screening of prostate specific antigen levels. But much work still remains to be done. Not until all of these three opportunities are perfected will we have the potential to fully prevent and treat this dreaded disease.

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Conclusion

The prostate is a small accessory sex gland that is composed of three zones, peripheral, transitional, and central. Each of these zones can be associated with different diseases of the prostate. With increasing age, the prostate gland has an increasing tendency to develop prostatic diseases, including benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and prostate cancer. Thus, with the continually increasing life span in society today, prostatic diseases have become one of the most important health concerns in men. While the exact causes of these diseases have yet to be elucidated, a variety of risk factors have been identified and associated with them, most importantly, age, hormonal activity, race, and diet. These factors, coupled with a thorough knowledge of the prostate gland, can lead to a better understanding of the causes and natural history of the disease processes. This, in turn, could result in better treatment and prevention methods for prostatic diseases and possibly improve the quality of life among the aging male population.

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Chapter 2

Epidemiology of Prostate Cancer and Possible Relationships with Vitamin D

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Abstract

Prostate cancer is the most common non-skin cancer in American men. Its incidence increases sharply with age from about the age of 60 onwards and approximately 1 out of every 7 men over the age of 60 will develop this disease. More than 80% of prostate cancer is found in men over the age of 65. It is estimated that prostate cancer will have accounted for 33% of all new cancer cases in men in 2003, with 220,900 new cases expected to be diagnosed. Second only to lung and bronchus cancer, prostate cancer will be responsible for approximately 10% of all cancer deaths among men in 2003, accounting for an estimated 28,900 deaths (**Figure 2-1**). While the exact cause of prostate cancer remains obscure, several factors have been implicated in giving rise to this disease. Old age, race, environment, and diet are the most noticeable of these factors but genetic predisposition and socioeconomic status are other factors that may be associated with risk for prostate cancer. In recent years, observations have been made that show an inverse relationship between prostate cancer mortality rates and the geographically incident ultraviolet (UV) radiation exposure from the sun. Ultraviolet light is essential for vitamin D synthesis in the skin. These observations have led to the suggestion that vitamin D may play a role in decreasing prostate cancer incidence. This chapter will examine the epidemiology of prostate cancer and show how it relates to vitamin D levels.

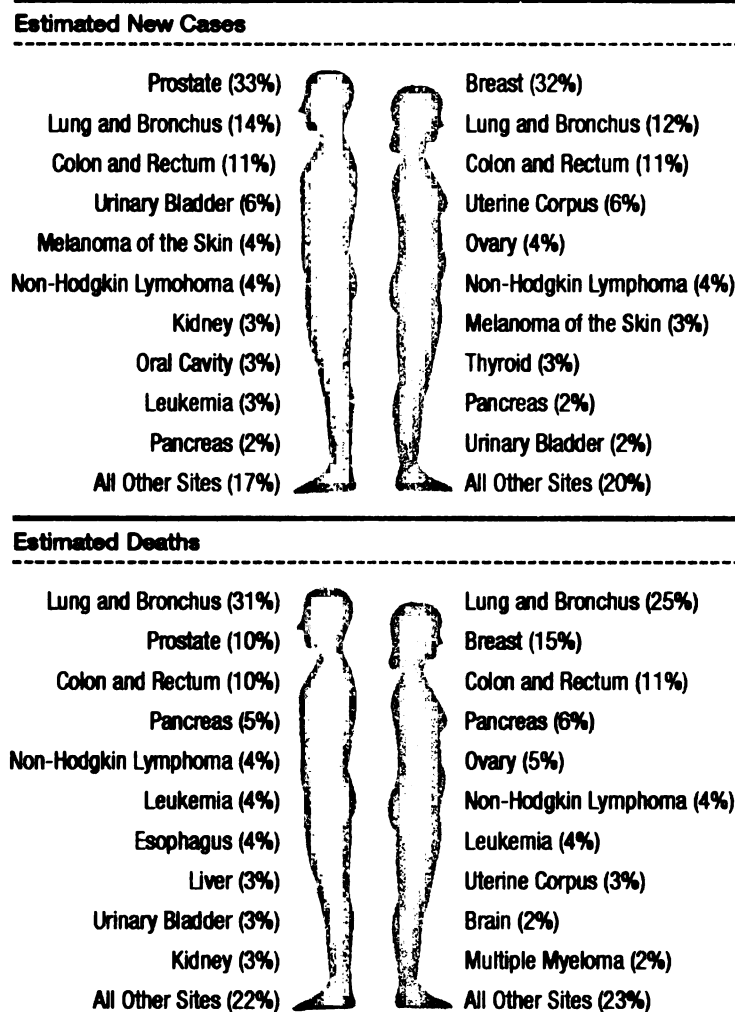


Figure 2-1: Estimated new cancer cases and deaths by gender, US, 2003 (Jemal et al., 2003).

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Introduction

Over the past two decades there has been an increase in prostate cancer incidence and mortality. Countless studies examining the epidemiology of prostate cancer and its possible relationships with vitamin D have been conducted since Schwartz and Hulka (1990) first hypothesized that vitamin D deficiency increases the risk of prostate cancer. Dietary intake provides a small amount of the daily requirement of vitamin D, but the major source of vitamin D for Americans is UV light-induced conversion of 7-dehydrocholesterol, a vitamin D precursor, as a result of sunlight exposure. Vitamin D deficiency due to a reduction in endogenous synthesis is a common link among three of the major risk factors for prostate cancer (Blutt and Weigel, 1999). First, older men generally have less sunlight exposure than younger men and their ability to synthesize vitamin D decreases with age. Second, African-American men have a reduced efficiency of vitamin D synthesis due to the high melanin content in their skin. Third, prostate cancer mortality rates show an inverse correlation with sunlight availability. This paper will explore several risk factors that could possibly lead to an increase in prostate cancer incidence and mortality. Such factors include old age, race, diet, environment, occupational hazards, socio-economic status, and vitamin D levels.

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Prostate cancer incidence and mortality rates increase with age. Clinical prostate cancer has been mainly a disease of older men, with the median age of diagnosis being 72 years (Lin and Lange, 2000). Unlike many other cancers, a steady rise occurs after the age of 50 but is rare under the age of 50 (Webber et al., 1988). Ninety-five percent of prostate cancer cases are diagnosed in men between the ages of 45 and 89 (Lin and Lange, 2000) but less than one case per 100,000 occurs in men under the age of 40 (Quader, 1999). Because of the increase in the life expectancy of the male population, prostate cancer has become an increasing concern.

An increase in life expectancy also causes a decrease in vitamin D metabolism, which begins around midlife and is due to several factors. There is a decrease in dietary calcium, cutaneous production of vitamin D, and serum concentration of 25-hydroxyvitamin D₃ (25OHD₃). Calcium absorption in the intestine diminishes causing a decrease in dietary calcium and VDR concentration, and calcium bioavailability to meet serum demands declines (Halloran and Portale, 1997). Several studies have shown vitamin D to inhibit growth, proliferation, and differentiation of prostate cells (Zhao et al., 2000; Blutt et al., 1997; and Skowronski et al., 1993). These data suggest a possible link between the decreasing vitamin D levels in elderly men and prostate cancer. As life expectancy increases so does the increased burden of this disease on society. This increase in risk is enhanced even more in cases of high risk, such as in African- American men.

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Race

The incidence of latent cancer, or cancer discovered at the time of autopsy, is similar throughout all countries and races. However, there are marked racial and ethnic differences in the mortality and incidence of prostate cancer in the United States (**Figure 2-2**). African-American men have the highest incidence rate of prostate cancer. An African-American man has an 85% greater chance of being diagnosed with, and 114% greater chance of dying from prostate cancer than a white man in the United States (Brawn et al., 1993). The chances of African-American men developing cancer are about 10% higher than in Whites, 50-60% higher than in Hispanics and Asian/Pacific Islanders, and more than 200% higher than in American Indians (Jemal et al., 2003). African-American men have approximately 30% higher mortality rates from cancer than whites and their death rate is more than double when compared to Asian/Pacific Islanders, American Indians, and Hispanics (Jemal et al., 2003) (**Figure 2-3**).

In attempts to decipher the differences in prostate cancer incidence among races, recent studies have tested the levels of prostate specific antigen (PSA) in black and white men without prostate cancer. These studies have found that black men tend to have higher levels and greater densities of PSA than do age-matched white men (Abdalla et al., 1998 and Eastham et al., 1998).

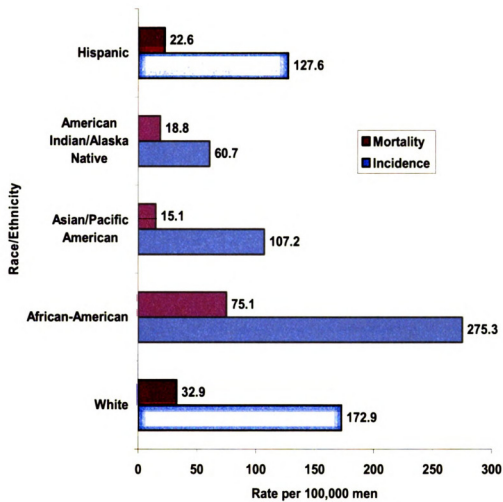


Figure 2-2: Prostate cancer incidence by race and ethnicity, US, 1992 to 1999 (data from Jemal et al., 2003).

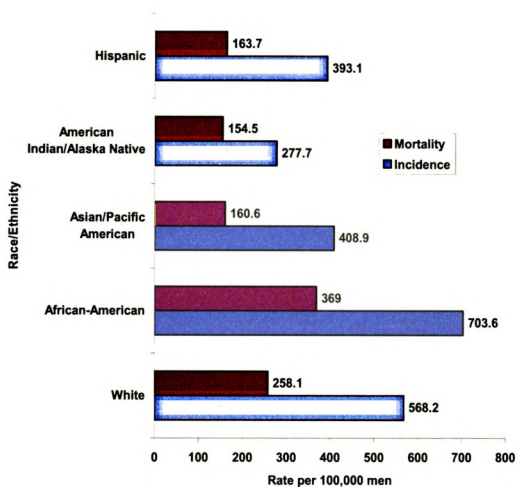


Figure 2-3: Average cancer mortality and incidence rate by race and ethnicity, US, 1992 to 1999 (data from Jemal et al., 2003).

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One factor that may relate these differences in prostate cancer incidence to vitamin D deficiency is the fact that high melanin levels in darkly pigmented skin inhibit the formation of previtamin D₃, a precursor to 1,25(OH)₂D₃. The melanin in the skin absorbs UV light and prevents the previtamin D₃ formation. Similarly, a recent study (Corder et al., 1995) demonstrated the possibility that men at elevated risk of prostate cancer have, on average, lower summer levels of serum 1,25(OH)₂D₃ compared to other men, for whom aggregate levels of 1,25(OH)₂D₃ remained constant throughout the year. This is significant when you consider the fact that black men, because of the melanin levels in their skin, tend to have lower levels of previtamin D₃. Another factor that could play a role is the genetic polymorphism that occurs in the vitamin D receptor (VDR) between different races. The effect of VDR polymorphism on prostate cancer risk may be the result of differential activity of the variant VDR alleles in transactivation of vitamin D target genes.

On the other hand, white men tend to have a higher level of prostate cancer incidence than Asian-American men in the United States. Even with the variation in incidence between specific Asian-American ethnicities, the incidence in each of the groups is significantly less than it is in white American men (American Cancer Society, 1998). One reason for these differences could be related to the genetic polymorphism in the VDR gene. It is also believed that differences in diet may contribute to these differences in prostate cancer incidence between different ethnic groups, which could also relate dietary habits of different ethnic groups to the vitamin D levels in corresponding groups.

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There has been considerable interest in the role of dietary components in the etiology and epidemiology of prostate cancer. Many epidemiological studies demonstrate the relationship between diet and prostate cancer. Prostate cancer is a hormone-associated malignancy and international data suggest that it is a cancer that may be causally associated with nutrition (Webber et al., 1988). The differences in incidence between different racial groups strongly suggest a link between diet and prostate cancer.

The incidence of prostate cancer is high in the United States compared to that in Asian countries (**Figure 2-4**). But, it is interesting to note that within a few generations, the incidence of this disease in Asian immigrants to the United States begins to approach the risk of American men (Hernandez et al., 1999). Kristal et al (2002) demonstrated that energy intake was associated with increased risk of prostate cancer, whereas high consumption of fat and calcium was associated with the development of more aggressive disease only. The possible mechanism explaining a deleterious effect of calcium on prostate cancer risk is that high calcium intake leads to lower levels of active $1,25(\text{OH})_2\text{D}_3$. In the Western diet, fat intake accounts for approximately 40% of the calories while in Japan it accounts for only 10 to 15% (Webber et al., 1988). The Western diet is high in animal fat and protein and refined carbohydrates and low in fiber-rich foods. Conversely, the low-fat diet of the Asian communities is rich in starches, legumes, fruit and vegetables (many of which have high fiber content), and soy products. One explanation of how dietary fats increase the risk of prostate cancer is hormonal. High-fat diets increase circulating androgen levels, and high testosterone levels may influence prostate cancer risk.

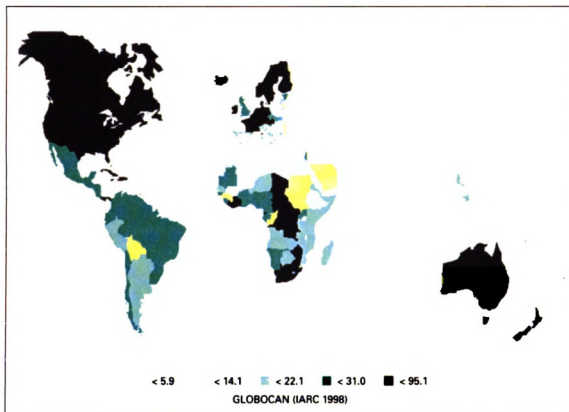


Figure 2-4: Number of prostate cancer cases world-wide, per 100,000 men, age standardized using the World standard population. (modified from Quinn and Babb, 2002; Webber et al., 1988).

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Soy is rich in the isoflavones genistein and daidzein, both of which have been shown to play a role in the inhibition of human cancer cell growth (Hernandez et al., 1999). The typical Asian diet also contains large amounts of fish oil which is high in vitamin D₃ content. This high level of vitamin D₃ in the Asian diet, compared to the Western diet, may play a role in the observed reduced prostate cancer incidence rates between Asian men and American men.

The traditional Asian diet also consists of large amounts of yellow and green vegetables that contain high levels of vitamin A, which may help protect against the induction of prostate cancer. Vitamin A has raised considerable interest as a cancer prevention agent because of its important role in cellular differentiation, inhibition of invasion, induction of apoptosis, and reversion of prostate cancer cells to the normal phenotype. Webber et al. (1999) examined the ability of *N*-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid, to reverse the malignant characteristics of DU-145 cells towards normal phenotype. They found that 4-HPR inhibited growth by 31% and 96% at concentrations of 1μM and 10μM, respectively. Treatment with 1μM 4-HPR also caused a 53% decrease in the expression of vimentin, an intermediate filament protein associated with invasion, as well as a 32% and 52% decrease in cell motility and invasive ability, respectively.

In 2001, Quader et al. examined the chemopreventative potential of *all-trans*-retinoic acid (ATRA) and 4-HPR on WPE1-NB14 and WPE1-NB11 cell lines. Both RA and 4-HPR inhibited anchorage independent growth and *in vitro* invasion of both the WPE1-NB14 and WPE1-NB11 cell lines. The effects of 4-HPR were tested on the RWPE-1, WPE1-7, WPE1-10, and DU-145 cell lines (Sharp et al., 2001). In this

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study, 4-HPR was shown to inhibit growth and decrease vimentin expression in all cell lines. 4-HPR induced the expression of cytokeratin 18, a differentiation marker for prostate epithelial cells, and it decreased the expression of the tumor suppressor proteins p53 and pRb.

Vitamin E is also believed to influence the development of prostate cancer. A form of vitamin E, α -tocopherol, was given to male smokers in Finland, ages 50-69. After receiving α -tocopherol for 5-8 years, the men showed a decrease in incidence and mortality rates of prostate cancer by 32% and 41%, respectively (Heinonen et al., 1998). Vitamin E is also believed to exert its effects by preventing free radical damage to DNA and proteins, by enhancing immune functions, and by reducing the activity of protein kinase C, which regulates cell proliferation (Heinonen et al., 1998).

Seasonal and Geographic Variations and Exposure to Sunlight

If exposure to sunlight is adequate, endogenous synthesis of vitamin D in the skin makes a greater contribution to vitamin D adequacy than any other factor. Ultraviolet (UV) light is required for the conversion of 7-dehydrocholesterol to vitamin D in the skin. Vitamin D levels show variations dependent on seasons of the year and geographic location. In a study done on pre-school children in Great Britain, Davies et al. (1999) demonstrated that vitamin D levels were much lower in winter months, when sunlight exposure tends to be much lower, than in summer months. This suggests that vitamin D status is dependent on season.

A similar experiment was conducted on men in Finland (Tuohimaa et al., 2001). Finnish men receive practically no effective UV light during the several winter months of

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the year, thereby making Finland an optimal country for an epidemiological study of serum vitamin D levels. Tuohimaa et al. (2001) found that there was a high seasonal variation in the serum 25OHD₃ concentration, with the highest values being found in September. Men with higher levels of serum 25OHD₃ had a 1) lower relative risk of developing prostate cancer, 2) less aggressive prostate cancer, and 3) delayed clinical verification of prostate cancer by 1.8 years. Taken together, these data suggest that vitamin D has beneficial effects at all stages of cancer development (**Figure 2-5**) and its levels are season-dependent.

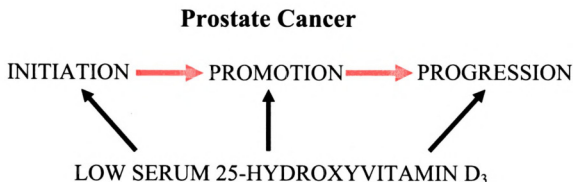
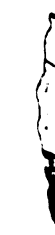
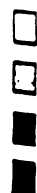


Figure 2-5: Summary of putative effects of serum 25-hydroxyvitamin D₃ levels on all three stages of prostate carcinogenesis (modified from Tuohimaa et al., 2001).

Using the data from the 3073 contiguous United States counties, Hanchette and Schwartz (1992) demonstrated that the incidence rates of prostate cancer follow a gradient with UV light intensity (**Figure 2-6**). This pattern shows that UV radiation and prostate cancer mortality are inversely correlated. Kafadar (1997) showed mortality rates in white men are also higher in northern than in southern counties. White men living in



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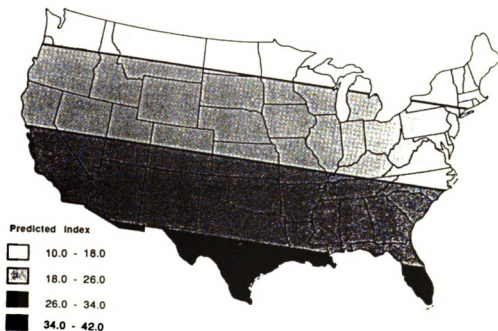
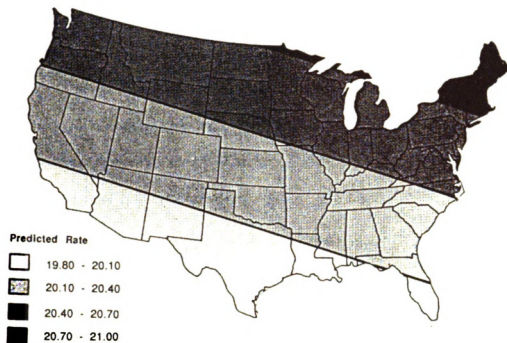


Figure 2-6: Linear trend surface maps. Top: Prostate cancer mortality among white men. Bottom: UV radiation using epidemiological index. An inverse correlation between prostate cancer mortality rates and UV radiation can be seen when comparing the two maps. (Hanchette and Schwartz, 1992).

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northern areas of the United States had mortality rates of approximately 26 in 100,000 population compared to 18-19 in 100,000 population in the southern most areas. Prostate cancer is also rare in native Africans but it is common in African-Americans, who have less exposure to sunlight than native Africans. Taken together, these data demonstrate a correlation between low vitamin D₃ levels (caused by low exposure to UV light) and higher incidence rates of prostate cancer.

Other Factors

There are several factors other than the ones described above that are related to prostate cancer development. Men with higher than normal plasma testosterone levels are believed to have a higher risk of developing prostate cancer since the prostate is dependent upon androgen to grow (American Cancer Society, 1998). When compared to white men, African-American men, who are at the highest risk of prostate cancer in the world, have 10-15% higher circulating testosterone levels (Coetzee and Ross, 1994). There may also be a genetic predisposition to the development of prostate cancer. Recent studies have suggested that familial predisposition may be responsible for 5-10% of prostate cancer cases (American Cancer Society, 2002).

Another factor that has an effect on prostate cancer risk is occupation. Occupational exposure may lead to differences in incidence of prostate cancer. For example, the overall risk in farmers for developing any type of cancer is lower than that in other occupational groups. But, farmers have a 20 to 50% higher chance of developing prostate cancer than other age-matched non-farmers (Williams, 1997). Compared to other occupations, the lifetime risk of a farmer for developing prostate cancer is

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approximately 7% higher and almost 1 out of 4 farmers will develop prostate cancer in their lifetime (Williams, 1997). It is believed that the increased risk can be attributed to the exposure to herbicides and insecticides. Cadmium is another occupational hazard associated with prostate cancer risk. Cadmium is a toxic metal known to be a carcinogen and most of its exposure comes from industrial settings, such as nickel-cadmium battery manufacturing or welding (Achanzar et al., 2001).

Conclusions

In recent years prostate cancer has become the most common cancer in men. It will account for 33% of all new cancer cases in men in 2003, and approximately 10% of all cancer deaths will be a result of this disease. Prostate cancer incidence in men under the age of 65 is 250/100,000 compared to 1,000/100,000 in men over the age of 65 (Kristal et al, 2002). While the exact cause of prostate cancer remains an enigma, several factors have been associated with the incidence of this deadly disease. The most prominent of these factors are old age, race, and diet. Other potential factors that demonstrate an association with prostate cancer are genetic predisposition, occupation, and socioeconomic status. With the recent discoveries showing an inverse relationship between prostate cancer cell growth, differentiation, and proliferation and vitamin D levels, the fact that vitamin D is somehow associated with most of these factors can not be overlooked. Thus, vitamin D may hold great promise as an important addition to the agents currently available for prostate cancer prevention and treatment.

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Chapter 3

The Physiological Role and Properties of Vitamin D

Abstract

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Abstract

A vitamin is an essential substance that our bodies cannot manufacture and can only be obtained from food. Because the body can manufacture vitamin D by exposing the skin to sunlight, by definition it is not really a vitamin. Vitamin D is actually a steroid hormone best known for its activity in regulating calcium and bone metabolism. It is one of the most important biological regulators of calcium metabolism and, along with the peptide hormones calcitonin and parathyroid hormone, it is responsible for the maintenance of calcium and phosphorus homeostasis. Vitamin D is also required for the normal development of bones and teeth.

Vitamin D exists in two forms: vitamin D₂ (ergocalciferol), found in yeast, and vitamin D₃ (cholecalciferol), which is found in animal skin. Precursors of vitamin D are synthesized in the skin and are subsequently converted in the liver and kidneys, to the biologically active form, the hormone 1,25-dihydroxyvitamin D₃. From the kidneys this hormone is transported, via the bloodstream, to its target organs.

A lack of vitamin D could potentially cause a variety of problems. These include inhibition of growth, rickets and osteomalacia, hypocalcemia, and increased production of the parathyroid hormone. Too much vitamin D could also cause problems, including hypervitaminosis D, calcification of soft tissues, loss of appetite and weight, and elevated blood calcium. With the obvious dependence of normal life on vitamin D, it is not surprising that major efforts are being made to understand the physiological role and molecular biology of this vital molecule.

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Introduction

Vitamin D is one of four fat-soluble vitamins that have been recognized as having important biological functions. Vitamin D is one of the most important and essential regulators of calcium and phosphorus metabolism in higher animals. Calcium and phosphorus homeostasis are both regulated by the combined action of vitamin D and two peptide hormones, calcitonin and parathyroid hormone (Chen, 1999).

Vitamin D exists in two molecular forms: vitamin D₂ and vitamin D₃, known as ergocalciferol and cholecalciferol, respectively (Figure 3-1). These closely related compounds both possess antirachitic activity (Collins and Norman, 1991). Ergocalciferol is derived from a common plant steroid, ergosterol, and is the form usually used to fortify foods with vitamin D. Cholecalciferol is formed when ultraviolet light from the sun converts the biological precursor 7-dehydrocholesterol into vitamin D₃. Since cholecalciferol can be produced in the body, vitamin D is not really a vitamin. A more accurate description of vitamin D is that of a steroid hormone.

Vitamin D deficiency may cause inadequate absorption of calcium from the intestinal tract and retention of phosphorus in the kidneys, leading to a deficient deposition of hydroxyapatite in the bones. This causes rickets in children and osteomalacia in adults. Vitamin D deficiency can also lead to inhibition of growth, hypocalcemia, and increased production of the parathyroid hormone. An excess of vitamin can lead to hypervitaminosis D, calcification of bones and soft tissues, kidney stones, weakness, nausea, polydipsia, and polyuria. This chapter will describe the many properties of vitamin D, including its physiological role, synthesis and metabolism, and its molecular biology.

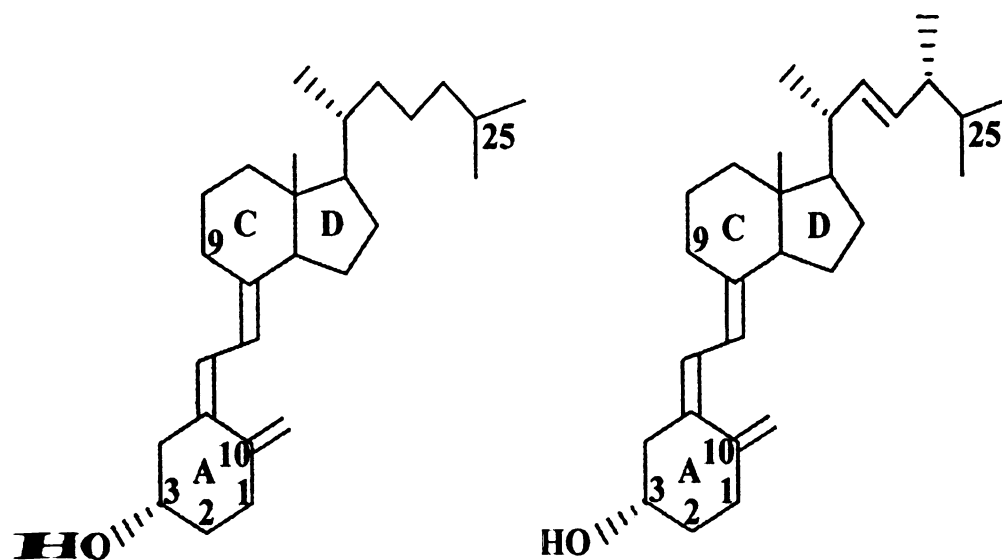


Figure 3-1: Molecular structures of nutritional forms of vitamin D, cholecalciferol (left) and ergocalciferol (right). Numbers designate carbon atom positions and letters designate the rings of the molecules.

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The Physiological Role of Vitamin D

Vitamin D is essential in maintaining a positive calcium balance and skeletal integrity. This hormone first gained recognition because of its antirachitic properties but is now recognized as an important regulator of calcium metabolism. Together with calcitonin and parathyroid hormone, vitamin D maintains calcium and phosphorus homeostasis in the body.

Vitamin D is a precursor of the calcium-regulating hormone, $1,25(\text{OH})_2\text{D}_3$, also known as calcitriol. This steroid hormone acts in the nuclei, cytoplasm, and plasma membrane of target cells and its production is subject to feedback regulation. Calcitriol is produced in the kidneys and is released into the circulation exerting its effects on mineral homeostasis by acting on the intestine, bone, kidney, and the parathyroid gland (Hendy, 1997) (Figure 3-2). In the intestine, the calcium and phosphorus ions enter into the physiological environment. In the bone the ions are stored and are made available for the minute-to-minute regulation of their respective serum levels, and their principal rate of excretion can be regulated in the kidney. Thus, the relationships between the absorption of calcium and phosphorus ions by the intestine, their increase and reabsorption by bone, and their urinary excretion by the kidneys all play a role in calcium and phosphorus homeostasis in the body.

In the small intestine, calcitriol stimulates the active transport of calcium and phosphorus from the lumen of the intestine to the blood. An independent active phosphate transport is also stimulated throughout the small and large bowels (Hendy, 1997). A dose of calcitriol causes a biphasic calcium transport response that has two phases, the first peaking at 6 hours and the second maintaining elevated calcium transport

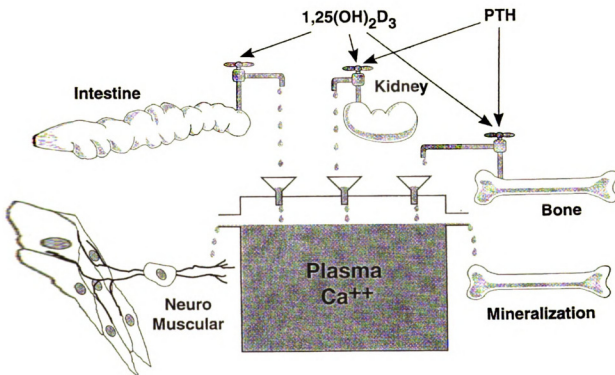


Figure 3-2: Diagrammatic representation of the physiological actions of vitamin D showing different organs and hormones involved in calcium homeostasis (McCrary and DeLuca, 1999).

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for several days (Hendy, 1997). The mechanism responsible for the movement of calcium ions is not clear, but it is known that calcitriol causes a bi-directional flux of calcium ions throughout the small intestine, a process composed of three components:

- 1) uptake of calcium ions from the intestinal lumen at the microvillus border;
- 2) translocation of ions across the cell to the basolateral membrane; and 3) active *extrusion* of ions into the circulation (Combs, 1998). Through these actions of calcium *and* phosphorus transport, complete skeletal mineralization occurs due to the indirect *anabolic* effects of calcitriol on bone (McCrary and DeLuca, 1999).

The role of vitamin D on bone is complex. Accumulating more than one-quarter *of a* single dose of vitamin D within a few hours of its administration, bone is the largest *target* organ for vitamin D (Combs, 1998). The metabolites of vitamin D are essential for *bone* formation because hypovitaminosis D can lead to rickets or osteomalacia, both of *whic*h can be corrected by exogenous administration of the vitamin (Civitelli and Avioli, 1995). However, an excess of calcitriol can induce bone resorption. Accordingly, bone *mineralization* and demineralization are both affected by vitamin D.

Because of their high levels of vitamin D receptors (VDR), osteoblasts, cells that *lay down* bone mineral, are probably the main targets of calcitriol in bone (Civitelli and Avioli, 1995). The synthesis of several noncollagenic proteins that are involved in bone *matrix* formation, can be stimulated by calcitriol. Vitamin D also stimulates osteoclast-*mediated* bone resorption, a process also mediated by calcitriol (Combs, 1998). The *hormone* appears to play a role in the differentiation of macrophages to osteoclasts, the *multinucleated* bone-degrading cells. When calcium levels are extremely low, both *calcium* and phosphorus are mobilized from bone by calcitriol and parathyroid hormone

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(PTH) (McCrary and DeLuca, 1999), a process that helps maintain the homeostasis of those minerals in the plasma.

The kidney is the major site of calcitriol synthesis and of several other vitamin D derivatives (**Figure 3-3**). In the kidneys, increased reabsorption of calcium, but not **p**hosphorus, from the urine into the serum is mediated by calcitriol and PTH (McCrary and DeLuca, 1999). PTH causes the kidneys to excrete phosphorus. Calcitriol indirectly **d**ecreases the levels of PTH in the kidneys thereby activating the reabsorption of **p**hosphorus from the urine to the serum (McCrary and DeLuca, 1999). There is no net **s**erum increase of phosphorus through the action of calcitriol and PTH when both are **p**resent in the kidneys, but there is a net increase in serum calcium levels. Perhaps the **m**ost important effect calcitriol has on the kidneys is its feedback inhibition of **1 α -h**ydroxylase activity and the associated stimulation of 24-hydroxylase, which is the **f**irst step in the inactivation of vitamin D metabolites (Civitelli and Avioli, 1995). This **m**echanism controls hormone synthesis and inactivates circulating metabolites, **m**aintaining adequate levels of vitamin D in the body.

Vitamin D deficiency and excess

Deficiency of Vitamin D

A number of factors could result in vitamin D deficiency: inadequate UV **i**rradiation of the skin, insufficient dietary intake of vitamin D, failure of the liver 25-**h**ydroxylase, or failure of the renal hydroxylase. Although sunlight is the main source of **v**itamin D, it is well know that many people do not receive enough solar radiation to

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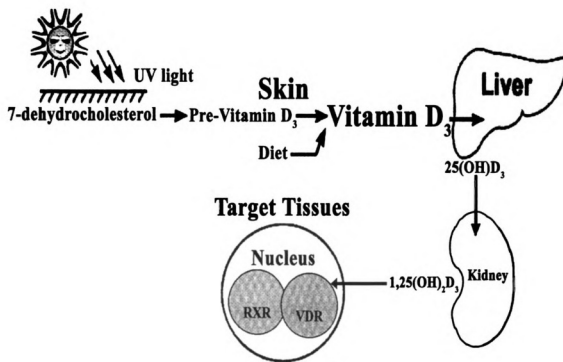


Figure 3-3: Diagram of vitamin D₃ metabolism showing metabolites involved and organs where they are synthesized (modified from Ohmdahl and May, 1997).

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support adequate vitamin D status. If a person's lifestyle keeps them indoors or if air pollution or clothing reduces the exposure to UV light, even people in sunny climates may not produce adequate vitamin D. As a result, most people show seasonal variations in 25OHD₃ levels, which, if not corrected by dietary means, could lead to periods of sub-optimal vitamin D status. Since most foods contain only a small amount of vitamin D, it is difficult to obtain enough vitamin D from the diet without the consumption of fortified foods.

Vitamin D deficiency leads primarily to skeletal diseases, such as rickets in children and osteomalacia in adults. The appearance of a typical bone disease, vitamin D-resistant rickets, also called vitamin D-dependent rickets, is a result of a genetic disorder related to point mutations in the gene for the VDR (Bouillon et al., 1995). When these diseases are due to inadequate vitamin D supplies, they are described as privational rickets and privational osteomalacia (Combs, 1998). Rickets first appears between 6 and 24 months old (Friedrich, 1988). Symptoms include impaired bone mineralization, bone pain, muscular tenderness, hypocalcemic tetany, delayed tooth eruption, nervousness, increase in plasma alkaline phosphatase, and a decrease in plasma calcium concentration.

Osteomalacia is characterized by loss of calcium and phosphate from the already formed bone. Symptoms are similar to rickets, i.e. muscle weakness and bone tenderness, particularly in the spine, shoulder, ribs, or pelvis, bone pain and possible broken bones. Osteomalacia is characterized by an increase in the ratio of non-mineralized bone to mineralized bone (Combs, 1998) and is common in women after they have given birth to several children (Friedrich, 1988). Hypoparathyroidism (lack or

reduced production of PTH) can be caused by removal of or disease of the parathyroid gland. People with hypoparathyroidism lack the signal (PTH) to respond to hypocalcemia and are unable to convert 25OHD_3 to $1,25(\text{OH})_2\text{D}_3$ resulting in decreased levels of serum calcium and elevated levels of serum phosphate, otherwise known as hyperphosphatemia. The deficiency of $1,25(\text{OH})_2\text{D}_3$ in individuals with hypoparathyroidism is the result of the absence of two important stimuli for the 1α -hydroxylase: PTH and hypophosphatemia (Friedrich, 1988). Osteoporosis, characterized by decreased bone mass with retention of normal histological appearance, has also been linked to vitamin D deficiency in women (Combs, 1998). Certain drugs, such as anticonvulsives used by epileptic patients, have been shown to cause functional vitamin D deficiency. These drugs catabolize 25OHD_3 and $1,25(\text{OH})_2\text{D}_3$ resulting in reduced circulating levels of 25OHD_3 .

Vitamin D Toxicity

Excessive amounts of vitamin D are not available from natural sources and vitamin D generated by the action of light on the skin has never led to vitamin D toxicity (Friedrich, 1988). Vitamin D toxicity is not a common cause of hypervitaminosis. However, vitamin D intoxication is a concern in patients taking vitamin supplements and/or those being treated with vitamin D for hypoparathyroidism, renal osteodystrophy, vitamin D-resistant rickets, psoriasis, osteoporosis, and some cancers.

Vitamin D taken in excess can cause pathological changes in the body and can result in irreversible calcification of soft tissues including the heart, lungs, and kidneys. If allowed to go unchecked, death could eventually occur. Therefore, it is very important

to detect for early signs of vitamin D intoxication. Symptoms of vitamin D toxicity include excess calcification of bone, kidney stones, headaches, weakness, nausea, vomiting, polyuria (passing of large volumes of urine in a given time), polydipsia (excessive or abnormal thirst), hypercalcemia (elevated blood calcium levels), hypercalciuria (elevated calcium levels in urine), anorexia, joint pain, and disorientation.

There are several forms of exogenous vitamin D toxicity. Hypervitaminosis with hypercalcemia may be secondary i) to excessive intake of vitamin D metabolites 25OHD₃ and 1,25(OH)₂D₃, or vitamin D analogs; ii) to increased production of 25OHD₃ or 1,25(OH)₂D₃ from exogenous substrates; iii) and even to topical applications of potent vitamin D analogs (Thys-Jacobs et al., 1997). Although rare, vitamin D toxicity can also be caused by endogenous factors, as seen in Williams syndrome, an idiopathic infantile form of hypercalcemia. Patients with granulomatous diseases are said to be hypersensitive to vitamin D and can develop hypercalcemia without the intake of exogenous hormone. Poorly regulated extrarenal synthesis of calcitriol by the granulomatous tissue is the main cause of this type of toxicity (Thys-Jacobs et al., 1997). Diseases associated with this form of vitamin D toxicity include sarcoidosis and tuberculosis. Endogenous etiologies may also result from ectopic production of calcitriol in lymphoma. This is associated with diseases such as Hodgkin's disease and non-Hodgkin's lymphoma.

The vitamin D parent compound, vitamin D₃ (cholecalciferol), 25OHD₃ (25-hydroxycholecalciferol), or 1,25(OH)₂D₃ (calcitriol) are three forms of vitamin D that could cause vitamin D toxicity in individuals. Because of their extensive lipid solubility in the liver, muscle, and fat tissues, vitamin D₂ and vitamin D₃ toxicity is more difficult

to manage than toxicity of the metabolites 25OHD₃ and 1,25(OH)₂D₃. As a result, the half-life of vitamin D ranges from several days to months, whereas the half-lives of 25OHD₃ and 1,25(OH)₂D₃ are 25 days and 10 hours, respectively (Thys-Jacobs et al., 1997). Since the duration of toxicity is related to the half-life of the compound, hypercalcemia can persist for weeks due to an overdose of the parent vitamin D compound for as long as 18 months due to its slow release from fat depots and an overdosage of 25OHD₃. In contrast, 1,25(OH)₂D₃ is not stored in appreciable amounts in the body, therefore, excessive toxicity of this metabolite is more rapidly reversed (Thys-Jacobs et al., 1997). Multiple factors, including the concentration of the vitamin D metabolites, vitamin D receptor (VDR) number, activity of 1 α -hydroxylase, the metabolic degradation pathway, and the capacity of the vitamin D binding protein (DBP), all influence the susceptibility of an individual to vitamin D toxicity.

Synthesis and Metabolism of Vitamin D

Vitamin D can be acquired by ingestion of either vitamin D₃ from foods that naturally contain it (i.e. fish liver oil, sardines, or eggs) or from milk and dairy products, which are often fortified with vitamin D₂ or D₃. But the main source of vitamin D₃ in the body comes from exposure of the skin to near-UV (wavelength 290-315 nm) light. Half of the pro-vitamin D₃ content in human skin is found in the dermis and the other half is found in the epidermis. However, during sunlight exposure the high-energy photons are absorbed in the epidermis and greater than 90% of the pre-vitamin D₃ synthesis occurs in the epidermis (Holick, 1988). Pre-vitamin D₃ is then converted to vitamin D₃, which is translocated to the dermal capillary bed via the vitamin D-binding protein.

The reaction begins upon exposure to sunlight causing endogenous synthesis in the skin (see Figure 3-3) where 7-dehydrocholesterol (pro-vitamin D₃) is converted to pre-vitamin D₃ through a photosynthetic reaction (**Figure 3-4**). This reaction causes a cleavage of the B ring of the steroid structure to form pre-vitamin D₃, which is a thermally unstable compound. This is followed by a heat-catalyzed process that takes approximately three days to complete. During this process, pre-vitamin D₃ undergoes rearrangement of its double bond to form a more stable isomer, vitamin D₃. This new structure is temperature-dependent and favored at body temperature. Because this conversion takes three days, pre-vitamin D₃ is sensitive to photodegradation by sunlight. Two other biologically inert products, lumisterol and tachysterol (**Figure 3-5**), are produced by the photolysis of pre-vitamin D₃. Vitamin D₃ can also be photolyzed to 5,6-transvitamin D₃, suprasterol 1, and suprasterol 2 if it is not able to escape into the circulation (Holick, 1988). Thus, by causing photodegradation of pre-vitamin D₃ and vitamin D₃, sunlight appears to be the major factor that regulates the output of vitamin D₃ in the skin.

Vitamin D₃ must bind with the vitamin D-binding protein (DBP) before it can be translocated into the circulation. The DBP then transports the vitamin D₃ to the liver where it is hydroxylated at the 25-carbon position, forming 25OHD₃. From the liver, 25OHD₃ is transported, via the DBP, to the kidneys where it gets hydroxylated at the 1-carbon position. This final form, 1,25(OH)₂D₃, is the most biologically active form of vitamin D. 1,25(OH)₂D₃ is then transported to the intestinal mucosal cells, bone, and skeletal muscle, or back to the kidney where it is either stored, used in the regulation of calcium absorption and mobilization, or catabolized into 24-hydroxylated metabolites.

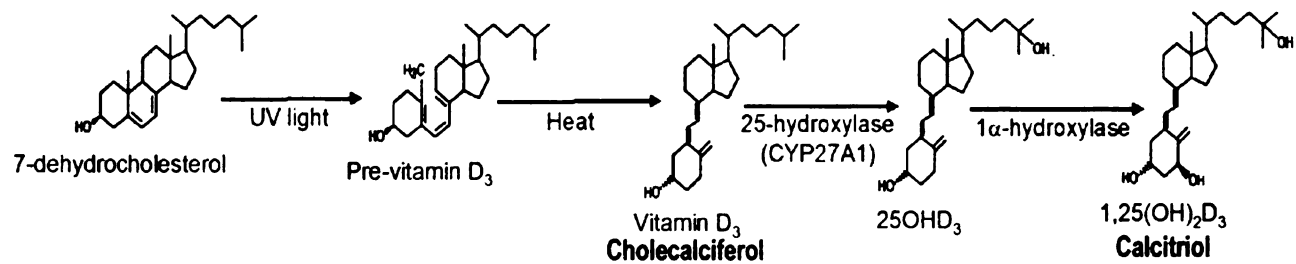


Figure 3-4: Metabolism of vitamin D₃ showing molecular structures of metabolites and the enzymes involved.

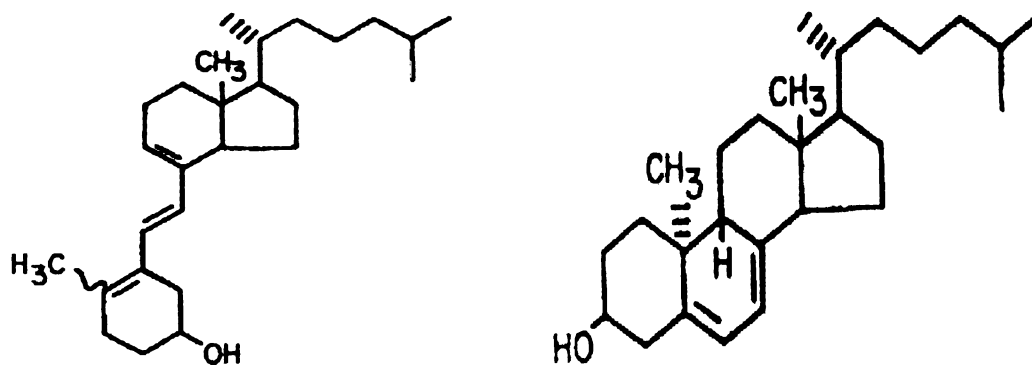


Figure 3-5: Structures of tachysterol (left) and lumisterol (right).

Conversion to 25OHD₃

Once vitamin D₃ enters the circulation, it is immediately taken up by adipose tissue (Jones et al., 1998) or transported to the liver where it is hydroxylated at the 25-carbon position. Early data suggested the liver to be the only location for 25-hydroxylation, although some reports indicate this activity also takes place in intestinal and renal extracts (Jones et al., 1998).

The 25-hydroxylation of vitamin D is the initial step in vitamin activation. The result is the formation of the metabolite 25OHD₃. The production of 25OHD₃ occurs in the liver and is catalyzed by the enzyme 25-hydroxylase. Similarly, vitamin D₂ is metabolized to 25OHD₂. The 25-hydroxylase, a cytochrome P-450 based enzyme, is found in liver microsomes and mitochondria. There is still some controversy as to whether 25-hydroxylation is carried out by one or two enzymes and whether these enzymes are found in the mitochondrial or microsomal fractions of the liver (Jones et al., 1998). The activity of the 25-hydroxylase enzyme appears to be poorly regulated by the hepatic concentration of vitamin D and there is little or no inhibition of 25OHD₃ formation.

The major circulating form of vitamin D₃, 25OHD₃, is the best indicator of vitamin D status. It is present in plasma at concentrations that range from 10-40 ng/ml (Hollis, 1997). The stability of the metabolite is dependent upon its high affinity for DBP (Jones et al., 1998). An urgent need for calcium or an abundance of calcium results in the 1 α -hydroxylation or the 24-hydroxylation of this metabolite, respectively. Therefore, the metabolic fate of 25OHD₃ is dependent on the calcium requirements of the animal. It was recently discovered that megalin, a member of the LDL receptor superfamily which

is present in the apical surface renal proximal tubular cells and neuroepithelium, is essential to deliver 25OHD₃ for the generation of 1,25(OH)₂D₃ (Christakos et al., 2003).

Conversion to 1,25(OH)₂D₃

The second step of activation, 1 α -hydroxylation, occurs mainly in the kidney (Jones, 1999). 25OHD₃ gets hydroxylated at the 1-carbon position of the A ring to yield 1,25(OH)₂D₃. The reaction is catalyzed by the 1 α -hydroxylase enzyme, which is located primarily in the renal cortical mitochondria (Combs, 1998) of the proximal and straight tubules in the kidney (Christakos et al., 2003). Several studies indicate extrarenal 1 α -hydroxylase activity in noncancerous prostatic cells and some prostate carcinoma cells (Schwartz et al., 1998), lymphocytes and alveolar macrophages (Ylikomi et al., 2002), skin (Horst and Reinhardt, 1997), bone, liver, pancreas, colon, parathyroids, adrenal medulla, and cerebellum (Hewison et al, 2000).

The 1 α -hydroxylase belongs to a class of enzymes known as the mixed-function oxidases, which use molecular oxygen as the oxygen source instead of water. It has been known for some time that this enzyme is composed of three proteins: 1) a cytochrome P-450, 2) ferredoxin, and 3) ferredoxin reductase for activity (Jones, 1999).

The synthesis of 1,25(OH)₂D₃ from 25OHD₃ by 1 α -hydroxylase is a strictly controlled process. Thus, activity of the 1 α -hydroxylase enzyme regulates the vitamin D endocrine system. This is done by several factors, including 1,25(OH)₂D₃, parathyroid hormone (PTH), calcitonin (CT), and circulating levels of calcium and phosphate (Combs, 1998). The 1 α -hydroxylase enzyme is feedback-inhibited by 1,25(OH)₂D₃ and is stimulated by prolactin, hypocalcemia, and hypophosphatemia (Combs, 1998). This

means that when circulating levels of $1,25(\text{OH})_2\text{D}_3$ are low, the production of $1,25(\text{OH})_2\text{D}_3$ in the kidney is high, and when circulating levels of $1,25(\text{OH})_2\text{D}_3$ are high, the synthesis of $1,25(\text{OH})_2\text{D}_3$ is low (Combs, 1998; Collins and Norman, 1991). Parathyroid hormone (PTH) treatment has been shown to stimulate 1α -hydroxylase (Brenza and DeLuca, 2000), which increases the production of $1,25(\text{OH})_2\text{D}_3$ in response to hypocalcemia (**Figure 3-6**). Taken together, these data indicate that the modulation of the hormone $1,25(\text{OH})_2\text{D}_3$ is based on the calcium needs of the organism. Synthesis of $1,25(\text{OH})_2\text{D}_3$ can also be regulated by prolactin, calcitonin, sex steroids, thyroid hormone, and glucocorticoids (Henry, 1997).

Conversion to $24, 25(\text{OH})_2\text{D}_3$

Also produced in the kidney, $24,25(\text{OH})_2\text{D}_3$ is a second dihydroxylated metabolite of vitamin D_3 . This metabolite is the second most abundant circulating metabolite of vitamin D (Friedrich, 1988). The enzyme that catalyzes the reaction of vitamin D to this metabolite, known as 24-hydroxylase, has been found in all tissues that contain VDRs. The 24-hydroxylation of 25OHD_3 and $1,25(\text{OH})_2\text{D}_3$ is the first step in a metabolic pathway to inactivate and degrade these vitamin D metabolites (Horst and Reinhardt, 1997). Similar to the 1α -hydroxylase enzyme, 24-hydroxylase is a P-450-dependent enzyme that requires NADPH. Unlike 1α -hydroxylase, 24-hydroxylase is increased by hypercalcemia and hyperphosphatemia and is strongly induced by $1,25(\text{OH})_2\text{D}_3$ (Combs, 1998). It is produced under conditions of vitamin D adequacy and normal calcium

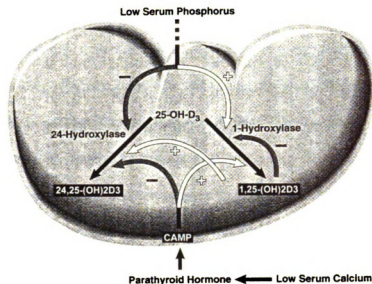


Figure 3-6: Regulation of 1α -hydroxylase and 24-hydroxylase in the kidney. The reciprocal regulation of the 1α - and 24-hydroxylase enzymes by serum calcium, serum phosphorus, PTH, and $1,25(\text{OH})_2\text{D}_3$ (modified from McCrary and DeLuca, 1997).

homeostasis and it also inhibits the stimulatory effect of PTH on bone resorption (Combs, 1998). It has recently been shown that PTH regulates 24-hydroxylase activity by reducing its half-life 4.2-fold, thereby decreasing the stability of the enzyme (Zierold et al., 2003).

Chemical and Physical Properties

Structure

Vitamin D is the inclusive term that refers to steroids that possess antirachitic activity. The structures of ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) are previously shown in **Figure 3-1**. The cleavage of the 9,10 bond (ring B) must occur in order for the formation of the pre-vitamin D forms from the pro-vitamins,

7-dehydrocholesterol and ergosterol. These vitamin structures with open rings are known as secosteroids. The only structural differences between ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) are a double bond and an additional methyl group in the side chain of vitamin D (Eitenmiller and Landen, 1999). The similarity in chemical and physical properties is due to their structural similarity.

Through x-ray crystallographic work it was discovered that the diene system of vitamin D that extends from C-5 to C-8 is transoid and nearly planar but the C-6 to C-19 diene system is cisoid and is not planar (Collins and Norman, 1991). The C-10 to C-19 double bond is twisted out of the plane by 60° resulting in the A ring undergoing rapid interconversion between the two possible chair conformations, a characteristic unique to the vitamin D molecule (Collins and Norman, 1991). It is also known that the C and D rings are rigid and that the side chain prefers an extended configuration (Collins and Norman, 1991).

Nomenclature

The nomenclature rules of the International Union of Pure and Applied Chemists (IUPAC) for steroid structure are used to characterize the ring system. Cholesterol serves as the parent compound and the A, B, C, and D rings are derived from the cyclopentanoperhydrophenanthrene steroid structure (**Figure 3-7**). Because of the fission of the B ring, vitamin D is designated as a *seco*-steroid. The formal name for vitamin D₃

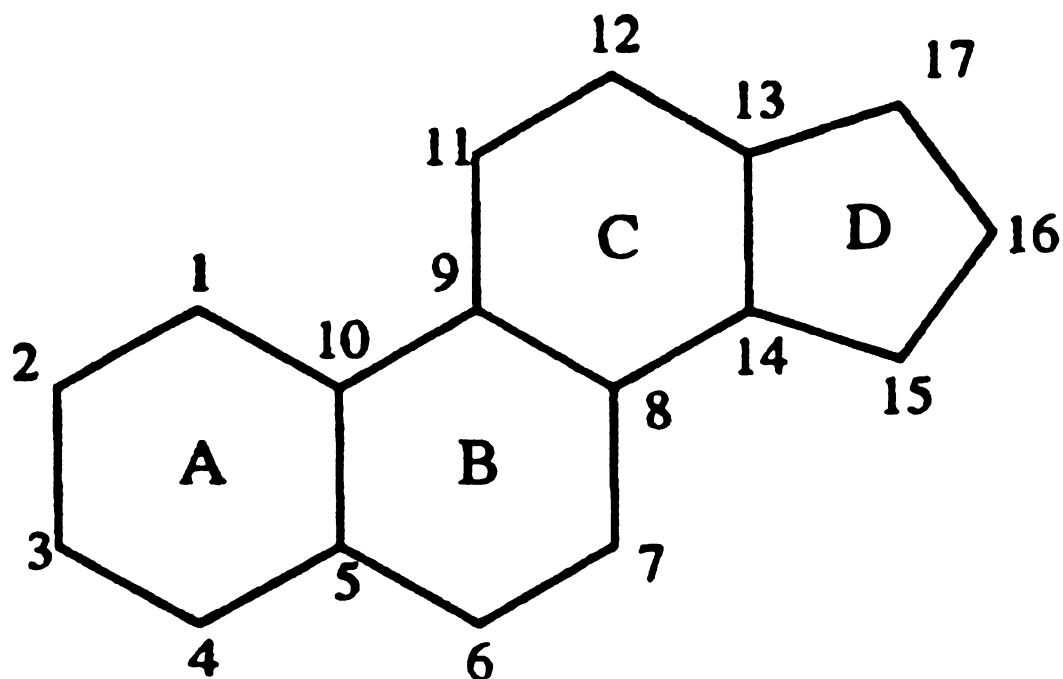


Figure 3-7: Steroid nucleus structure of cyclopentanoperhydrophenanthrene (Eitenmiller and Landen, 1999).

is 9,10-seco(5Z,7E)-5,7,10(19)-cholestatriene-3 β -ol and the formal name for vitamin D₂ is 9,10-seco(5Z,7E)-5,7,10(19),21-ergostatetraene-3 β -ol.

Spectral Properties

Vitamin D does not fluoresce. All forms of vitamin D show a characteristic broad UV spectrum with maximum absorption near 264 nm and a minimum near 228 nm (Collins and Norman, 1991; Friedrich, 1988).

Stability, Light Sensitivity, and Solubility

Vitamin D is stable in the absence of water, light, acidity, and at low temperatures. When exposed to light or under acidic conditions, vitamin D isomerizes to the 5,6-*trans*-isomer and isotachysterol (Eitenmiller and Landen, 1999). Vitamin D is stable in alkaline conditions. Saponification is often used as the initial treatment of sample for extraction of complex matrices. Vitamin D is less susceptible to oxidative losses than vitamin E, β -carotene, and retinol but oxidation can be a predominant route for decomposition at the conjugated double bonds at the 5, 6 and 7,8 positions. Vitamin D will undergo oxidation if exposed to air for 24-72 hours and it is best stored in evacuated ampules at 0°C.

Analytical methods account for all biologically active forms to accurately assess vitamin D activity since various environmental conditions can cause isomerization of vitamin D₂ and vitamin D₃ to the pre-vitamin D forms. Vitamin D in fortified foods is quite stable. Studies have shown that when vitamin D₃ in fortified foods was exposed to light, air, and extreme temperatures (up to 149°C), the vitamin showed only slight loss, was not affected, and did not produce statistically significant losses, respectively (Eitenmiller and Landen, 1999).

Vitamin D is soluble in a variety of solvents including acetone, ethyl alcohol, benzene, chloroform, and ether and petroleum ether. It is moderately soluble in fats and oils. Vitamin D is insoluble in water.

General Properties of Vitamin D

Vitamin D₂ and D₃ are white (Collins and Norman, 1991) to yellowish (Friedrich, 1988) powders. Vitamin D₂ has a melting point of 121°C and contains four double bonds while vitamin D₃ contains three double bonds and has a melting point of 84-85°C. Vitamin D₃ has a molecular weight of 384.65 and is in the form of fine needles.

Conclusion

Through its biologically active metabolite 1,25(OH)₂D₃, vitamin D is known to act on bone and intestine to maintain calcium homeostasis. It is one of the most important biological regulators of calcium metabolism and, along with the peptide hormones calcitonin and parathyroid hormone, it is responsible for the minute-to-minute maintenance of calcium and phosphorus homeostasis in the body. Vitamin D exists in two forms, vitamin D₂ and vitamin D₃, both of which possess antirachitic activity. Precursors to vitamin D are synthesized in the skin and, subsequently, in the liver and kidneys, to the hormone 1,25-dihydroxyvitamin D₃. From the kidneys this hormone is transported, via the bloodstream, to its target organs. Several diseases can arise from both a vitamin D deficiency and vitamin D toxicity. Numerous studies have been conducted in attempts to fully characterize the vitamin D steroid hormone. Vitamin D is a remarkable molecule but it is taken for granted by most individuals. Hopefully with all the recent interest in vitamin D people will realize how important it is and begin to appreciate this gift from the sun.

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Chapter 4

Intermediate Filaments

Abstract

The cytoskeleton is a dynamic structure that fills the cytoplasm of all eukaryotic cells. It is composed of three main classes, known as the microfilaments, microtubules, and the intermediate filaments. Intermediate filament expression is generally cell-type specific which makes these filaments very useful tools in cell type determination. Two intermediate filaments that are important cell type markers in the prostate are the cytokeratins and vimentin. Vimentin is normally a mesenchymal cell intermediate filament but its expression is sometimes observed in normal, hyperplastic, and cancerous prostate tissue. Vimentin has been shown to be associated with increased cell proliferation, a decrease in cell differentiation, as well as increased metastatic ability and invasiveness of cancer cells. Cytokeratins are expressed in prostatic epithelial cells. Cytokeratins 5, 14 and 15 are expressed in the basal cells while cytokeratins 8, 18, and 19 are expressed in the secretory cells of the normal prostate. The expression of these cytokeratins is a good prognostic indicator of prostate cancer. Intermediate filaments are also involved in cellular apoptosis. Intermediate filaments are useful tools in cell type determination as well as the determining the metastatic ability and level of invasiveness of cancer cells.

Introduction

The cytoskeleton fills the cytoplasm of eukaryotic cells. It is a dynamic three-dimensional structure that acts as both “muscle and skeleton,” for movement and stability. This structure is comprised of three main classes of fibers: 1) microfilaments, 2) microtubules, and 3) intermediate filaments. Each class of fiber is distinctly different with regard to its subcellular organization, characteristic morphology, relationship to different cell functions, relative stability, a discrete polypeptide composition, and specific physiochemical properties. Intermediate filaments have a diameter of approximately 10 nm and, as indicated by their name, are thicker than microfilaments (7 nm) but thinner than microtubules (25 nm) (Bershadsky and Vasiliev, 1988). The major structural components of the microfilaments and microtubules are highly conserved in different cell types, whereas the proteins of the intermediate filaments can vary greatly in amino acid sequence and length. This chapter will concentrate on only one of these classes, the intermediate filaments.

Intermediate Filaments

Intermediate filaments are an important part of the cytoskeletal system of a cell. They stabilize organelles, like the nucleus, and they form a network that gives shape and mechanical integrity to the cell (Prasad et al., 1998). They are also involved in cell locomotion and receptor mediated endocytosis. The intermediate filament network spreads from the cell periphery to the nucleus and forms dynamic linkages between nuclear matrix and the extracellular matrix.

The intermediate filaments are comprised of a large family of greater than 50 gene products (Goldman et al., 1999). The polymerization of these protein subunits to form the intermediate filament network is complex, with some being able to form a single type of protein chain (homopolymer) and others forming filaments only when they are combined with more than one type of protein chain (heteropolymers) (Goldman et al., 1999). If denatured, intermediate filaments can also rapidly and spontaneously refold and coil back into their original shape, indistinguishable from those formed in living cells (Franke, 1993).

The largest family of the cytoskeletal proteins, the intermediate filament proteins, shares a common structure (**Figure 4-1**). The most conserved region of this structure is the long, α -helical rod domain that consists of approximately 310 amino acids (Goldman et al., 1999). This domain, considered to be the building block of the intermediate filament, forms coiled-coils in assembled intermediate filaments and is subdivided into four blocks of helical structure (1A, 1B, 2A, and 2B) by short linker domains (L1, L12, L2) (Paramio and Jorcano, 2002). This rod domain is flanked by non-helical domains at the N terminus (head) and C terminus (tail). These domains are not well conserved and have been postulated to protrude from the core of the filaments and are assumed to specify the possible polypeptide-specific functions (Paramio and Jorcano, 2002).

Based on the pattern of cell type specific expression (Goldman et al, 1999), immunological and/or assembly properties (Paramio and Jorcano, 2002), gene structure, and sequence homology, intermediate filaments can be subdivided into 6 categories. Types I and II, the cytokeratins, contain the largest number of intermediate filament proteins and are expressed mainly in epithelial cells. Type III contains vimentin, which is

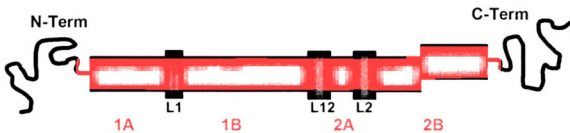


Figure 4-1: Common structure of the intermediate filament with a central domain with four α -helical domains (1A, 1B, 2A, 2B) disrupted by linker non-helical domains (L1, L12, L2), and two non-helical divergent domains at the amino and carboxy terminus (modified from Paramio and Jorcano, 2002).

expressed in mesenchymal cells, undifferentiated cells and cells in culture, (Capetanaki, 1984), desmin is characteristic of muscle cells, glial fibrillary acidic protein (GFAP) is found in glial cells, and peripherin is expressed in cells of the peripheral nervous system. Type IV intermediate filament proteins, which are found in the neurons, are the neurofilaments proteins and α -internexin. Although the majority of the intermediate filament proteins are expressed in the cytoplasm, the Type V proteins, the lamins, are localized to the nucleus where they polymerize to form the nuclear lamina (Goldman et al., 1999). Type VI is the least characterized intermediate filament protein, nestin. The cytokeratins and vimentin will be the only two intermediate filaments discussed in this chapter.

Cytokeratins

Cytokeratins (**Figure 4-2**) are the first intermediate filament proteins to appear during embryonic development. They are usually arranged into bundles called tonofibrils, which form a cage-like structure around the nucleus and extend to the cell periphery. Cytokeratins are the hallmark of intermediate filament proteins of epithelial and mesothelial cells. Identification of cytokeratins in cultured cells is a standard method to verify the epithelial nature of the *in vitro* population.

The cytokeratins are a multigene family of about 20 biochemically and immunologically distinct yet closely related polypeptides, which range in molecular weight from 40 to 78 kDa. The cytokeratin filaments are assembled from heterodimeric subunits of acidic type I cytokeratins (cytokeratins 9 to 20) and basic type II (cytokeratins

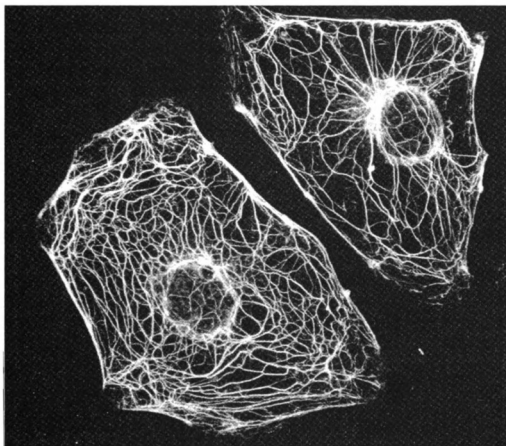


Figure 4-2: Culture PtK cells stained with cytokeratin antibody (Schwila, 1986)

1 to 8) intermediate filament proteins (Paramio and Jorcano, 2002). Different types of epithelial cells can be characterized based on these acidic and basic cytokeratin combinations. Simple epithelia express cytokeratins 8 and 18 with variable levels of cytokeratin 19 and 20 (Yoon et al., 2001) and in some instances 17 and 7 (Paramio and Jorcano, 2002). Stratified epithelia express cytokeratins 5 and 14 in the basal layer, and when these cells begin terminal differentiation they move upwards and begin expressing cytokeratins 1 and 10 in the skin, cytokeratins 4 and 13 in internal stratified epithelia and cytokeratins 3 and 12 in corneal cells (Paramio and Jorcano, 2002). These patterns reveal clues to the lineage of a cell and its differentiation status and also imply that cytokeratins might carry out specific functions in epithelial cells.

The prostatic luminal, secretory cells express cytokeratins 8, 18 and 19, and cytokeratins 5, 14, 15 and 16 are expressed in the prostatic basal cells (Traub, 1985; Peehl et al., 1994; Sherwood et al., 1990). The cytokeratin profiles in the prostate gland are an extremely useful diagnostic tool in the determination of prostate cell differentiation and the level of prostate cancer. Alberti *et al.* (2000) found that several constitutive cytokeratins (8, 18, and 19) showed a strong down regulation in poorly differentiated prostate cancer cells, and their expression significantly decreased with respect to both normal human prostate cells and benign prostatic hyperplasia and also with respect to moderately and well differentiated tumors. A decrease in the expression of cytokeratins 8 and 18 in prostate carcinoma, when compared to normal prostate, was also observed in animal models (Ramaekers et al., 1994). Sherwood et al. (1990) showed that established prostate cancer cell lines expressed cytokeratins 8 and 18 but not cytokeratins 5, 7, and 15 which were observed in the benign prostate. These data support the belief that

prostate cancer arises from the luminal cells of the prostate, which express cytokeratins 8, 18, and 19, and not from the basal cells, which express cytokeratins 5, 14, 15 and 16.

Coexpression of two different intermediate filaments is known to occur. It has been shown in prostate and breast cancer that the down-regulation of cytokeratin is associated with the expression of the type III intermediate filament vimentin. Vimentin will be discussed in the next section.

Vimentin

Vimentin (**Figure 4-3**) is a 54 kDa intermediate filament protein that is normally expressed in cells of mesenchymal origin, such as fibroblasts and endothelial cells (Paramio and Jorcano, 2002) and cells derived from mesoderm. Vimentin is typical of osteocytes, chondrocytes, melanocytes, Langerhan cells of the skin, and most but not all lymphatic cells (Osborn, 1993). It is expressed in normal adult epithelial cells, neuronal or muscle cells (Osborn, 1993). Its expression precedes the expression of other type III proteins during the differentiation and development of neural cells (later replaced by GFAP) and muscle (later replaced by desmin) (Paramio and Jorcano, 2002). Unlike cytokeratins, vimentin is coded for by a single gene. Vimentin is the first intermediate filament expressed during cell differentiation. It can be expressed in epithelial cells during early development, wound healing, during cell culture, in ovarian and mesothelial epithelium, and in carcinomas (Fraga et al., 1998). Several early studies suggested that vimentin expression in epithelial cells was induced by growing the cells *in vitro*, but

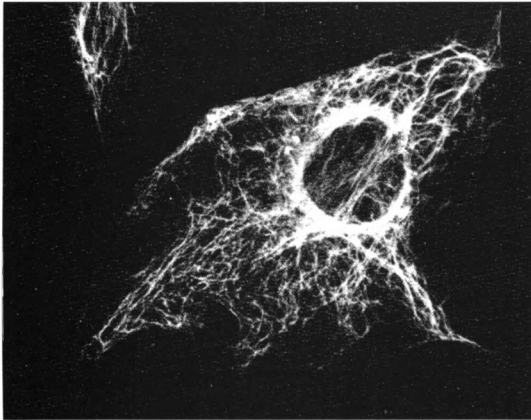


Figure 4-3: Culture PtK cells stained with vimentin antibody (Schwila, 1986)

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data are now accumulating to suggest instead that vimentin expression in epithelial cells *in vitro* correlates with a high degree of transformation and might be related to tumor progression (Gilles et al., 1996). Increases in vimentin expression are associated with a decrease in cellular differentiation.

There have been numerous studies to show the effects of vimentin expression on the motility and invasiveness of cancer cells. Lang *et al.* (2002) demonstrated that vimentin expression increased with increasing motility of prostate cancer cells and that vimentin-positive cells were more likely to be associated with poorly differentiated tumors. Fraga and colleagues (1998) showed that vimentin expression increased significantly in epithelial cells of benign prostatic hyperplasia (BPH) when compared to the cells in the adjacent normal epithelium. Since several epithelia, which do not express vimentin in their slowly proliferating epithelium, do express this filament when grown as rapidly dividing cells in culture, it was suggested that the difference in proliferative activity between BPH and normal tissues causes the difference in vimentin expression, with vimentin activity being proportional to proliferative activity (Fraga et al., 1998).

A relation between vimentin expression and the invasiveness of other types of cancer has also been shown. Vimentin appears to be a good prognostic indicator in breast cancer; it is associated with increased cell proliferation rates and increased invasive ability in breast cancer *in vitro* and *in vivo*. Vimentin expression has also been shown to be associated with hormone-independent breast cancer cells lines, all of which were more invasive *in vitro* than cell lines that did not express vimentin (Sommers et al., 1994). Zajchowski et al (2001) also demonstrated that breast cancer cells with the greatest motility and invasive activity over-expressed the mesenchymal marker vimentin. An

association between invasive and migratory potential and vimentin expression has also been shown in cervical cancer *in vivo* (Gilles et al., 1996). In this study, vimentin expression was detected in all invasive carcinomas and lymph node metastases but not in the CIN III (pre-invasive) lesions, which is consistent with its absence in non-invasive cell lines *in vitro*.

Generally, fully differentiated cells express only one class of intermediate filament subunits typical for that cell type. However, vimentin is unique from other intermediate filaments in that it shows coexpression with other types of intermediate filaments. Vimentin has been found to be coexpressed with desmin in muscle cells, GFAP in certain adult glial cells, neurofilament polypeptides during neuronal differentiation, and cytokeratins during epithelial differentiation (Capetanaki et al., 1984).

Coexpression of Vimentin and Cytokeratins

Normal differentiated epithelial cells express cytokeratins and not vimentin. However, coexpression of vimentin and cytokeratins has been reported in several types of tumors, including breast, renal, thyroid, ovarian, and prostate carcinomas (Leong et al., 1988). This observation has led to studies examining the possible reasons behind this coexpression phenomenon and to answer the question of whether or not this coexpression is advantageous to tumor cells.

Hendrix et al (1992) tested the coexpression of vimentin and cytokeratins 8 and 18 in melanoma cells. They showed that poorly metastatic cells (A375P), moderately metastatic cells (A375M), and highly metastatic cells (C8161) showed no staining, low levels of staining, and high levels of staining, respectively, for cytokeratins 8 and 18.

These data show an obvious correlation between the coexpression of vimentin with cytokeratins 8 and 18 and the invasive ability and metastatic behavior of these melanoma cells. Since intermediate filaments can act as signal transducers that can regulate gene expression, the coexpression of vimentin and cytokeratins may be advantageous to tumor cells by facilitating the transcription of genes that would promote tumor development. Hendrix et al. (1992) also suggest that the phenomenon of “switching” phenotypic expression and coexpression of two phenotypic markers in cells confers a selective advantage toward transformation.

Recent studies have suggested differences in the motility and dynamic activities of vimentin and cytokeratins during coexpression in epithelial cells. By tagging vimentin and cytokeratins with green fluorescent protein, Yoon et al. (2001) showed that these intermediate filaments possess surprisingly different properties within the same cytoplasmic regions. Vimentin fibrils demonstrate changes in shape, extending and shortening over short periods of time, and bidirectional translocation, whereas cytokeratin tonofibrils exhibit slow oscillations or undulations and unidirectional fluctuations of diffuse and particulate cytokeratin from the surface of the cell towards the nucleus. These data suggest the dynamic properties of different types of intermediate filaments within the same cytoplasmic region are regulated by different factors. The further study of these factors may shed more light on the specific roles of these intermediate filaments in cancer progression, differentiation, and levels of metastatic ability of different cancers.

The Role of Cytokeratins and Vimentin in Apoptosis

Apoptosis, or programmed cell death, allows for the loss of cells that either are not needed during development or may be dangerous to the organism because of mutations or other damage. Several changes must occur in the cytoskeleton of a cell to allow the structural and mechanical integrity of the cell to be destroyed during apoptosis. During early apoptosis there are many changes that occur to the cytoskeletal organization of the cell, including the disassembling and proteolytic cleavage of intermediate filaments vimentin and the cytokeratins.

Vimentin filaments regulate cell attachment and subcellular organization. During early apoptosis, vimentin function is altered when the cells become detached from the surrounding tissue. The filaments in the detached cells undergo proteolytic cleavage into several different sized fragments, which is part of the execution phase of apoptosis. (Prasad et al., 1998). During apoptosis, cytokeratin 8 and 18 filaments undergo reorganization and the phosphorylation and fragmentation of cytokeratin are independently responsible for intermediate filament reorganization (Prasad et al., 1998).

Activation of caspases is the final common event that occurs during apoptosis. Caspases cleave a variety of substrates, including structural proteins, proteins involved in signal transduction, and components of the cell cycle machinery. Cytokeratins exhibit caspase-sensitive motifs and fragments derived from them have been found in apoptotic cells (Prasad et al., 1998). Caspases are thought to be the primary method of protein degradation utilized in apoptosis. These data suggest that there may be important roles for cytokeratins and vimentin in the process of apoptosis.

Conclusions

Intermediate filament proteins are an important part of the cytoskeletal system of a cell. They stabilize the organelles, they form the network that gives shape to the cell and provides mechanical integrity, and they are involved in cell locomotion and receptor mediated endocytosis. The network extends from the nucleus, through the cytoplasm, and ends at the plasma membrane. There are six categories of intermediate filament proteins: Types I and II are the cytokeratins (epithelial cells), type III includes vimentin (mesenchymal cells), desmin (muscle cells), and GFAP (glial cells), type IV are the neurofilaments and α -internexin (neurons), type V proteins, lamins, are localized to the nucleus, and type VI proteins are nestin.

Cytokeratins 8 and 18 are expressed in the prostatic luminal, secretory cells of the prostate and cytokeratins 5 and 14 are expressed in the less differentiated basal cells of the prostate. This makes cytokeratins very useful prognostic indicators of prostate cancer and cell differentiation. Several studies have shown that prostatic expression of cytokeratins 8 and 18 decreases as the cells become less differentiated and more cancerous. The same decrease in expression of cytokeratin 8 and 18 has been shown *in vivo* in prostate carcinoma when compared to normal prostate. These data support the observation that prostate cancer arises from the luminal epithelial cells on the path to luminal cell differentiation, where cytokeratins 8 and 18.

Abnormal expression of vimentin may be observed in the normal, hyperplastic, and cancerous prostate. Vimentin plays significant role in the determination of the invasive levels of cancer cells and in cell differentiation and proliferation. It is well documented that the increase in vimentin expression is associated with an increase in the

invasive and metastatic ability of prostate cancer cells. Vimentin is a very useful prognostic indicator of the progression level of prostate cancer.

Coexpression of cytokeratins and vimentin is associated with a less differentiated phenotype and it appears to give tumor cells, showing such coexpression, an advantage over normal cells. The inhibition of coexpression results in a decrease in invasive potential of melanoma cells. Studies have shown that cytokeratins and vimentin possess different properties within the same cytoplasmic regions suggesting that the dynamic properties of these filaments are regulated by different factors. More work needs to be done to elucidate the mechanism(s) of action of these different factors have on intermediate filaments. Intermediate filaments are good targets for the study of apoptosis due to the important role the filaments play in cell integrity and stability.

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Chapter 5

Matrix Metalloproteinases (MMPs)

Abstract

Cancer invasion and metastasis are the result of tissue remodeling that involves a proteolytic degradation of the extracellular matrix. Metastatic spread continues to be the greatest barrier to cancer cure. Matrix metalloproteinases (MMPs) are proteases implicated in the degradation of the extracellular matrix during invasion by cancer cells. Clarifying the mechanisms of action of MMPs and MMP inhibitors, and discovering new treatments and/or methods to control their activity, could potentially lead to the development of important therapeutic strategies against cancer invasion and metastasis. This chapter describes the classification and regulation of MMPs and their association with different cancers. Also discussed in this chapter are the effects of vitamin D on members of the MMP family.

Introduction

The extracellular matrix (ECM) is a complex structural entity that surrounds and supports cells in mammalian tissues. It contains signaling molecules responsible for maintaining tissue form and function. The ECM is composed of three main classes of biomolecules: 1) structural proteins (collagen, and elastin); 2) specialized proteins (fibronectin, laminin, and fibrillin), and 3) proteoglycans. The primary function of the ECM is support but it also plays a role in directing development and morphogenesis by influencing cellular processes such as proliferation, differentiation, migration, and adhesion. It is a dynamic matrix of structural proteins, growth factors, and latent enzymes that is constantly being remodeled.

In the 1960s and 1970s, investigators noticed a correlation between the invasive behavior of tumor cells and the expression levels of different proteases (Liotta et al., 1979; Poole et al., 1978). It is now known that a wide variety of proteases can cleave ECM molecules. These proteases can be grouped into four different classes, the serine-, cysteine-, aspartyl-, and metallo-proteinases (Stetler-Stevenson and Yu, 2001). Members from all four groups have been associated with cancer cell invasion and metastasis, and it is likely that they have overlapping functions in tumor-mediated ECM degradation (Fingleton and Matrisian, 2000).

The three step theory of cell invasion proposed by Liotta and colleagues (1982) involves the following basic steps: 1) formation of appropriate cell:matrix interactions via cell-surface receptors (the integrins) and specific glycoproteins (i.e. laminin, type IV collagen, and fibronectin) and disruption of contacts between cell:cell receptors (the cadherins); 2) proteolytic destruction of matrix barriers to clear a path for the cell; and

3) directed movement of cells into the area of ECM remodeling, which probably depends on specific chemotactic factors. Cells are then able to escape from primary tumors, intravasate into blood vessels and lymphatics, and get transported to and arrest in distant organs. After which time the cells extravasate and grow to form secondary tumors in the new organ. Early studies on invasion focused primarily on interaction of tumor cells with the ECM, in particular the basement membrane. The basement membrane (BM) is a specialized matrix consisting of type IV collagen, laminin, entactin, proteoglycans, and glycosaminoglycans (Nelson et al., 2000). Loss of BM continuity and lack of BM around the invasive tumor cells were found to be characteristics of invasive carcinomas (Liotta et al, 1991; Stetler-Stevenson et al., 1993).

Proteolytic degradation of the ECM is a crucial step in the process of tumor invasion; it plays an important role in the metastatic cascade. As mentioned above, several proteases are involved in this process, but matrix metalloproteinases and members of the serine protease family are considered to be the most important. Serine proteases, such as prostate specific antigen (PSA) and urokinase-type plasminogen activator (uPA), are involved in ECM degradation and prostate cancer cell invasion (Webber et al., 1995; Waghray and Webber, 1995).

There are several reasons why MMPs appear to be the most important and physiologically relevant of all the proteases in terms of matrix turnover. First, they are secreted proteins, placing them in the ideal location for ECM degradation. Second, they are most potent at pH levels close to neutral. And, third, they are regulated at several levels, which suggests that a tight control over normal processes is required.

Matrix metalloproteinases (MMPs) have been repeatedly implicated in cancer cell invasion and the metastatic process. They appear to be involved in the initial step of ECM degradation as well as in facilitating the breakdown of other physical barriers to metastasis (Stetler-Stevenson and Yu, 2001). Initially, this was believed to be their most important role in the metastatic process. But recent data suggest that MMPs may play a more complex role than was first thought, and they may also make important contributions at other steps in the metastatic process. It is now believed that MMPs are important in contributing to the process of angiogenesis and in creating and maintaining an environment that supports the initiation and maintenance of growth of both primary and metastatic tumors (Chambers and Matrisian, 1997; Stetler-Stevenson and Yu, 2001).

The Matrix Metalloproteinase Family

Bacterial collagenases were discovered long before Gross and Lapiere (1962) described the first mammalian collagenase. They discovered this enzyme while studying the dissolution of the tadpole tail which occurs during the morphogenesis into a frog. This collagenase, now called interstitial collagenase or MMP-1, was the first evidence for the existence of an animal collagenase (Gross and Lapiere, 1962). After this discovery, the study of animal collagenases, and other enzymes involved in ECM degradation, grew at an almost exponential rate.

Matrix metalloproteinases (MMPs) are the predominant group of enzymes that degrade the ECM and cell surface molecules. MMPs play a critical role in a variety of physiological and pathological process, including embryonic development, tissue morphogenesis, wound healing, arthritis, and cancer (Sternlicht et al., 2000). They were

initially classified as proteinases capable of degrading the ECM but their targets have since expanded to include several other extracellular proteins. These proteins include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, cell surface receptors, and cell:cell and cell:matrix adhesion molecules (Coussens et al., 2002).

MMPs are a family of secreted or cell-surface bound zinc endopeptidases. There are now more than 20 enzymes classified as MMPs, and they are recognized by a descriptive name (e.g. gelatinase A, which degrades non-fibrillar collagens) and an MMP number. All MMPs share a catalytic domain that is responsible for binding zinc, which is essential for catalytic function. They also share a “pro” domain that is responsible for maintaining the latency and a “pre” domain to target for secretion. The MMP family members differ from each other structurally by the presence, or absence, of additional domains responsible for activities such as inhibitor binding, matrix binding, cell-surface localization, and substrate specificity (Chambers and Matrisian, 1997). The MMPs are subdivided into four main groups based on their substrate specificity: gelatinases, collagenases, stromelysins, and MT-MMPs. Further classification of the MMPs can be made according to domain structures (**Figure 5-1**).

Gelatinases

There are two members in this group, gelatinase A (MMP-2) and gelatinase B (MMP-9). Both of these enzymes readily degrade heat-denatured collagens (gelatins) and share an almost identical substrate preference. Substrates for these MMPs include gelatin, types I, IV, and V collagen, and elastin. MMP-2 is a 72 kDa protein that is

MMP	Domain Structure	Major Substrates
Matrilysin (MMP-7)		Proteoglycans, glycoproteins, type IV collagen, gelatin, elastin
Interstitial Collagenase (MMP-1)		Fibrillar collagens
Neutrophil Collagenase (MMP-8)		Fibrillar collagens
Collagenase-3 (MMP-13)		Fibrillar collagens
Stromelysin-1 (MMP-3)		Proteoglycans, glycoproteins type IV collagen, gelatins
Stromelysin-2 (MMP-10)		Proteoglycans, glycoproteins type IV collagen, gelatins
Metalloelastase (MMP-12)		Elastin
MMP-18		N.D.
MMP-19		N.D.
Stromelysin-3 (MMP-11)		Laminin and fibronectin (weakly)
MT1-MMP (MMP-14)		Gelatinase A, proteoglycans, glycoproteins
MT2-MMP (MMP-15)		N.D.
MT3-MMP (MMP-16)		Gelatinase A
MT4-MMP (MMP-17)		N.D.
MT5-MMP (MMP-21)		N.D.
Gelatinase A (MMP-2)		Gelatins, types I and IV collagen
Gelatinase B (MMP-9)		Gelatins, types I and IV collagen

Figure 5-1: The Matrix Metalloproteinase Family. Groups are arranged by domain structure and separated by dashed line. Family members with distinct but subtle structural features are separated by dotted line. PRO = prodomain; PRE = leader sequence; CAT = catalytic domain; H = hinge domain; HEM = hemopexin-like domain; F = furin site; FN = fibronectin-like domain; TM = transmembrane domain; and ND = not determined (modified from Chambers and Matrisian, 1997).

expressed in a variety of tissues and often elevated in malignant tumors (Liotta et al., 1979). MMP-9 is a 92 kDa protein expressed in macrophages, neutrophils, epithelial cells, and keratinocytes (Matrisian, 1992). It contains an additional “collagen-like” domain in its structure. Both MMP-2 and MMP-9 contain three fibronectin type II-like repeats inserted into the catalytic domain which allow them to bind to gelatin, the main substrate of both enzymes.

Collagenases

The collagenase group includes interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenases-3 and -4 (MMP-13 and MMP-18, respectively) (Nagase, 2000). These enzymes degrade fibrillar collagen types I, II, III, VIII and X, proteoglycans and gelatin. The ability to cleave fibrillar collagens is limited to this group of MMPs (Matrisian, 1992). All collagenases degrade interstitial collagens, but each collagenase exhibits a preference for different collagen types. MMP-1 prefers type III, MMP-8 type I, and MMP-13 has a preference for type II collagen.

Stromelysins

Stromelysin 1 (MMP-3), stromelysin 2 (MMP-10), and stromelysin 3 (MMP-11) are the three members of the stromelysin group of MMPs. Because the enzymatic activity and amino acid sequence of MMP-11 is significantly different from MMP-3 and MMP-10, it is often placed in a separate subgroup. MMP-3 and MMP-10 both degrade proteoglycans and ECM glycoproteins (i.e. laminin and fibronectin), type IV collagen and gelatin. MMP-11 is secreted in the active form and weakly degrades stromelysin-like

substrates. Both MMP-3 and MMP-10 play a role in the activation of several proMMPs (Nagase, 2000).

MT-MMPs

Currently five membrane-type MMPs (MT-MMPs) have been identified: MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), and MT5-MMP (MMP-21). Each has a single cytoplasmic domain and a transmembrane (TM) domain. The TM domain results in the MT-MMPs residing at the cell membrane. This characteristic is in contrast to the other MMP family members, which are all secreted as pro-enzymes into the extracellular environment. Along with MMP-11, the MT-MMPs are activated intracellularly with the help of a furin sequence located in their structural domains. Localization of MT-MMPs at the cell membrane restricts their actions on extracellular proteins near the cell surface. The major substrates of some of the MT-MMPs include gelatinase A, fibrillar collagens, proteoglycans and glycoproteins (Chambers and Matrisian, 1997).

Other MMPs

Several other MMPs have been identified but do not fall into one of the four main subgroups due to differences in substrate specificity or domain structures. Several of these “other” MMPs are described in this section. Matrilysin (MMP-7) is the smallest member of the MMP family (Nagase, 2000). This MMP is occasionally grouped into the stromelysin family. MMP-7 digests several ECM components including proteoglycans, glycoproteins, type IV collagen, gelatin, and elastin. The elastase group so far has only

one member. Metalloelastase (MMP-12) digests elastin and nonfibrillar collagen. This MMP may represent a new class of elastin-degrading MMPs that includes MMP-18 and MMP-19. MMP-12 shares the same domain structure as stromelysins and collagenases. The last of the MMPs include MMP-19, MMP-20, MMP-21, MMP-22, and MMP-23. These MMPs have been more recently discovered and, as a result, their characterization is very limited.

It is important to note that there is another family of proteins that have metalloproteinase activity. The ADAM (A Disintegrin And Metalloproteinase) proteins contain enzyme-like regions and a disintegrin region that can mediate cell adhesion and detachment. Of the approximately 30 members of the ADAM family, only 16 are known to have metalloproteinase activity (Nelson et al., 2000).

Association of MMPs and Cancer

In order for a cell to invade and metastasize, there are a series of collagen-containing structural barriers that it must be able to cross. Extracellular matrix (ECM) and basement membrane (BM) barriers must be passed during intravasation and extravasation into and out of, respectively, the lymphatic and blood vascular systems. The collagen-containing BM underlying endothelial cells presents a final barrier that must be breached during the metastatic process. Tissue degradation is required to permit tumor cell invasion at both the primary and secondary tumor sites. While some of the proteolytic activity may be derived from stromal cells and tumor-infiltrating immune cells from host tissues (Chambers and Matrisian, 1997), it is obvious that cancer cells require a sufficient amount of enzymatic ability to degrade these barriers. MMPs were

prime candidates for these degradative activities because the family members collectively degrade all components of the ECM. The first evidence to support the requirement for elevated levels of proteolytic activity in cancer cells and the importance of MMPs in this process came from experiments carried out in the early 1980s (Barsky et al., 1983; Liotta et al., 1979; Liotta, et al., 1980). MMPs have been associated with the malignant phenotype for a number of years, and there is now an extensive amount of evidence that shows elevated MMP levels in cancer tissues compared to normal tissues.

Most MMPs have been detected in human tumor cell lines. MMPs may be expressed by malignant cells as well as the adjacent stromal cells. Many MMPs are expressed in connective tissue cells (e.g. fibroblasts and inflammatory cells) in response to the tumor (Nelson et al., 1997). In several tumors systems there is a differential expression of MMPs in tumor cells and in the surrounding tumor stroma. This suggests that the remodeling of the tumor and surrounding tissues may be facilitated by the stromal response to the tumor (Stetler-Stevenson and Yu, 2001). There is also a positive correlation between tumor aggressiveness and the expression of high levels of multiple members of the MMP family.

The overexpression of MMPs usually correlates with more aggressive tumor behavior and poor prognosis (Sternlicht et al., 2000). Gelatinase A (MMP-2) has been shown to have increased levels in several different human tumors (Montironi, et al., 1992; Stetler-Stevenson et al., 1993; Nelson et al., 2000). Stearns and Wang (1993) demonstrated with immunoperoxidase labeling, Northern blot analysis, and enzyme-linked immunosorbent assays (ELISAs) that MMP-2 levels increased with increasing Gleason scores in human prostate cancer. Stearns and Stearns (1996) had similar results

when they studied MMP-2 levels in prostatic cancer tissue sections. These studies suggest MMP expression levels may be good indicators of the grade or progression of prostate cancer.

Using SDS-PAGE zymography, increased presence of both MMP-2 and MMP-9, was demonstrated in the urine of patients with prostate, breast, colon, renal, and bladder cancers (Moses et al., 1998). Gokaslan and colleagues (1998) tested MMP-2 and MMP-9 expression in various primary carcinomas that were metastatic to the spine (from breast, lung, renal cell, and thyroid carcinomas) to assess the role of these enzymes in spinal invasion. They discovered that the more aggressive lung carcinomas and melanomas, expressed the highest levels of MMP-2 and MMP-9 (**Figure 5-2**), suggesting that increased levels of MMPs are correlated with enhanced invasive ability. These results also suggest that increased MMP activity is important in degrading the BM at the primary tumor site as well as at distant metastatic sites.

MMP-2 and MMP-9 are not the only members of the MMP family that show elevated expression levels in cancer. Stromelysin-3 (MMP-11) has been associated with malignant breast cancer, but is not expressed in normal breast tissue or benign fibroandenomas (Wolf et al., 1993). Investigators have also demonstrated increased expression of MMP-1, MMP-10, MMP-7, and MT1-MMP in a variety of human cancers (Stetler-Stevenson and Yu, 2001). Several generalizations about the association of MMPs and cancer can be made. First, the number of different MMP family members that can be detected tends to increase with tumor progression. Second, there is a positive correlation between the levels of any MMP family member and tumor stage; MMP levels

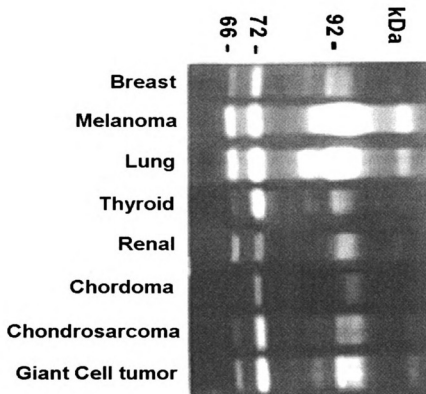


Figure 5-2: Gelatin zymography of tissue extracts obtained from primary and metastatic spinal tumor samples. Equal amounts of protein (50 μ g) were fractionated by SDS-PAGE using gelatin as the substrate. Molecular size of major MMPs is denoted in kilodaltons (kDa). MMP-9 at 92 kDa, MMP-2 at 72 kDa (modified from Gokaslan et al., 1998).

tend to increase with increasing tumor stage. Third, since MMPs can be produced by tumor cells as well as by host organ stromal cells, as a host response to the tumor, the expression pattern of MMPs supports a role for these enzymes in later stages of tumor progression. While MMPs and their role in tumor progression are the focus of this chapter, it is important to note that they are not the only contributors to the proteolytic degradation that occurs during this process. Interactions between members of the other protease families provide additional levels of regulation and complexity to tumor cell invasion and metastasis (Webber et al., 1995; Waghray and Webber, 1995; Webber et al., 1996).

MMP Regulation

The regulation of MMPs can occur at several levels: gene expression (transcription), activation, and activity. These multiple levels of regulation allow for a wide range of approaches to control the functions of the MMP family members.

Gene Expression

As previously mentioned, MMP expression is often elevated during tissue remodeling or cancer invasion and metastasis. Several agents are known to induce the expression of MMP genes, including cytokines, growth factors, oncogenes, tumor promoters, chemical agents (phorbol esters, actin stress fiber-disrupting drugs), ultraviolet B irradiation, and physical stress (Stearns and Wang, 1993; Nagase and Woessner, 1999). Several suppressive factors (transforming growth factor- β , retinoic acids, and glucocorticoids) can down-regulate MMP gene expression (Nagase and Woessner, 1999).

Activation

Once the MMP is expressed and secreted into the extracellular environment, the next regulatory point is activation. With the exception of MMP-11 and the MT-MMPs, all MMPs are initially synthesized and secreted from the cell as inactive pro-enzymes, or zymogens. The latent enzymes can be activated *in vitro* and *in vivo*. In all cases, activation requires the removal of a portion of the PRO domain. Removal of this domain destabilizes the association between the cysteine residue with zinc and results in a conformational change. The conformational change, referred to as the “cysteine switch,”

allows the newly activated catalytic site to interact with zinc. The zinc ion is the catalytic element of MMPs.

MMP activation *in vitro* can be accomplished by proteinases and non-proteolytic agents such as mercurial compounds, reactive oxygen, and denaturants (Nagase and Woessner, 1999). Four main mechanisms for the *in vivo* activation of MMPs have been proposed: 1) the extracellular activation of MMPs by non-MMP proteins (i.e. uPA/plasmin system, thrombin, and trypsin); 2) the extracellular activation of MMPs by other MMPs; 3) the intracellular activation of MMP-11 and MT-MMPs by furin; and 4) the MT-MMP membrane-associated activation of MMP-2.

Activity

After MMPs have been activated, they are subject to inhibition by a family of endogenous tissue inhibitors of metalloproteinases (TIMPS) as well as by α 2-macroglobulin, an inhibitor in the plasma. TIMPs are a family of secreted proteins whose primary function is to inhibit the proteolytic activity of MMPs (Liotta et al., 1982). Studies done on TIMPS helped provide evidence that increased MMP activity was a direct cause of the enhanced invasive ability of cancer cells. In these studies, TIMPs specifically inhibited degradation of matrices, invasion, and tumorigenesis. However, the protease inhibitors had no effect on cell proliferation, attachment to amnion in the chick amnion *in vivo* invasion assay, and migration through non-coated filters in the Boyden chamber *in vitro* invasion assay.

To date there are four members of the TIMP family, numbered TIMP-1 through TIMP-4, based on their order of discovery. TIMPs specifically and reversibly inhibit MMPs, but they are not known to inhibit any other metalloproteinases (Murphy and

Willenbrock, 1995). The inhibitory ability of the TIMPs is central to their effectiveness as tumor and invasion suppressors. All four TIMPs have 12 absolutely conserved cysteine residues that form six intramolecular disulfide bonds. Three of these bonds are located in the N-terminal domain, which contains the MMP-inhibitory activity (Stetler-Stevenson and Yu, 2001). TIMPs bind to latent and active MMPs in a 1:1 stoichiometry. This balance between active proteases and inhibitors would result in an optimal non-invasive phenotype. Complete dissolution of matrix proteins would prevent tumor cells from being able to form attachments to each other or to the matrix proteins, both of which are necessary for invasion to occur. But, pathological tissue destruction, such as in cancer, does occur in the cases of reduction or ablation of TIMP gene expression. Conversely, any imbalance of the ratio in favor of TIMPs can lead to abnormal ECM accumulation and a decrease in invasion of tumor cells.

The role of TIMPs has proven to be more complex and multifaceted than just as inhibitors of MMP activity. There are several reports demonstrating that TIMPs can both stimulate and inhibit cell growth (Edwards, 2000; Stetler-Stevenson and Yu, 2001). They have also been shown to promote and inhibit apoptosis. Members of the TIMP family have also been associated with cancer. In several cases, malignant tumors have been shown to express increased rather than decreased levels of TIMPs (Grignon et al., 1996; Jung et al., 1997). Whether or not these increased levels of TIMPs were in response to increases in MMP levels was not determined. There can also be variable expression patterns of MMPs and TIMPs within tumors versus surrounding stromal cells. It is obvious that the interplay between MMPs and TIMPs *in vivo* is complex and poorly understood and further studies need to be done to fully understand their relationship.

Emerging Roles for MMPs in Tumor Progression and Invasion

Matrix metalloproteinases (MMPs) and their inhibitors have been strongly linked to the proteolytic activity associated with metastasis and as contributors to the process. However, recent studies suggest that MMPs are also important in creating and maintaining an environment that supports the initiation and growth maintenance of both primary and metastatic tumors. The extracellular matrix (ECM) can have effects on cellular proliferation, differentiation, and migration. Evidence suggests MMPs play a role in these processes, at least in part, by specifically altering the integrity and composition of the ECM.

One emerging role for MMPs is in tumor initiation. The first evidence supporting this role came from Sternlicht and colleagues (1999). They showed that MMP-3 induces tumors in animals with and without an intact immune system. Using mammary epithelial cells that expressed MMP-3, they demonstrated that MMP-3 converts normal mammary epithelial cells into highly invasive mesenchymal-like tumors *in vivo*. They also showed that this transition could be blocked by over-expression of TIMP-1, which further supported the role of MMPs in tumor initiation. The initiation of tumor growth may involve the regulation of the growth environment by regulating access to growth factors from the ECM surrounding the growing tumor.

MMPs also play a role in tumor initiation and growth at secondary sites. Studies have shown that TIMPs can block MMP activity during this process. Intravital video microscopy (IVVM) permits direct observation of the microcirculation *in vivo*. Using this technique and quantitative analysis of extravasation, Koop et al (1994) challenged the idea that tumor cell extravasation was the rate limiting step in metastasis. They

determined that B16F10 melanoma cells transfected with TIMP-1 showed no difference in the rate of extravasation compared to the parent cell line. However, after seven days there was a significant reduction in both size and number of tumor nodules in the TIMP-expressing cells compared to the parent cells. Similar results were shown using a tumorigenic, metastatic rat bladder cell line (Kawamata et al., 1995). Kawamata and colleagues demonstrated that transfection of cells with TIMP-1 or TIMP-2 cDNA only slightly affected primary tumor growth and local invasion, but significantly inhibited extravascular growth at distant metastatic sites. Taken together, these studies suggest that MMP activity may be more important to the establishment and growth of tumor deposits than in the invasive steps of metastasis. These studies also suggest the rate-limiting step of metastasis may occur after extravasation.

The modulation of ECM proteins by MMPs has been shown to play a role in cellular migration and invasion. The integrins are a family of cell:matrix adhesion molecules that provide adhesive traction and contraction of actin filaments during cell migration. Recent data suggest that MMP-integrin interactions may modulate integrin function on the cell surface. Brooks and colleagues (1996) showed that the $\alpha_v\beta_3$ integrin can bind MMP-2. This suggests that MMP-integrin interaction may localize MMPs at sites proximal to important ECM ligands that would facilitate invasion. MMP-2 was also shown to cleave the γ_2 chain of laminin-5 to reveal a cryptic site on the α_3 chain that has chemotactic properties and stimulated cell migration (Giannelli et al., 1997).

MMPs have also been shown to play a role in angiogenesis, the process by which new blood vessels are formed. Several studies have shown the need for MMP-2 and MMP-9 in the process of angiogenesis in mice (Fingleton and Matrisian, 2000).

Angiogenesis is essential for tumor growth and survival, and its regulation by MMPs most likely plays an important role in the regulating of sustained tumor growth. It can also serve as a method to transfer metastatic cells to secondary sites. Johnson et al. (1994) demonstrated that an up-regulation of MMPs and a down-regulation of their inhibitors (TIMPs) are features of the angiogenic process. Conversely, MMP-9 and MMP-7 have been shown to inhibit angiogenesis by cleaving plasmin to yield angiostatin, a potent inhibitor of angiogenesis. This MMP activity is now believed to be the principle protease activity responsible for angiostatin generation (Patterson and Sang, 1997).

There is much evidence to support these emerging roles for MMPs, but much still remains to be elucidated. A further understanding of these new roles for MMPs is vital to the design and development of new and effective cancer therapeutics. **Figure 5-3** is a diagram showing the classic as well as emerging roles of MMPs in tumor progression and metastasis.

Effects of Vitamin D on MMPs

Considerable attention has been focused on the role of vitamin D in cancer prevention and treatment. There is substantial evidence to show that vitamin D can induce apoptosis and differentiation, and inhibit proliferation and invasion in several types of cancer. The mechanisms of action of vitamin D in these processes are being discovered but still remain somewhat unclear. This section will focus on the effects of vitamin D on matrix metalloproteinases (MMPs). The effects of vitamin D on several other processes will be reviewed in chapters 6, 8 and 9. Currently there is limited data

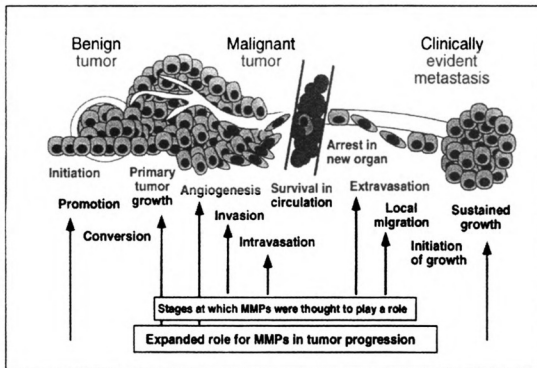


Figure 5-3: Role of MMPs in the metastatic process. MMPs have been classically thought to contribute to the tissue destruction required for cells to invade, intravasate, extravasate, and migrate. More recent evidence suggests these enzymes can also play a role in the growth of tumors, angiogenesis, and the sustained growth of metastatic nodules (Nelson et al, 2000).

describing the effects of vitamin D on MMPs. This is an important area of study given the previously described roles MMPs play in tumor progression and metastasis.

Schwartz et al. (1997) studied the effects of 1,25-dihydroxyvitamin D₃ (calcitriol) on human prostate cancer cell lines: DU 145, and LNCaP. They reported that the poorly invasive LNCaP cell line was hardly affected by treatment with calcitriol. However, the highly invasive DU 145 cells showed a significant decrease in invasive ability when exposed to calcitriol treatment. In order to determine the possible mechanism responsible for this decrease in invasion, Schwartz and colleagues examined secreted MMP-2 and

MMP-9 levels with gelatin-based zymography in the presence of calcitriol. They demonstrated a significant decrease in activity levels of both enzymes in the DU 145 cell line. Similar results were obtained using the human breast carcinoma cell line MDA-MB-231 (Hansen et al., 1994; Koli and Keski-Oja, 2000). In an experiment, using Copenhagen rats injected with MAT LyLu prostate cancer cells, a significant decrease in the size and number of lung metastases was observed in rats treated with calcitriol (Lokeshwar et al., 1999). It should be noted that this study did not test MMP levels in the rats. However, taken together these data demonstrate that calcitriol has an inhibitory effect on MMP-2 and MMP-9 activity. This decrease in activity could decrease basement membrane degradation, resulting in an inhibition of invasive ability of the cells. They also suggest that calcitriol could inhibit and/or prevent the initiation and growth maintenance of tumors at both primary and ectopic sites, such as, lungs.

Calcitriol can also have a stimulatory effect on MMP activity. It has been shown to increase the activity of MMP-9 in tibias of mice (Lin et al., 2002), MMP-1 and MMP-3 in cells of the rheumatoid lesion (Tetlow and Woolley, 1999), and MMP-13 in mouse osteoblast cells (Uchida et al., 2001). Most of the effects of MMPs in these studies are on bone during the skeletal formation at early and late stages of osteogenesis and/or during skeletal remodeling during bone growth. These processes require much bone resorption, which would help explain the observed stimulatory effects of calcitriol.

Interestingly, investigators in another study demonstrated that vitamin D receptor (VDR) expression in human osteoarthritic cartilage was often associated with sites of prevalent MMP expression, which is in contrast to the virtual absence of VDR in normal age-matched cartilage (Tetlow and Woolley, 2001). Indeed, in all of the studies

mentioned in this section, the effects by vitamin D on MMPs appear to be receptor-mediated because VDRs are expressed in all these tissues. This indicates that vitamin D may be effective in MMP regulation during tumor progression and metastasis.

Conclusion

Matrix metalloproteinases (MMPs) are the predominant family of enzymes that degrades the extracellular matrix. It is well known that they are a contributing factor in several complex physiological processes and they give malignant cancers their defining ability to invade and metastasize. With the significant amount of attention MMPs have received in tumor growth and metastasis, classical roles for these enzymes are becoming more defined and new roles are being discovered. MMPs have recently been implicated in the initiation and sustained growth of tumors in both primary and secondary sites. However, much still needs to be learned about MMPs before we can fully comprehend these intriguing enzymes. With further understanding of the roles and methods of regulating MMPs, and with the development of effective therapeutic agents, such as vitamin D, we will begin to realize the therapeutic benefit to cancer treatment by the inhibition of MMPs.

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Chapter 6

Cell Proliferation and Differentiation and the Effects of Vitamin D

Abstract

The structural and functional modification of an unspecialized cell into a specialized one is known as differentiation. This process most often takes place during embryogenesis when a fertilized egg differentiates into one of several different cell types, but it also occurs continuously during adult life with the differentiation of stem cells into specialized cell types. Several factors regulate cell differentiation, including hormones, vitamins, growth factors, and the extracellular matrix. The differentiating actions of these factors on the target tissues can be carried out independently or concomitantly with other factors. In recent years, vitamin D has gained a considerable increase in interest because of its ability to modulate and regulate cell differentiation. In this chapter I will discuss the process of differentiation and examine how vitamin D and its metabolites, including its biologically active hormone, $1,25(\text{OH})_2\text{D}_3$ (calcitriol), work independently or synergistically, with other agents, to modulate and regulate the differentiation of certain cell types.

Introduction

Differentiation is the generation of cellular diversity that allows cells to become structurally or functionally specialized. It pertains to single or groups of cells and it is most often observed during embryogenesis. A fertilized egg can give rise to many differentiated cell types, including muscle cells, hematopoietic cells, nerve cells, epidermal cells, blood cells, fat cells, and so on. But differentiation is not limited to embryogenesis. During adult life certain cells (i.e. blood cells and epithelial cells of the small intestine) can differentiate from stem cells (Muramatsu, 1990), allowing for a continuous regeneration of certain cells throughout the lifetime of an organism.

Differentiated cell types are found in all animals, but bilaterians, animals with bilateral symmetry (dorsal-ventral and left-right axes), are the only ones that produce organs, or three-dimensional arrays of differentiated cells (Davidson, 2001). Layers of differentiated mesodermal cell types comprise major components of all body parts in the adult body forms of bilaterians. Once differentiated, cells exhibit specialized functions and their transcription patterns are locked down by diverse stabilization mechanisms. These mechanisms include: local DNA methylation and demethylation rates, feedback circuits that regulate differentiation, a decrease in mRNA turnover, and changes in chromatin structural domains that include the regulatory systems of the relevant genes (Davidson, 2001). These cells are said to be terminally differentiated and can be marked by a precise set of differentially expressed protein coding genes. Only in unusual cases, like transdifferentiation, will cells differentiate and then re-differentiate in a second direction (Davidson, 2001).

Since all cells in the body contain the same genetic make-up, differentiation must be the result of alteration(s) and/or suppression of gene function, not the loss of genes. In other words, cells become differentiated due to differences in their functioning genes. Several agents contribute, either independently or synergistically with other agents, to the alteration and suppression of gene function during differentiation. These agents include hormones, growth factors, components of the extracellular matrix, vitamins, steroids, cytokines, and protein kinases.

One type of these agents that has been under intense investigation in recent years is vitamin D. Vitamin D is essential for calcium and phosphorous homeostasis and for proper bone development. The role of vitamin D in the prevention and treatment of rickets has been recognized for several years. Vitamin D and its biologically active form, $1,25(\text{OH})_2\text{D}_3$ (calcitriol), have been shown, experimentally, to play significant roles in the modulation and regulation of cell growth and differentiation. Induction of differentiation by vitamin D has been demonstrated in several normal cell types, including, but not limited to, prostate, breast, colon, bone, hematopoietic, skin, B-cell progenitors, keratinocytes, and epithelial cells. Vitamin D receptors (VDR) have been found in the classical target organs (kidney, bone, intestine, parathyroid glands) as well as in a number of nonclassical organs, such as, breast and prostate epithelium, skin, muscle, pancreas, hematopoietic and immune systems, and reproductive organs (Dusso and Brown, 1998). These classical and nonclassical actions have expanded the therapeutic applications of vitamin D and its biologically active metabolite, calcitriol. This chapter will discuss cell differentiation in general and examine the role of vitamin D in the process of differentiation in several different cell types.

Differentiation During Early Development and Growth

During early development of an organism, cell differentiation must be carefully regulated in order for normal growth and development to take place. Differentiation takes place as early as in the preparation for fertilization, when male and female germ cells undergo meiosis and cytodifferentiation. The purpose of these processes is to reduce chromosome numbers from a diploid to a haploid number, and to alter germ cell shape in preparation for fertilization.

During the process of gonad formation primordial germ cells arrive at the gonad of the genetic female and differentiate into oogonia. The cells then undergo several mitotic divisions and arrange in a cluster that is surrounded by a layer of epithelial cells. The majority of oogonia continue to divide and differentiate into primary oocytes, which replicate their DNA and enter into meiosis. Similar processes occur in the male, with the main difference being that differentiation of primordial germ cells begins at puberty instead of during the third month of development as in the female. The cells continue to differentiate through the formation of gametes and up to fertilization. This example demonstrates how early in development differentiation occurs and how important it is to strictly regulate this process.

But most people do not think of differentiation occurring at such an early stage of development. They are more familiar with the differentiation that begins to take place after zygote formation. The first cell division results in the formation of two cells from the zygote. The zygote, which is a giant store house of materials, divides or cleaves into several smaller cells termed blastomeres. The blastomeres remain loosely clumped until the 3rd cleavage when they maximize contact with each other and form a compact ball

called the morula (**Figure 6-1**). The inner cells of the morula make up the inner cell mass and the surrounding cells constitute the outer cell mass. The inner cell mass and outer cells mass give rise to the embryo proper and the trophoblast, respectively. The embryo is now at the blastocyst stage. Cells of the inner cell mass, or the embryoblast, are located at one pole while the cells of the trophoblast flatten and form the epithelial wall of the blastocyst.

During the next stages of development, perhaps the most characteristic process of differentiation takes place. This process, known as gastrulation, establishes all three germ layers in the embryo and begins with the formation of the primitive streak on the surface of the epiblast. Cells of the epiblast migrate in the direction of the primitive streak to form the mesoderm and intraembryonic endoderm. When the cells arrive at the epiblast, they become flask-shaped, detach from the epiblast, and invaginate inward (**Figure 6-2**). Some of these invaginated cells form the embryonic endoderm, while others form the mesoderm by lying between the epiblast and newly formed endoderm. The ectoderm is formed from cells remaining in the epiblast. Through the process of gastrulation, the epiblast is able to form all three germ layers in the embryo: the ectoderm, mesoderm, and endoderm.

The cells of the three germ layers are destined to differentiate into more than 200 different cell types that form specific tissues and organs. The ectoderm gives rise to the skin; central and peripheral nervous systems; hair; nails; enamel of the teeth; and the pituitary, mammary, and sweat glands. The mesoderm gives rise to the spleen, muscle, bone, connective tissue, the adrenal cortex, cells of the blood and lymph systems and the

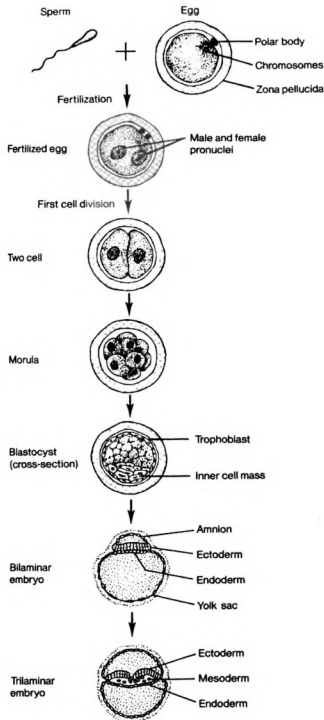


Figure 6-1: Fertilization and early stages of embryonic development through the formation of the germ layers (Cooper, 1992).

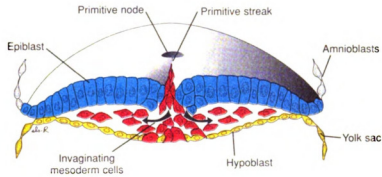


Figure 6-2: Cross section through the primitive streak showing invagination of the epiblast (modified from Sadler, 1995).

dermis. The endoderm provides the epithelial lining of the gastrointestinal and respiratory tracts, and the urinary bladder. It also forms the parenchyma of the thyroid and the epithelial lining of the tympanic and auditory tubes, and portions of the liver, pancreas, tonsils, and parathyroid gland.

Differences between the cells of an embryo arise in various ways. Sister cells can be born different as a result of asymmetric cell division (**Figure 6-3**). In asymmetric division, some significant set of molecules is divided unequally between two daughter cells at the time of division. The unequal division of molecules acts as a determinant for the differentiation of one of the cells by altering the gene expression in the daughter cell that receives it.

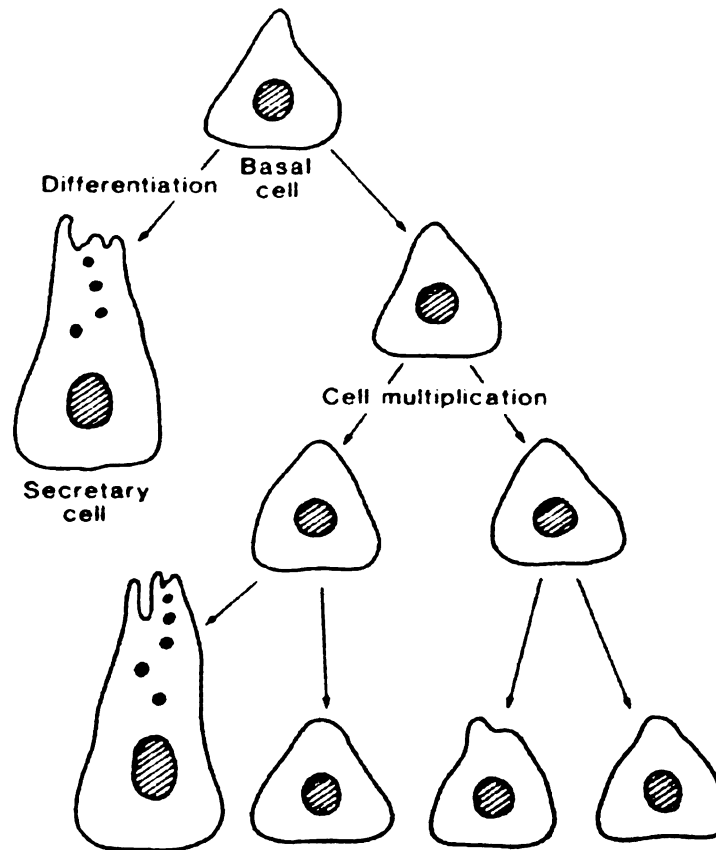


Figure 6-3: Diagram showing cell multiplication and cell differentiation, both of which can occur simultaneously in the same culture where different cells may have different fates. Similar phenomena occur in the acinar epithelium *in vivo* (Webber, 1981).

During the process of differentiation similar cells can become different through a competitive interaction with one another (cell:cell contact) or they may receive different exposures to inductive signals from cells outside the group. These inductive signals may be in the form of hormones or growth factors. Cell memory allows transient signals to have a lasting effect on the internal state of a cell. The ability of a cell to remember which genes have been turned on and which gene expressions to maintain allows the cell to become determined for a specific differentiated fate. With these cellular abilities, sequences of different signals acting at different times and places in growing cell arrays can give rise to elaborate and varied multicellular organisms.

Relationship Between Cell Proliferation and Differentiation

Cell proliferation and differentiation are two closely linked processes in the development of an organism. In normal development there is homeostasis between these two processes, meaning cell gain from proliferation equals cell loss from differentiation and apoptosis. This balance between proliferation and differentiation of cells is regulated by a number of factors, several of which will be discussed later. Some of these factors will have a negative effect (growth inhibitors) on proliferation and others will have a positive effect (growth stimulators). In normal growth regulation, growth stimulators must be countered by the effects of growth inhibitors, thereby giving a stable population where cell loss will equal cell gain. Normal tissues generally have a decrease in their ability of cell division as they differentiate. The more differentiated the cells become, the greater the level of growth inhibitors, which leads to a decrease in proliferation. In abnormal tissues, such as a tumor, the cells retain the ability to proliferate and there is a decrease in cell differentiation. The decrease in differentiation is associated with a decrease in growth inhibitors.

On average, a normal stem cell division generates one daughter stem cell and one cell that will undergo terminal differentiation and stop dividing. If the stem cell divides more rapidly, differentiated cells will be produced and discarded (i.e. through apoptosis) more rapidly, but the balance between the two is maintained. In abnormal growth (i.e. hyperplasia or a tumor), more than 50% of the daughter cells remain as stem cells. As a result, the process of differentiation is altered so that daughter cells that follow this route retain the ability to carry on dividing indefinitely and avoid apoptosis (**Figure 6-4**).

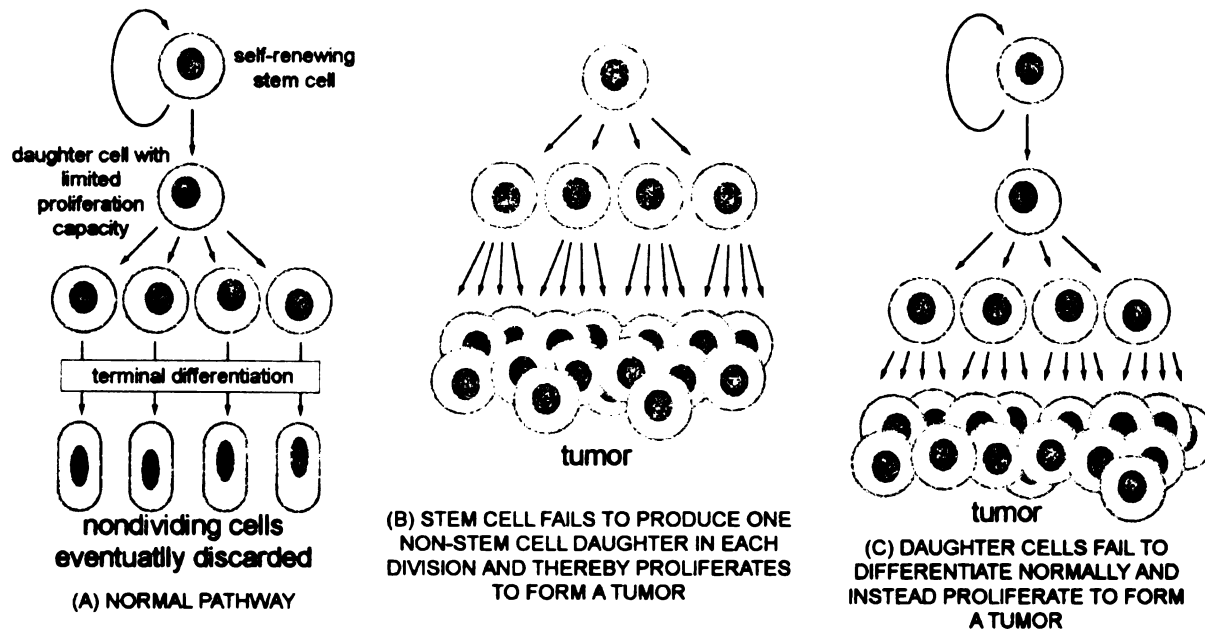


Figure 6-4: Normal and deranged control of cell production from stem cells. (A) The normal strategy for producing differentiated cells. (B and C) Two types of derangement that can give rise to the unbridled proliferation characteristic of cancer. Excessive cell division rate for the stem cell in (A) will not by itself have this effect, as long as each stem cell division produces only one daughter that is a stem cell (modified from Alberts et al., 2002).

Presumably, several cancers arise from an alteration in the normal differentiation program, such that a cell committed to differentiation does not terminally differentiate and die, but rather continues to divide indefinitely. In other words, changes that block the normal development of cells toward a nondividing, terminally differentiated phenotype, or those that prevent apoptosis, play a crucial role in many cancers.

Markers of Differentiation and Differentiated Function in Epithelial Cells

Intermediate Filaments

Intermediate filaments that are important cell type markers in epithelial cells are the cytokeratins. Vimentin is a mesenchymal cell intermediate filament.

Cytokeratins:

Cytokeratins (**Figure 6-5**) are the first intermediate filament proteins to appear during embryonic development. They are usually arranged in bundles called tonofibrils, which form a cage-like structure around the nucleus and extend to the cell periphery. Cytokeratins are the hallmark intermediate filament proteins of epithelial and mesothelial cells. Identification of cytokeratins in cultured cells is a standard method to verify the epithelial nature of the *in vitro* population.

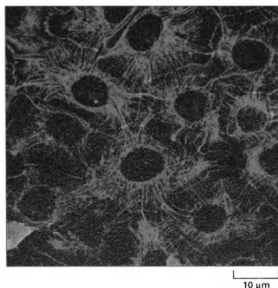


Figure 6-5: Cytokeratin filaments in epithelial cells. Immunofluorescence micrograph of the network of cytokeratin filaments (green) in a sheet of epithelial cells in culture. The filaments in each cell are directly connected to those of its neighbors by desmosomes. A second protein (blue) has been stained to reveal the location of the cell boundaries (Alberts et al., 2002).

The cytokeratins are a multigene family of about 20 biochemically and immunologically distinct yet closely related polypeptides, which range in molecular weight from 40 to 78 kDa. The cytokeratin filaments are assembled from heterodimeric subunits of acidic type I cytokeratins (cytokeratins 9 to 20) and basic type II (cytokeratins 1 to 8) intermediate filament proteins (Paramio and Jorcano, 2002). The expression of these cytokeratins is a good prognostic indicator of cancer progression. Intermediate filaments are useful tools in cell type determination as well as in determining the level of invasiveness and metastatic ability of cancer cells.

The prostatic luminal, secretory cells express cytokeratins 8, 18 and 19, and the prostatic basal cells express cytokeratins 5, 14, 15 and 16 (Traub, 1985; Peehl et al., 1994a; Sherwood et al., 1990). Different types of epithelial cells can be characterized based on these acidic and basic cytokeratin combinations. Simple epithelia express cytokeratins 8 and 18 with variable levels of cytokeratin 19 and 20 (Yoon et al., 2001) and in some instances 17 and 7 (Paramio and Jorcano, 2002). Stratified epithelia express cytokeratins 5 and 14 in the basal layer, and when these cells begin terminal differentiation they move upwards and begin to express cytokeratins 1 and 10 in the skin, cytokeratins 4 and 13 in internal stratified epithelia and cytokeratins 3 and 12 in corneal cells (Paramio and Jorcano, 2002). These patterns reveal clues to the lineage of a cell and its differentiation status and also imply that cytokeratins might carry out specific functions in epithelial cells.

The cytokeratin profiles in the prostate gland are an extremely useful diagnostic tool in the determination of prostate cell differentiation and the level of prostate cancer grade. Alberti et al. (2000) found that several constitutive cytokeratins (8, 18, and 19)

showed a strong down regulation in poorly differentiated prostate cancer cells, and their expression significantly decreased as compared to normal human prostate cells, benign prostatic hyperplasia, and to moderately and well differentiated tumors. A decrease in the expression of cytokeratins 8 and 18 in prostate carcinoma, when compared to normal prostate, was also observed in animal models (Ramaekers et al., 1994). Sherwood et al. (1990) showed that established prostate cancer cell lines showed expression of cytokeratins 8 and 18 but not cytokeratins 5, 7, and 15 which were observed in the benign prostate. All these data support the belief that prostate cancer arises from the luminal cells of the prostate, which express cytokeratins 8, 18, and 19, and not from the basal cells, which express cytokeratins 5, 14, 15 and 16.

In a recent study, Hudson et al. (2001) attempted to identify subpopulations of cells in the basal layer of the prostate by examining the relationship between cell proliferation and differentiation in benign prostatic hyperplasia (BPH) tissue. By staining basal and luminal cells with fluorescence-labeled cytokeratins, Hudson et al. (2001) showed that cytokeratin 19 is found basally and in a position intermediate between the basal and luminal cytokeratin 8-positive layer. Cytokeratin 19 has been implicated in the process of differentiation, for example, in prostate epithelial cells as they progress from basal to differentiated secretory cells (**Figure 6-6**). This suggests that cytokeratin 19 could be a possible marker for an amplifying, non-stem-cell, basal population in the prostate. **Figure 6-6** shows a hypothetical differentiation pathway for human prostate epithelial cells based on cytokeratin expression.

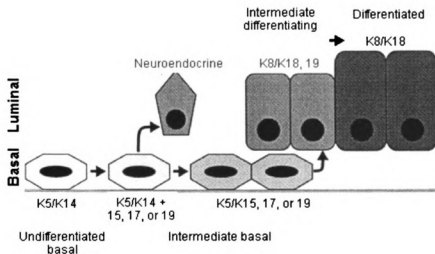


Figure 6-6: Hypothetical differentiation pathway for human prostate epithelial cells based on patterns of cytokeratin staining. Basal stem cells (K5 and K14 only) give rise to an intermediate transit amplifying population expressing K19. These cells differentiate into luminal cells with transient co-expression of K19, 8, and 18 before complete differentiation into cells expressing K8 and K18 only (modified from Hudson et al., 2001).

Vimentin:

Vimentin is a 54 to 58 kDa intermediate filament protein that is normally expressed in cells of mesenchymal origin, such as fibroblasts, myofibroblasts, and endothelial cells (Paramio and Jorcano, 2002; Webber et al., 1999a) and cells derived from mesoderm. It is the first intermediate filament associated with cell differentiation. An increase in vimentin expression occurs during differentiation into three germ layers during embryonic development. Vimentin is typical of osteocytes, chondrocytes, melanocytes, Langerhan cells of the skin, most but not all lymphatic cells, and it is expressed in neuronal or muscle cells (Osborn, 1993).

The expression of vimentin has also been shown to be associated with increased cell proliferation and increased metastatic ability and invasiveness of cancer cells. Its expression is observed in many tumors of epithelial origin. Vimentin is often expressed

in epithelial cells in culture. Several early studies suggested that this expression was induced by *in vitro* cultivation of these cells. Some data suggest that vimentin expression in epithelial cells *in vitro* correlates with malignant transformation. Several *in vivo* studies suggest that vimentin expression in epithelial cells may be related to tumor progression (Gilles et al., 1996).

Lang *et al.* (2002) demonstrated that vimentin expression increased with increasing motility of prostate cancer cells and that vimentin positive cells were more likely to be associated with poorly differentiated tumors. When testing vimentin expression in hyperplastic versus normal prostate epithelium cells, Fraga et al. (1998) showed that vimentin expression increased significantly in benign prostatic hyperplasia (BPH) cells when compared to the cells in the adjacent normal epithelium. Since several epithelia, which do not express vimentin in their slowly proliferating epithelium, do express this filament when grown as rapidly dividing cells in culture, Fraga et al. (1998) suggest the difference in proliferative activity between BPH and normal tissues causes the difference in vimentin expression, with vimentin activity being proportional to proliferative activity.

Generally, fully differentiated cells express only one class of intermediate filament subunits typical for that cell type. However, vimentin is unique in that it shows co-expression with other types of intermediate filaments. Vimentin has been found to be co-expressed with desmin in muscle cells, with glial fibrillary acidic protein (GFAP) in certain adult glial cells, with neurofilament polypeptides during neuronal differentiation, and with cytokeratins during epithelial differentiation (Capetanaki et al., 1984).

Recent studies have suggested differences in the motility and dynamic activities of vimentin and cytokeratins during coexpression in epithelial cells. By tagging vimentin and cytokeratins with green fluorescent protein, Yoon et al. (2001) showed that these intermediate filaments possess surprisingly different properties within the same cytoplasmic regions. Vimentin fibrils demonstrate changes in shape, extending and shortening over short periods of time, and bidirectional translocation, whereas cytokeratin tonofibrils exhibit slow oscillations or undulations and unidirectional fluctuations of diffuse and particulate cytokeratin from the surface of the cell towards the nucleus. Further study of these factors may shed more light on the specific roles of these intermediate filaments in differentiation, cancer progression, and metastatic ability of different cancers.

Secretory Proteins:

One very good marker of differentiation in prostate cells is prostate specific antigen (PSA). The differentiated, secretory cell lining the prostatic glands, secrete PSA into the lumen of the glands. The synthesis of PSA occurs in response to androgen stimulation. PSA represents the best serum marker for prostatic carcinoma and is arguably the best tumor marker available today. It is useful in the diagnosis and staging of prostate cancer, and for monitoring tumor progression in men with prostate cancer. Rising serum levels are an indication of prostate cancer and result from an increase in total number of secretory cells.

There are also markers of de-differentiation once normal cells become malignant. Cancer cells may produce an array of products not normally expressed in adult tissues, or are only transiently expressed in normal tissue. During development, embryonic cells

express certain hormones and other proteins known as fetal antigens, including α -fetoprotein, carcino-embryonic antigen, and human chorionic gonadotropin. Once tissue is fully developed and differentiated, these proteins are no longer expressed and are replaced by new gene products. De-differentiation in cancer is associated with re-expression of these fetal antigens where genes that are normally silent in the adult tissue are turned on. When produced by cancer cells they are known as fetal markers of malignancy.

Modulators of Cell Differentiation

There are several factors that can modulate cell differentiation. These factors include vitamins, hormones, growth factors, and the extracellular matrix. Several of these factors, and their mechanisms of action and effects on cell differentiation are discussed in the next section. The effects of vitamin D on cell differentiation are discussed later in this chapter.

Modulation of Cell Differentiation by Retinoids

Vitamin A is required for normal development and for maintaining normal differentiation of epithelial cells. Retinoids are derivatives of vitamin A. Many natural and synthetic retinoids have been shown to induce differentiation and apoptosis in a wide variety of cell culture models. By modulating cell differentiation and apoptosis, retinoids have the potential to reverse changes associated with malignant transformation and tumor progression towards a normal phenotype. This would make retinoids a novel agent for cancer treatment and prevention.

In a recent study, Sharp et al. (2001) examined the effects of *N*-(4-hydroxyphenyl) retinamide (4-HPR), a synthetic retinoid, on cell proliferation, and on the expression of intermediate filament proteins, cytokeratin 18 and vimentin, on three immortalized, but non-tumorigenic (RWPE-1, WPE1-7 and WPE1-10), and one human prostate cancer cell line (DU-145). Sharp et al. (2001) demonstrated that 4-HPR caused a significant decrease in the expression of vimentin as well as an increase in cytokeratin 18 expression in all cell lines. A dose-dependent inhibition of growth was also seen in all cell lines. This study is very significant in that it shows 4-HPR, a synthetic retinoid, induces differentiation by increasing cytokeratin expression and decreasing vimentin expression in pre-neoplastic cells (RWPE-1, WPE1-7, WPE1-10) as well as in a malignant cell line (DU-145). In a similar experiment (Webber et al., 1999b), it was shown that treatment with 4-HPR causes a dose-dependent decrease in vimentin expression in DU-145 cells (Figure 6-7). Webber et al. (1999b) also demonstrated that *all-trans* retinoic acid (ATRA), another retinoid, caused growth inhibition of DU-145 cells in a dose dependent manner.

Retinoids have also been shown to play a role in the differentiation of stem cells into cardiomyocytes (Wobus et al., 1997), and in the differentiation of normal bronchial epithelial cells (Moghal and Neel, 1998), neuroblastoma cells (Ferrari et al., 1998), several ovarian cancer cell lines (Zhang et al., 2000), and leukemic blast cells (Manfredini et al., 1999). Interestingly, ATRA has been shown to block the pro-differentiating effects of calcitriol in normal human keratinocytes (Gibson et al., 1998). This blocking effect could possibly be due to: 1) a competition between the vitamin D

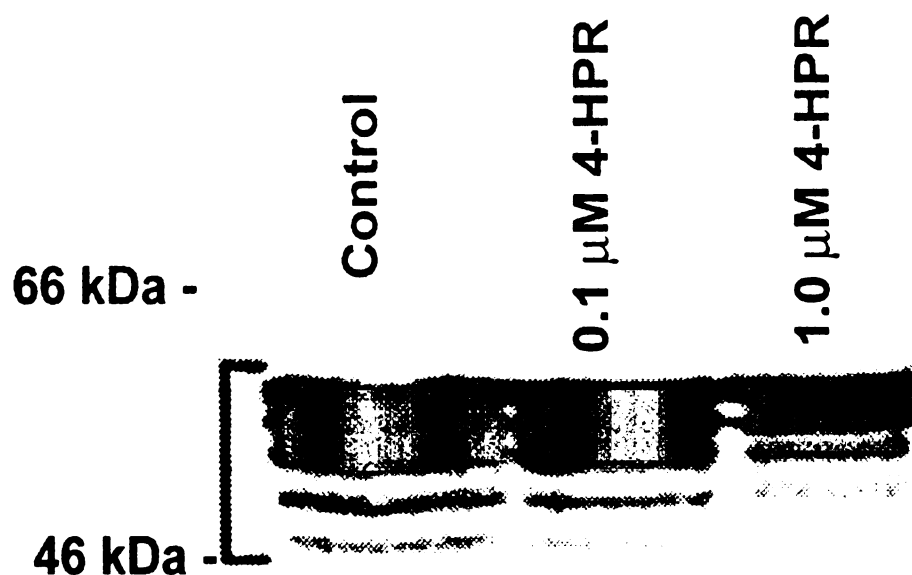


Figure 6-7: Treatment with 4-HPR causes a marked decrease in vimentin expression in DU-145 cells. Western blot analysis shows a decrease in vimentin (V) expression by 4-HPR in a dose-dependent manner (modified from Webber et al., 1999b).

receptor (VDR) and the retinoic acid receptor (RAR) for heterodimerization with retinoid X receptor (RXR); 2) RAR binding to VDR thereby blocking the ability of VDR to heterodimerize with RXR; 3) the inhibition by retinoic acid of calcitriol binding to the VDR; or 4) interactions via other transcription factors.

Retinoids are also able to induce the expression of TGF- β . TGF- β is an important negative regulator of epithelial cell proliferation in that it inhibits epithelial cell proliferation and induces differentiation. Abnormalities in response to this growth factor are a major step in the process of carcinogenesis. Several other growth factors are involved in modulating cell differentiation.

Modulation of Cell Differentiation by Growth Factors

Growth factors are potent mediators of cellular proliferation, differentiation, and cell death. Growth factors have been shown to be negative or positive regulators of growth and function of several different types of cells. Polypeptide growth factors, including epidermal growth factor (EGF), transforming growth factor (TGF) α and β , fibroblast (FGF), and insulin-like growth factors (IGF), have been extensively studied in the prostate gland. Polypeptide growth factors are generally locally produced and exert their effects in either an autocrine or paracrine manner.

TGF- β inhibits growth of epithelial cells in the normal prostate, BPH, and probably early phases of prostatic carcinoma. The ability of TGF- β to antagonize the mitogenic effects of other, positive growth factors (Sporn et al., 1986) could be one mechanism responsible for this growth inhibition. The finding that the TGF- β receptor is under negative androgen control is also consistent with the role of TGF- β as a growth-inhibiting factor in normal prostates (Culig et al., 1996). Conversely, TGF- β stimulates tumor growth in advanced stages of prostate cancer. Prostate cancer cells are able to activate latent TGF- β precursors, suggesting an autocrine stimulatory TGF- β loop in advanced stages of prostate cancer. TGF- β in normal prostate tissue is localized in the stroma, while it is homogeneously distributed in poorly differentiated tumors, indicating aggressive tumor cells may acquire the ability to produce their own TGF- β (Culig et al., 1996). Both of these factors probably play a role in the TGF- β -induced growth stimulation of advanced prostate cancer.

Several fibroblast growth factors have been identified in the prostate. They are mainly expressed by stromal cells and stimulate epithelial cell growth. Basic fibroblast

growth factor (bFGF), which is one of the main growth factors in BPH, also acts in an autocrine manner in hormone-independent prostate cancer (Culig et al., 1996). This observation shows how the growth factors mentioned above are able to regulate the growth and differentiation of the prostate. **Figure 6-8** depicts the actions of these growth factors on the stroma and epithelium of normal, hyperplastic, and cancerous prostate.

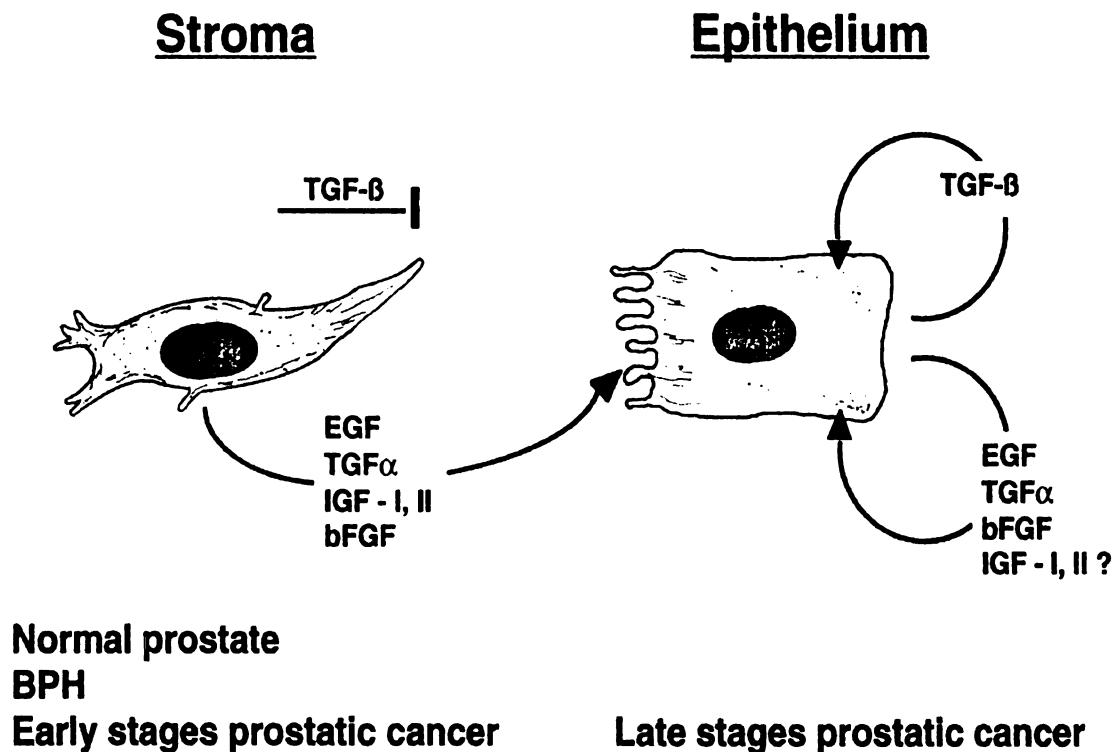


Figure 6-8: Mode of action of polypeptide growth factors in the normal, hyperplastic, and carcinomatous prostate showing the autocrine and paracrine manners of secretion of different growth factor at different stages of prostate cancer (Culig et al., 1996).

Development of the prostate is androgen-dependent, and is in part regulated by the inductive influence of the mesenchyme. Keratinocyte growth factor (KGF) is produced by fibroblasts and mesenchymal cells, has a high degree of specificity for epithelial cells, and promotes epidermal proliferation. During male urogenital development, androgens appear to act via mesenchymal androgen receptors (AR) to elicit synthesis and secretion of KGF which regulates prostatic epithelial growth and morphogenesis (Cuhna, 1996).

Growth factors have also been shown to play a role in the differentiation of several cell types other than prostate cells. While EGF induced the differentiation of mouse mammary epithelial cells (Taverna et al., 1991), in bronchial epithelial cells differentiation begins only upon the inhibition of EGF (Moghal and Neel, 1998). This suggests that the differentiating effects of certain growth factors may be cell-type specific. Interestingly, in the study by Moghal and Neel (1998), it was also demonstrated that inhibiting the proliferative effects of EGF was critical for retinoids to induce target genes needed to induce differentiation of the bronchial epithelial cells. This suggests that part of the mechanism of lung carcinogenesis, in which overexpression of EGF receptors is frequent, may involve antagonism of the differentiation-promoting activity of retinoids (Moghal and Neel, 1998). Other growth factors, such as, IGF-I and IGF-II have been implicated in the induction of differentiation in lung (Maitre et al., 1995) and intestinal epithelial cells (Jehle et al., 1998).

Modulation of Cell Differentiation by Extracellular Matrix

Certain components of the extracellular matrix (ECM) can also modulate the differentiation of certain cell types. The ECM is essential in the structural and functional (secretion of tissue specific proteins) differentiation of epithelial acini. An alteration of the ECM or any of its individual components could play a critical role in epithelial cell differentiation. Bronchial epithelial cells undergo mucosecretory differentiation only when grown on collagen gels (Moghal and Neel, 1998). Matrigel, a re-constituted basement membrane, has been extensively used to study the epithelial cell interactions with the basement membrane. The progression of prostate cancer is accompanied by the loss of normal epithelial architecture and the breakdown of the basement membrane. Webber et al. (1997) demonstrated that non-tumorigenic RWPE-1 cells undergo acinar differentiation as a result of the interaction with the ECM (Matrigel). Conversely, the malignant RWPE-2 and DU-145 prostate cancer cell lines have lost the ability to form acini when grown in Matrigel. PSA is an important, androgen-dependent marker of differentiated function of prostatic epithelial cells. When exposed to the synthetic androgen mibolerone the acini, formed by RWPE-1 cells cultured in Matrigel, synthesize and secrete high levels of prostate specific antigen (PSA) (Webber et al., 1997). The secretion of PSA mimics that of normal prostatic acini *in vivo*. Similar studies with breast and prostate epithelia have also shown the formation of acini that secrete proteins specific for functional differentiation (Barcellos-Hoff et al., 1989; Lang et al., 2001). These results show the effects of the ECM (Matrigel) on the structural and functional differentiation of prostate and breast epithelia. The crucial role of ECM and its

components in normal cell differentiation implies that its alteration may have serious consequences in malignancies and other diseases.

Modulation of Cell Differentiation by Hormones

Hormones can also play a significant role in the differentiation of certain tissues. A novel example of this is in the prostate. Androgens play a dual role in the development of this gland. They first direct the proliferation for growth of the prostate and then maintain the differentiated state of the prostate cells in the mature organ. They are essential for the homeostasis in normal prostate development and maintenance. Evidence of this is seen upon chemical or surgical castration, which results in a rapid onset of epithelial apoptosis in the prostate gland. Treatment with androgen has been shown to induce differentiation in several prostate cell lines (Webber et al., 1996; Webber et al., 1997; Ling et al., 2001; Whitacre et al., 2002).

Conversely, androgen stimulates proliferation in early-stage prostate cancer. This is proven in studies using finasteride, a 5-alpha-reductase inhibitor. The enzyme 5-alpha reductase converts testosterone to dihydrotestosterone (DHT), the major androgen involved in prostate growth. The administration of finasteride causes a reduction or prevention of prostate cancer growth. Finasteride administration is, therefore, one effective androgen-deprivation therapy used to treat prostate cancer. However, a fraction of prostate cancer cells escapes androgen-deprivation therapy. As a result, these cells can continue to grow and inevitably form tumors. These androgen-resistant cells have a way of avoiding or inhibiting the signaling mechanism used in the androgen-suppressed apoptotic pathway that is intrinsic to normal prostate epithelial cells. The growth and normal function of the prostate is dependent on DHT. The activity of DHT is mediated

by the androgen receptor (AR). Since prostate cancer cells express AR and advanced tumors are insensitive to androgen treatment, it is believed that an alteration (i.e. mutation) in AR structure and/or expression is the main contributing factor to androgen-resistant prostate cancer.

Abnormalities of Differentiation in Cancer

An important characteristic in cancer cells is that they generally fail, or have lost the ability to, differentiate into specialized cell types. When normal cells have progressed through a certain number of population doublings they simply stop proliferating, a process termed replicative cell senescence. Since most differentiated cells either no longer divide or divide slowly, the defective differentiation of cancer cells is closely linked to their uncontrolled proliferation indicating that they have escaped the restraints of cell senescence. Instead of progressing to a fully differentiated state, cancer cells continue to grow and divide due to arrest at an early stage of the differentiation process. The origins of a cancer can usually be traced to cell division of a single cell that has undergone some heritable change which allows it to outgrow its neighbors. This cell, and its progeny, no longer differentiate but can continue to proliferate at a more rapid pace than normal cells.

The relationship between cancer and abnormal differentiation can be well demonstrated in leukemia cells (**Figure 6-9**). All of the different kinds of blood cells are derived from a common pluripotent stem cell in the bone marrow. Some of these descendents become red blood cells and others become different types of white blood

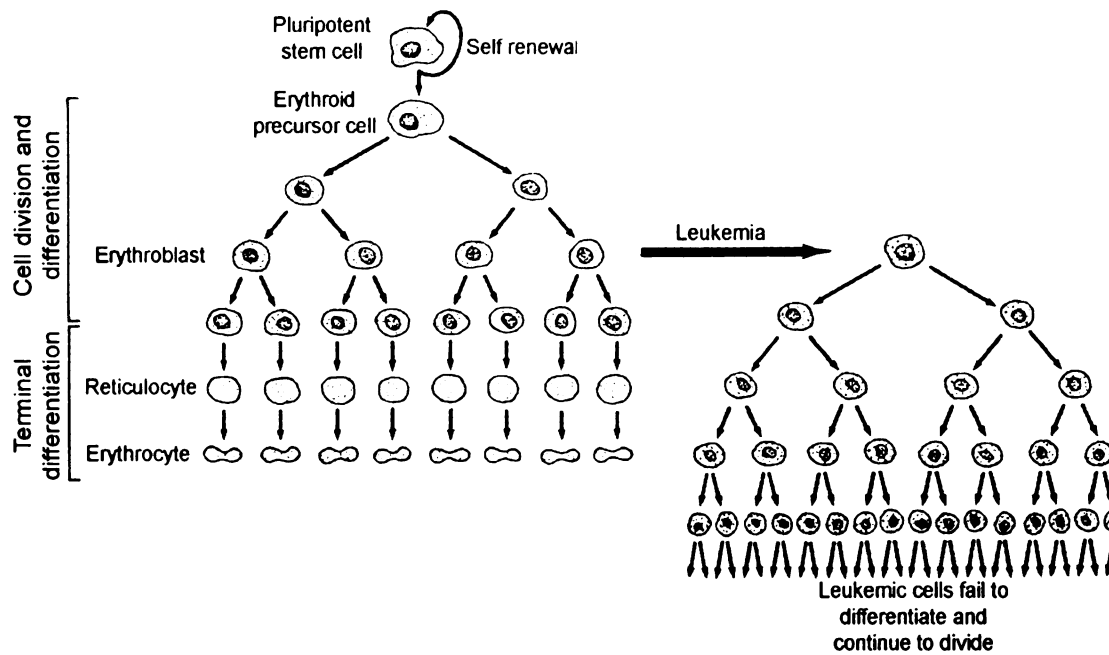


Figure 6-9: Defective cell differentiation in erythroleukemia. Erythrocytes are derived from a pluripotent stem cell in the bone marrow. Erythroblasts undergo several rounds of cell division as they differentiate, but cell division stops at terminal stages (erythrocytes). In contrast, the differentiation of leukemic cells is blocked at an early stage at which they continue to proliferate (modified from Cooper, 1992).

cells. Cell division stops after these cells go through several rounds of differentiation and become terminally differentiated. In contrast, leukemic cells become blocked at an early stage of differentiation, fail to differentiate normally, and undergo continued proliferation.

Apoptosis, or programmed cell death, is an integral part of the process of cell differentiation. Since many kinds of terminally differentiated cells survive for only a short time, stem cells are constantly dividing and replacing them in order to maintain homeostasis. Cancer cells generally fail to undergo apoptosis, and instead continue to proliferate indefinitely. When cancer cells gain this ability, they are said to have become immortalized.

Cell Cycle Checkpoints and Differentiation

Changes that block the normal maturation of cells toward a non-dividing, terminally differentiated state or block apoptosis play a crucial role in many cancers. The most obvious way a cell gains the ability to avoid normal differentiation and/or apoptosis is through mutations in key genes resulting in blocking one or both of these processes. The tumor suppressor genes, p53 and pRb, are perhaps the two genes most involved in these processes.

The p53 Tumor Suppressor Gene

The tumor suppressor gene, p53, has a fundamental role in growth control and neoplasia and may be the most important gene in human cancer. It is not required for normal development, but a lack, or mutation, of p53 usually leads to cancer (Alberts et al., 2002). This gene is mutated in approximately 50% of all cancers and is regarded as one of the most mutated genes associated with cancer development (Almog and Rotter, 1997); it is the most frequently mutated gene in human cancer (Hollstein et al., 1991). p53 stands at a critical point in the cell cycle and in the pathways governing the responses of a cells to DNA damage. It is involved in three very important processes: 1) cell cycle control, 2) apoptosis, and 3) genetic stability.

DNA damage activates p53 by an indirect mechanism. p53 is very unstable and present at low concentrations in undamaged cells. It stimulates the transcription of the p21 cdki (cyclin-dependent kinase inhibitors), thereby helping to block cell entry into S phase. This is because it interacts with another protein, Mdm2, that targets p53 for destruction by proteosomes. The binding to Mdm2 is reduced by the protein kinase mediated phosphorylation of p53. There is a marked increase in p53 in the cell as a result

of the decrease in p53 degradation. The ability of p53 to stimulate p21 transcription is enhanced by the decreased binding of p53 to Mdm2 (**Figure 6-10**). SV40 large T antigen has been shown to bind to and sequester p53, thereby blocking its ability to regulate cell proliferation. As a result, cells transformed with SV40 large T antigen are allowed to progress through the cell cycle, continuing to proliferate even when their DNA has been damaged.

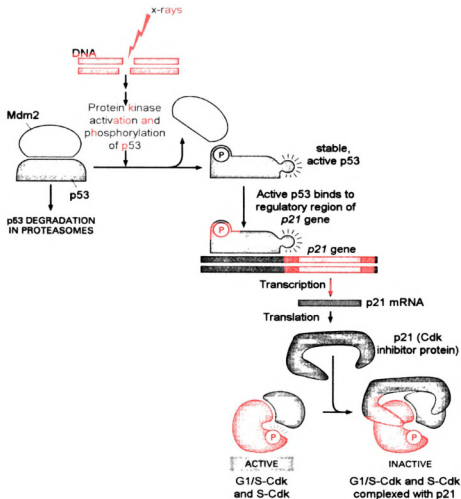


Figure 6-10: How DNA damage arrests the cell cycle in G1. When DNA is damaged, protein kinases that phosphorylate p53 are activated. Mdm2 normally binds to p53 and promotes its ubiquitinylation and destruction in proteasomes. Phosphorylation of p53 blocks it binding to Mdm2; as a result, p53 accumulates to high levels and stimulates transcription p21, thereby arresting the cell in G1 (modified from Alberts et al., 2002).

When normal cells are exposed to factors that cause DNA damage (radiation, oxygen deprivation, etc.), they raise their level of p53 by reducing the normally high degradation rate of the molecule. This either causes the cells to undergo apoptosis because of the DNA damage, or arrests the cells in the cell cycle until the damage can be repaired. Cells defective in p53 tend to escape apoptosis and continue to proliferate, allowing mutant cells to continually go through the cell cycle without becoming terminally differentiated.

The pRb Tumor Suppressor Gene

Another tumor suppressor gene, Rb, works in a way similar to p53. Many cancer cells proliferate inappropriately as a result of loss of Rb function due to a loss or mutations in the Rb gene, or in other genes in the regulatory pathway of Rb (**Figure 6-11**). Normal cells progress through the cell cycle when Rb is phosphorylated by an active protein kinase complex. The p16 protein is produced when cells become stressed, and inhibits the progression through the cell cycle by preventing the formation of the protein kinase complex. Many cancers have a deletion or inactivation of the p16 gene, or it can be silenced by methylation of its regulatory DNA. Some cancers have also been found to have amplified the genes encoding the protein kinase complex. These changes favor cell proliferation, and result in decreases in apoptosis and differentiation.

Important in the discussion of cell differentiation and its association with cancer is the “Chalone Theory of Cancer.” This theory states that “Mitotic control is lost when the chalone concentration falls so low that the distal cells remain mitotic, cell gain then exceeds cell loss and a tumor appears” (Webber, M.M. personal communication; Parkinson and Balmain, 1990). The more differentiated the cells, the more chalone

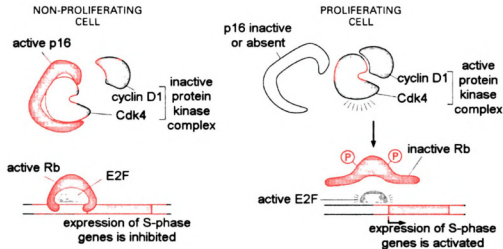


Figure 6-11: The pathway that controls cell cycling via Rb protein. All components of this pathway have been found to be altered by mutation in human cancers. In most cases, only one of the components is altered in any individual tumor. The Rb protein inhibits entry into the cell-division cycle when it is unphosphorylated. The complex of Cdk4 and cyclin D1 phosphorylates Rb, thereby encouraging cell proliferation. When cell is stressed, p16 inhibits the formation of an active Cdk4/cyclin D1 complex, preventing proliferation. Inactivation of Rb or p16 by mutation encourages cell division, thus each can be regarded as a tumor suppressor. Overactivity of Cdk4 or cyclin D1 encourages cell division, thus each can be regarded as a proto-oncogene (modified from Alberts et al., 2002).

(growth inhibitor) there is; the less differentiated the cells, the less the chalone. Chalone levels are low in some tumors when compared to normal tissues.

TGF- β and Cell Differentiation

Transforming growth factor- β (TGF- β) is a good example of a chalone, and it plays a critical role in cell differentiation. TGF- β is a cell-type specific growth factor and it acts as a negative autocrine or paracrine regulator of epithelial cell proliferation. It inhibits epithelial cell proliferation and its synthesis increases with differentiation in epithelial cells. Loss of growth inhibition through TGF- β -mediated pathways contributes to the formation of several types of cancer. The negative feedback control of TGF- β to

cause cells to differentiate is somehow blocked in cancer cells, possibly by mutations in its receptor or by the inactivation of Smads, which are key intracellular signal transducers in the TGF- β pathway (Roberts and Wakefield, 2003). TGF- β is a good example of how growth factors can also play a critical role in cell differentiation and cancer formation.

The Role of Vitamin D in Cell Proliferation

Cell proliferation, producing many cells from one, is a process fundamental to growth, development, and neoplasia. The processes by which calcitriol inhibits cell proliferation is an active area of study. Effects of vitamin D on changes in cell cycle progression and cell death have been investigated. To date, studies suggest that calcitriol modulates the proliferation of several different cell types through a number of mechanisms, including modulation of genes involved in cell cycle regulation, alterations in growth factors, and regulation of hormones and their receptors, for example, androgen in the prostate gland.

Effects of Vitamin D on the Cell Cycle

The concept of the cell cycle (**Figure 6-12**) is central to cell proliferation. Calcitriol-induced antiproliferative action is mediated predominantly through a G1/S phase block of the cell cycle. There is a decrease in the number of cells in the S phase and an accumulation of cells in the G1 phase due to calcitriol modulation of several cell cycle regulatory genes and reductions or increases in the kinase activities of cyclin-dependent kinases (cdks) (Zhuang and Burnstein, 1998; Ylikomi et al., 2002; Krishnan et al., 2003).

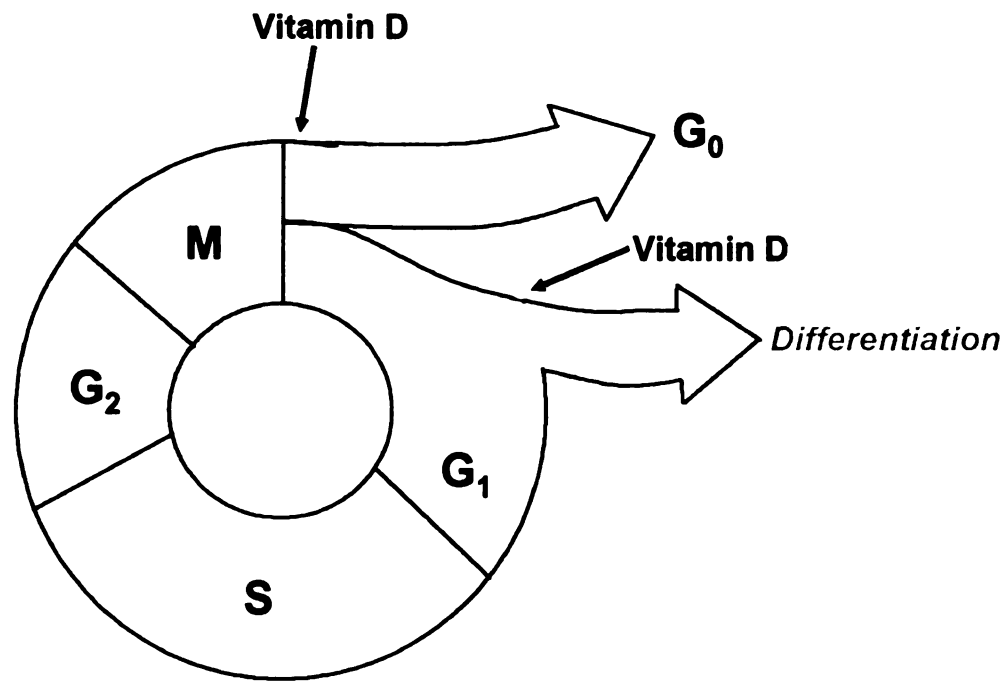


Figure 6-12: Diagram of the cell cycle showing points of antiproliferative and differentiating actions of vitamin D.

Treatment with calcitriol caused cells to accumulate in the G₁ phase of the cell cycle in leukemia cells (Zhang et al., 1995), some breast cancer cells (Simboli-Campbell et al., 1997), and LNCaP prostate cancer cells (Zhuang and Burnstein, 1998). Even though both are growth inhibited upon calcitriol treatment, neither the ALVA-31 cells nor the PC3 cells accumulate in the G₁ phase of the cell cycle (Zhuang and Burnstein, 1998), suggesting that calcitriol-mediated growth inhibition of these cells acts through alternative pathways. Calcitriol has also been shown to inhibit the proliferation of several other cell types (Bernardi et al., 2002; Consolini et al., 2001). The effects of calcitriol on some of these cell types will be discussed in further detail below.

Although not known for certain, several mechanisms have been proposed for vitamin D to induce cells to accumulate in the G₁ phase of the cell cycle. Much of the

data suggest a role for the Rb protein, which inhibits cell cycle progression from the G1 to S phase. During G1, Rb binds to and sequesters E2F, a gene regulatory protein critical in cell proliferation. This binding blocks the transcription of S phase genes. When the cells are stimulated to divide, Rb gets phosphorylated and thereby becomes inactive. Rb then dissociates from E2F and the cell progresses into S phase. The phosphorylation of Rb is regulated by cyclin-dependent kinases (cdk) and by their inhibitors, cyclin-dependent kinase inhibitors (cdki). Cyclins activate the kinases and are, therefore, the primary regulators cdk activity. Cyclin-dependent kinase inhibitors are primary inhibitors of kinase activity. This transcriptional control system includes the following feedback loops that sharpen the G1/S transition (**Figure 6-13**): 1) the liberated E2F increases the transcription of its own gene, 2) Rb phosphorylation and promotion of further E2F release is the result of E2F-dependent transcription of G1/S-cyclin and S-cyclin genes that lead to increased G1/S-cdk and S-cdk activities.

The DU-145 cell line, which does not have a functional Rb, and some prostate epithelial cells immortalized with SV40 large T antigen, which blocks Rb function, are not growth inhibited by calcitriol (Blutt and Weigel, 1999; Gross et al., 1996). However, the PWR-1E cell line, which was immortalized with SV40 large T antigen, does show growth inhibition when treated with cholecalciferol, otherwise known as vitamin D₃ (Tokar, 2004, unpublished data). Other vitamin D metabolites, including 25OHD₃, have been shown to decrease proliferation of colonic epithelial cells (Holt et al., 2002) and primary prostatic epithelial cells (Barreto et al., 2000). These data suggest that other factors, working alone or synergistically with Rb, may play a role in regulating the proliferation of immortalized, non-tumorigenic prostate epithelial cells and prostate

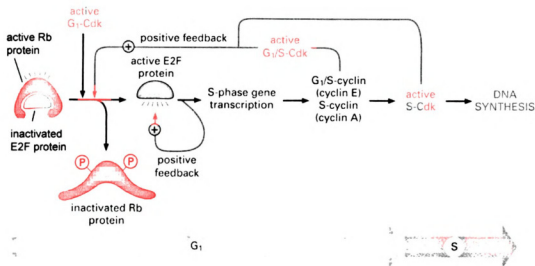


Figure 6-13: Mechanisms controlling S-phase initiation. G1-cdk activity initiates Rb phosphorylation. This inactivates Rb, freeing E2F to activate the transcription of S-phase genes, including the genes for a G1/S-cyclin and S-cyclin. The resulting appearance of G1/S-cdk and S-cdk activities further enhances Rb phosphorylation, forming a positive feedback loop. E2F acts back to stimulate the transcription of its own gene, forming another positive feedback loop (Alberts et al., 2002).

cancer cells. They also suggest that the effects of different metabolites of vitamin D may be cell type specific, which may be due to the levels and/or activity of certain vitamin D conversion enzymes in the different cell types. Block in cell cycle progression at the G2/M checkpoint in HL60 human leukemia cells, caused by calcitriol, has also been demonstrated (Godyn et al., 1994) but there are few other experiments conducted to examine the effects of vitamin D at this checkpoint in the cell cycle.

The blocking of cell cycle progression at the G1/S checkpoint is known to be associated with the alteration of protein levels or kinase activity of different cdks. A decrease in cdk2 activity in LNCaP cells and a decrease cdk2 and cdk6 activities has been shown in HL60 cells (Wang, et al., 1997; Zhuang and Burnstein, 1998). Treatment

of MCF-7 breast cancer cells with calcitriol caused a deregulation of cdk2 and cdk4 within 24 hours of initial treatment. After 7 days of treatment, growth inhibition of the MCF-7 cells was approximately 10% of control (Jensen et al., 2001). It was proposed that this growth inhibition of the MCF-7 cells may be due, in part, to an increase in the expression of p21, a cyclin-dependent kinase inhibitor (cdki). Since a vitamin D receptor (VDR) response element has been identified in the p21 promoter, this increase of the p21 transcript is probably a VDR-dependent mechanism (Jensen et al., 2001).

Cdk inhibitors (cdki) act as negative regulators of cell growth by causing cells to be arrested in G1 and withdraw from the cell cycle. Several of these genes have been found to be calcitriol-regulated (Freedman, 1999). One of the key regulatory proteins in the cell cycle, p27, has been shown to inhibit Rb phosphorylation and arrest cells in G1 by suppressing the activity of several cyclin/cdk complexes (Liu et al., 2002; Wang et al., 1996). Calcitriol has been shown to up-regulate p27 in several human cancer cell lines. Another cdki, p21, is also up-regulated by calcitriol in many human cancer cells (Wu et al., 1997; Seol et al., 2000). The increased activity of this cdki also leads to accumulation of cells in the G1 phase of the cell cycle. The *c-myc* proto-oncogene is a cell-cycle-related protein that is regulated by calcitriol. It has been shown to enhance cdk activity via inactivation of p21 and p27 (Ylikomi et al., 2002). There is also evidence to suggest that the effect of calcitriol on p21 may be secondary to an effect on transforming growth factor β 1 (TGF- β 1) (Osborne and Hutchinson, 2002).

Polek et al. (2003) examined the effects of p53 on the calcitriol-induced cell cycle arrest of three different prostate cancer cell lines, PC-3, LNCaP, and LN-56, which was derived from LNCaP cells. PC-3 and LN-56 cell lines lack a functional p53 gene. Upon

treatment with calcitriol, p53 is required for arrest of LNCaP cells in the G₀, and not in the G₁ phase of the cell cycle. It was also demonstrated that, even though calcitriol treatment resulted in growth inhibition of PC-3 and LN-56 cells, the cell cycle arrest was much shorter than that seen in LNCaP cells and it was not due to the p53 tumor suppressor gene. This suggests that the long-lasting effect of calcitriol in the LNCaP cells may result from cells exiting the cell cycle rather than simply being halted, or arrested, in the G₁ phase. This is probably due to the lack of a functional p53 in the PC-3 and LN-56 cell lines. To my knowledge, a substantial amount of work has not been done to examine the effects of vitamin D treatment on the p53 tumor suppressor gene or its ability to regulate cell proliferation. Additional work will undoubtedly elucidate the mechanisms of action of vitamin D on the p53 gene and its control of the cell cycle. A summary of the factors, in the cell cycle, regulated by vitamin D is shown in **Figure 6-14**.

Effects of Vitamin D on Growth Factors

Growth factors and hormones are important factors in regulating growth in normal cells. The expression and activity of these proteins are often disturbed in cancer cells, which lead to the further promotion of the malignant process. Normal growth factor receptors are activated by growth factor binding, upon which they signal the cell to divide. However, growth factor receptors of some cancer cells are active even in the absence of growth factor binding. This allows cancer cells to continuously signal cells to proliferate.

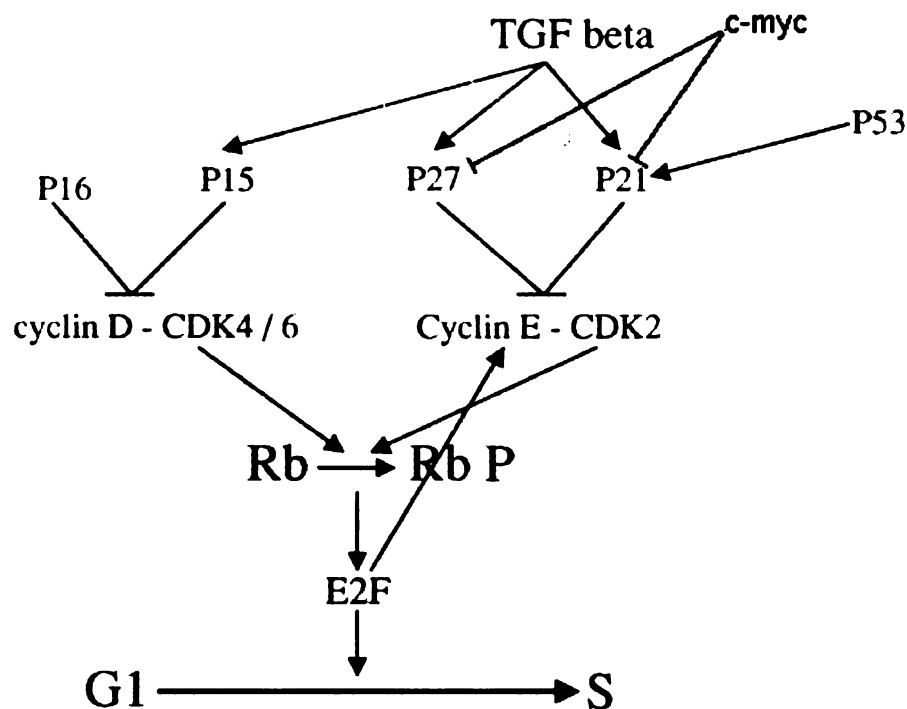


Figure 6-14: The G1/S check point of the cell cycle and the effect of calcitriol. Calcitriol regulates several proteins such as cyclins, cyclin-dependent kinases (cdks), and cyclin-dependent kinase inhibitors (cdki), that are involved in the the G1/S transition, especially in the phosphorylation (P) of retinoblastoma (Rb). Arrows show activation or upregulation. Blunt-ended lines represent inhibition or downregulation (Osborne and Hutchinson, 2002).

Growth factors, associated with increases in cell proliferation include epidermal growth factor (EGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), interleukins (Ylikomi et al., 2002), and keratinocyte growth factor (KGF), have been shown to be inhibited by calcitriol (Feldman et al., 2000). On the other hand, calcitriol has been shown to induce TGF- β in some cells. Transforming growth factor- β s (TGF- β) are a highly conserved 25 kDa dimers whose activities include regulation of extracellular matrix synthesis and cellular proliferation, enhancing proliferation at times and inhibiting

it at others, as well as modulating numerous activities of cells that relate to cell differentiation (Sporn and Roberts, 1990). The growth of prostatic epithelial cells is inhibited by TGF- β s both *in vitro* and *in vivo* (Danielpour, 1996). TGF- β exists in at least five different isoforms, TGF- β s 1,2,3 (found in mammals) and 4 and 5 (found in birds and amphibians, respectively) (Danielpour, 1996). Each of the TGF- β isoforms either upregulates or downregulates the expression of the other isoforms (Sporn and Roberts, 1990). TGF- β is a potent inhibitor of epithelial cell proliferation. An increase in TGF- β 1 expression was accompanied by growth inhibition of breast cancer cells treated with calcitriol (Ylikomi et al., 2002). It is believed that calcitriol exerts its effects on these growth factors by regulating mRNA levels of their respective binding proteins (Agarwal et al., 1999; Sprenger et al., 2001).

Effects of Vitamin D on Androgen and Estrogen Action

There is a substantial evidence to suggest a potential relationship between androgens and response to calcitriol. Androgens, such as testosterone and 5 α -dihydrotestosterone (DHT), act by binding to the androgen receptor. The androgen receptor mediates the action of these hormones. Because of the importance of androgens in prostate cancer, it is important to elucidate any interactions between androgens and calcitriol in normal and malignant prostate cells. Calcitriol has been shown to inhibit prostate cancer cell growth by both androgen-dependent and androgen-independent mechanisms. Zhao et al. (2000) examined the interactions of calcitriol and DHT on three human prostate cancer cells lines, LNCaP and MDA PCa 2a and MDA PCa 2b. The growth inhibitory effect of calcitriol was blocked by Casodex, a pure anti-androgen, in

the LNCaP cells. However, Casodex did not block the antiproliferative effects of calcitriol in either of the MDA cell lines. The differences could be due to the fact that the MDA cell lines have low affinity androgen receptors (ARs) due to mutations, in contrast to the LNCaP cell line. These data suggest that calcitriol acts to inhibit prostate cancer cell growth by androgen-dependent mechanisms and androgen-independent mechanisms in the LNCaP and MDA cell lines, respectively.

Calcitriol has also been shown to inhibit the growth of breast cancer cells through a modulation of the estrogen receptor (ER). In breast cancer cells, estrogen stimulates proliferation through a receptor mediated pathway. Treatment of MCF-7 cells, a estrogen positive breast cancer cell line, with calcitriol caused a dose-dependent decrease in ER in both protein and mRNA levels. The method by which calcitriol down-regulates the growth of MCF-7 breast cancer cells may be mediated through a down-regulation of the ER. This ER down-regulation would, in turn, decrease the responsiveness of the breast cancer cells to the growth stimulatory effects of estrogen.

The Role of Vitamin D in Differentiation

After the discovery of the vitamin D receptor (VDR) in tissues other than the intestine, major focus on the study of vitamin D centered on its effects on cell growth and differentiation (Halloran, 1992). It is unclear if vitamin D-induced cell differentiation is a consequence of a block of cell cycle progression or vice versa, and whether or not it is even possible to distinguish between the two processes. Studies on most cell lines support the idea that the two processes work together, with inhibition of cell growth being accompanied by stimulation of cell differentiation. However, there have been a

few studies to suggest that the two processes are uncoupled (Ransen et al., 2001). It is difficult to measure differentiation in *in vitro* experiments, but alterations in the expression of specific molecular, biochemical, and morphological markers are an indication that cells have reached a mature phenotype. Good examples of such markers are prostate specific antigen (PSA) in prostate cells and CD11b, CD14, and CD18 surface antigens in leukemia cells.

Prostate specific antigen (PSA) is often used as a differentiation marker because its secretion is characteristic of mature prostate cancer cells. Treatment of LNCaP prostate cancer cells with calcitriol resulted in an increase in the synthesis of PSA, which suggests that calcitriol may cause differentiation in prostate cells (Blutt and Weigel, 1999). Treatment of human leukemia cells with calcitriol caused a significant upregulation of several markers that indicate differentiation of these cells (Ransen et al., 2001). Vitamin D treatment has also been shown to cause differentiation in several other types of cancer cells, including breast, colon, and human squamous carcinoma cells (Ransen et al., 2001). These data demonstrate that vitamin D plays an important role in inducing cell differentiation in several cancer cell types. This has led to the examination of the role of vitamin D on the differentiation of normal cell types. The rest of this paper will discuss the effects of vitamin D on the differentiation of normal cell types.

Prostate Epithelial Cell Differentiation

Much attention is given to the prostate due to growth disorders that occur in this gland in men. Due to its dependence on a continuous supply of androgens, the normal prostate has been a frequently studied model for androgen-related gene expression and growth. The prostate requires androgens for development, growth, differentiation, and

maintenance of secretory function (Davies and Eaton, 1991). There may be several cell types in the normal prostate showing different responses to androgen, including cells that require androgens for survival; cells that can survive without androgen but grow more rapidly in its presence; cells indirectly dependent on androgen; and cells totally independent of androgen (Davies and Eaton, 1991). Several growth factors such as EGF, FGF, and TGF- β are also involved in the normal and abnormal growth of the prostate.

Vitamin D has been shown to have a regulatory effect on the prostate. Schwartz and Hulka (1990) first identified a potential relationship between prostate cancer and calcitriol, a finding that was further developed when a connection was found between ultraviolet light and prostate cancer mortality in the United States (Hanchette and Schwartz, 1992). Normal prostate epithelial and stromal cells in culture as well as prostate cell lines have been found to express vitamin D receptors (VDR) (Miller et al., 1992; Skowronski et al., 1993; Peehl et al., 1994a). These findings are important for the following reasons: 1) VDR must be expressed in target tissues in order for vitamin D to exert its effects, and 2) vitamin D can induce differentiation of prostate cells, which could possibly inhibit the formation of prostate cancer.

Increasing data suggest a role for vitamin D in prostate cancer prevention and treatment, but little is known about the role of the hormone in normal cells. Because vitamin D may play a role in the etiology of prostate cancer, Krill et al. (1999) designed a study to test the effects of calcitriol on normal primary prostate epithelial and stromal cells in the presence and absence of dihydrotestosterone (DHT), the active form of androgen in the prostate, in a chemically defined medium. Treatment of epithelial cells in culture with DHT alone did not affect cell proliferation. Treatment of epithelial cells

in culture with calcitriol alone reduced cell proliferation to 60% of controls; this inhibition was maintained in the presence of DHT. These results differ from those of Zhao et al. (1997) who saw a synergistic reduction in proliferation of LNCaP cells upon the combination treatment with calcitriol and DHT. The difference in results could be due to the presence of serum factors in the Zhao et al. (1997) study. Treatment of stromal cells with calcitriol had the opposite effect, these cells showed increased proliferation (Krill et al., 1999). This effect may be due to the use of chemically defined medium. The presence or absence of growth factors in the base media have been shown to affect the composition of the stromal cell population. Growth factors have also been correlated with VDR abundance and cell proliferation (Krishnan and Feldman, 1991). VDR expression and cell proliferation were stimulated in the presence of growth factors and serum was reported to exert a maximal effect since it contains a mixture of growth factors.

Castrated Sprague-Dawley rats were treated with calcitriol in the presence or absence of flutamide, an androgen receptor blocker, to test the effects of calcitriol on prostate growth *in vivo*. The prostatic weight of these rats increased with the administration of calcitriol and in the presence of calcitriol plus flutamide. This increase in weight was due to an increase in stromal cell numbers in the prostate. This report by Krill et al. (1999) shows that calcitriol has differential effects on prostate stromal and epithelial cells that do not appear to be androgen receptor-mediated.

A similar study was conducted by Konety et al. (1996) where both castrated and uncastrated Sprague-Dawley rats were treated with calcitriol, testosterone, or a combination of both hormones. A greater degree of cellular differentiation was observed

in rats that received treatment with both hormones compared to rats that received testosterone treatment alone. These results show that concomitant treatment with calcitriol and testosterone may play an important role in the growth and differentiation of the normal rat prostate. Because similar effects are not seen in the seminal vesicle, another sex accessory tissue, Konety et al., (1996) believe that the prodifferentiating effects of vitamin D may be restricted to the prostate. Future studies need to be performed to examine the effects of calcitriol treatment on androgen receptors.

While there is a large amount of information of the effects of calcitriol on normal and malignant prostate cells, few studies have been performed to test the effects of the hormone on the developing prostate. Administration of calcitriol *in utero* can influence prostate differentiation throughout the life of the animal (Konety et al., 1999). By focusing on the early stages, neonatal and prepubertal, of prostate development, Konety et al. (2000) examined the effects of calcitriol on the normal prostate *in vitro* and *in vivo*. The effects of calcitriol on growth of 267B-1, a normal human neonatal prostatic epithelial cell line, were tested in the presence and absence of DHT. Their results indicate that the administration of calcitriol just before puberty does not have a significant effect on prostatic growth in the presence of DHT, and has an inhibitory effect on neonatal epithelial cell growth in the absence and presence of DHT.

Several growth factors play a role in the effects of vitamin D on cell differentiation. Danielpour (1996) studied the role of TGF- β on the biological activities of all-trans-retinoic acid (ATRA) or calcitriol in NRP-152 cell line derived from a rat prostate. This study shows that ATRA or calcitriol both induced TGF- β s 2 and 3. These results show that the TGF- β s play a major role as local mediators of the effects of ATRA

and calcitriol on differentiation of prostatic epithelial cells. These studies were carried out on rat prostate cell models, therefore, it is important to explore similar effects on human prostate cells in order to have a reliable cell system that would mimic *in vivo* studies in humans.

Keratinocyte Differentiation

The observation made by Hosomi et al. (1983), proving that keratinocyte differentiation is induced by calcitriol, provided an explanation for the presence of VDR receptors in the skin. Calcitriol is produced from 25OHD₃ in cultured human keratinocytes, which correlates with early events in differentiation, such as transglutaminase activity. The transglutaminase enzyme is responsible for the cross-linking of proteins in the cornified envelope and the precursor proteins, involucrin and loricrin.. Calcitriol-induced terminal differentiation of cultured human keratinocytes is also reduced by insulin and enhanced by epidermal growth factor (EGF) (Chen et al., 1995). It is possible that EGF-specific protein tyrosine kinase catalyzes the phosphorylation of VDR, resulting in a more stable and/or more active VDR. This would explain the enhanced antiproliferative effect of calcitriol in the presence of EGF. Phosphorylation of VDR has been suggested as a primary event in calcitriol actions (Brown and DeLuca, 1990).

The pro-differentiating actions of calcitriol are antagonistically altered by ATRA in human keratinocytes (Gibson et al., 1998). Calcitriol and ATRA both had antiproliferative effects on normal human keratinocytes. However, when ATRA and calcitriol were added together, their antiproliferative actions were partially reversed (Gibson et al., 1998). Potential explanations for this effect are: 1) the competition

between VDR and RAR for RXR heterodimerization or 2) inhibition by ATRA of calcitriol binding to VDR.

Conversely, differentiation of keratinocytes can regulate calcitriol production (Bikle and Pillai, 1993). With adequate amounts of calcium, keratinocytes can go from a cell that is rapidly proliferating to one that is capable of making cornified envelopes. The cornified envelopes, one of the most distinctive features of epidermal terminal differentiation, are formed from the cross-linking of involucrin and loricrin by transglutaminase. Successive increases in cornified envelopes, involucrin, and transglutaminase correspond to an increase in differentiation. Two main enzymes that regulate calcitriol production, 25OHD₃-1-hydroxylase and 25OHD₃-24-hydroxylase, both found mainly in the kidney, also change with differentiation (Bikle and Pillai, 1993). This process consists of the following cascade of events: 1) a rise in the 25OHD₃-1-hydroxylase activity is followed by a rise in transglutaminase activity, 2) cornified envelopes appear as the 1-hydroxylase and transglutaminase activities fall, and 3) the appearance of cornified envelopes and fall in 1-hydroxylase activity corresponds with a rise in 24-hydroxylase activity. This 24-hydroxylase activity blocks the formation of the calcitriol metabolite.

Several studies have been performed to test the effects of vitamin D and its metabolites on keratinocyte differentiation. Itin et al. (1994) examined the effects of calcitriol on the differentiation of human keratinocytes grown in serum-free or defined culture medium. Their results showed an enhancement of differentiation when cells were grown in the presence of high calcium and treated with calcitriol at doses higher than

physiological concentrations (10^{-9} M). Doses lower than 10^{-9} M enhanced the proliferation rate of the keratinocytes.

The production of calcitriol and its receptor-binding affinity in human keratinocytes varies based on their level in differentiation. Pillai et al. (1988) demonstrated that pre-confluent and confluent cells contained higher numbers of VDRs than post-confluent cells and that the dissociation constant of these receptors was not consistently altered by differentiation. The number of receptors appears to fall as the cells differentiate. Since calcitriol is a potent stimulator of differentiation in the epidermis, these data suggest the possibility that endogenous calcitriol production may participate at early stages of keratinocyte differentiation in culture and *in vivo*. These prodifferentiating actions of calcitriol have led to the treatment of the hyperproliferative skin disease, psoriasis, as well as its use in the prevention and treatment of several cancer types.

Bone Cell Differentiation

The skeletal system in the body provides structural and mechanical support, protection of vital organs, and serves as a reservoir for calcium and phosphate ions (Lian et al., 1999). Vitamin D plays a key role in the homeostasis of calcium and phosphorus in the blood. During periods of reduced serum calcium levels, calcium transport is stimulated across the gut and into the bloodstream and calcium is mobilized from the bone by vitamin D acting on several cell types found in the bone. Osteoblasts (**Figure 6-15A**), bone-forming cells, and osteoclasts (**Figure 6-15B**), or cells that resorb bone, are both induced by vitamin D to undergo differentiation (**Figure 6-16**) by

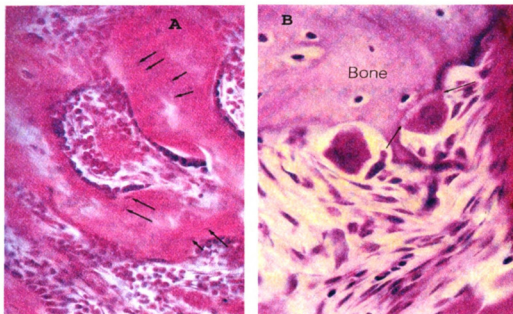


Figure 6-15: (A) Photomicrograph showing osteoblasts, the larger cells on the surface of the bone tissue. (B) Photomicrograph of bone tissue showing two osteoclasts, the large cells in the center of the figure (Ross et al., 1995a).

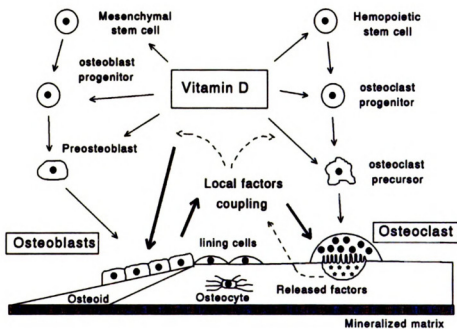


Figure 6-16: Schematic representation of the role of vitamin D in development of the bone cell types and regulation of bone remodeling (Lian et al., 1999).

modulation of the transcriptional activities of several genes related to bone cell phenotype.

Several studies have shown an interaction between vitamin D-induced bone cell differentiation and TGF- β . Since TGF- β signals through the Smad family of intracellular transducing proteins, Smads play an important role in this interaction. Smads are a family of transcriptional co-modulators that interact with several different DNA-binding proteins to regulate their activity in response to TGF- β (Wrana, 2000). Smads are divided into three classes: 1) the receptor-regulated Smads (which interact with TGF- β), 2) the common Smads, and 3) inhibitory Smads, each of which has a distinct function (Wrana, 2000). A recent study showed that in order for VDR and Smad3 to mediate cross-talk between calcitriol and TGF- β both proteins had to bind their respective DNA recognition elements.

Along with TGF- β , bone morphogenetic proteins (BMPs), are potent regulators of osteoblast differentiation, a crucial process in bone remodeling (Eichner et al., 2002). Both TGF- β 1 and BMP7 have been shown to modulate, in opposite ways, the differentiation capabilities of calcitriol on osteoblasts. TGF- β 1 had a stimulating and BMP7 had a inhibitory effect on calcitriol-induced osteoblast differentiation. This study is a good example of cross-talk between different signaling pathways and emphasizes the need for evaluation of the compatibility of TGF- β 1-, BMP7-, and calcitriol-based therapies (Eichner et al., 2002).

Very little is known about how differentiation of bone marrow stromal cells is regulated by hormones and growth factors. It is believed that these cells play a major role in bone formation as a source of osteoprogenitor cells (Liu et al., 1999). Liu et al.

(1999) examined the effects of concomitant treatment of secondary cultures of human bone marrow stromal cells with TGF- β and calcitriol. They observed a synergistically enhanced expression of alkaline phosphatase, a useful marker of active bone formation. No synergistic effect was seen with combinations of calcitriol and other growth factors, such as, BMP-2, insulin like growth factor-I (IGF-I), or basic fibroblast growth factor. This suggests that this action is specific for TGF- β . The treatment of bone marrow stromal cells with calcitriol and TGF- β had an inhibitory effect on cell proliferation. These data suggest that the concomitant treatment of human bone marrow stromal cells with TGF- β and calcitriol enhances the differentiation of these cells to the osteoblast lineage.

There is also evidence to suggest that hormones have different effects on osteoblasts based on their stage of phenotype development (Pockwinse et al., 1995). Fetal rat calvaria-derived osteoblasts in *in situ* hybridization experiments demonstrated the selective influences of calcitriol and glucocorticoids on subsets of cells that result from osteoblast differentiation. Based on the upregulation of osteopontin and osteocalcin, two genes that are highly upregulated during bone mineralization (Pockwinse et al., 1995), these hormonal effects are clearly osteoblast development stage-specific.

Cartilage is a form of connective tissue that is made up of chondrocytes and a specialized extracellular matrix, which is produced and maintained by the chondrocytes (**Figure 6-17**). Chondrocytes that are active in matrix production secrete collagen, glycosaminoglycans, and proteoglycans (Ross et al., 1995b). The terminal differentiation

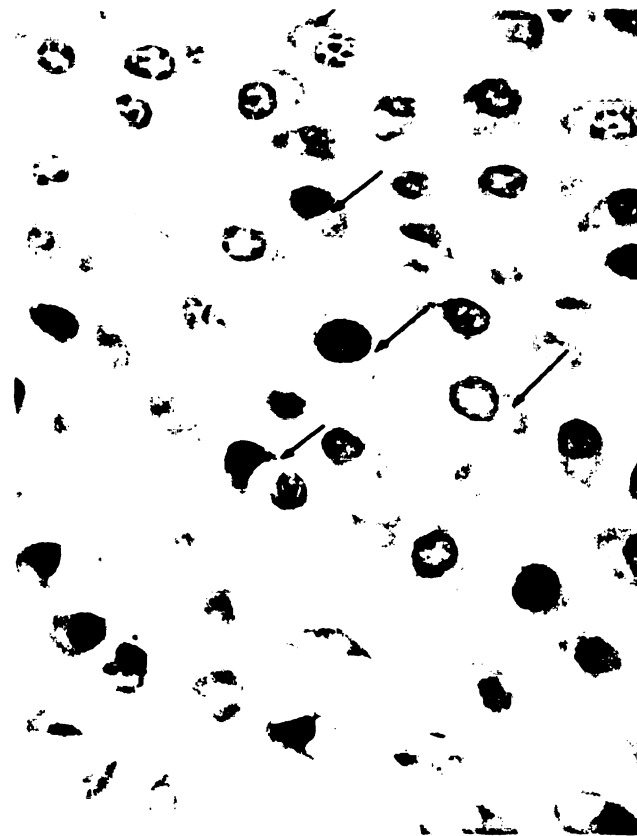


Figure 6-17: Photomicrograph of young, growing cartilage. Cells are chondrocytes. Arrows represent areas of Golgi apparatus. H&E staining. (Ross et al., 1995b).

of chondrocytes that were treated with ascorbic acid was recently examined. Ascorbic acid was shown to promote chondrocyte matrix production and upregulate VDR numbers in proliferating and hypertrophic chondrocytes *in vitro*. An elevation in plasma calcitriol levels in chicks fed an ascorbic acid-supplemented diet was also observed (Farquharson et al., 1998). Similarly, Cantore and colleagues (1991) demonstrated that dietary supplementation with ascorbic acid resulted in elevated plasma calcitriol levels in humans. Ascorbic acid is central to hydroxylation reactions in several biosynthetic pathways, including carnitine and norepinephrine biosynthesis (Levine, 1986) and may also enhance hydroxylation reactions in vitamin D synthesis (Sergeev et al., 1990). This

could be an explanation for the observed elevations in plasma calcitriol. These results show that ascorbic acid plays a role in chondrocyte matrix production and in the metabolism of vitamin D and the upregulation of its receptor. Both of these observations are important because they both can lead to chondrocyte terminal differentiation.

Mammary Gland Differentiation

Mammary gland development undergoes considerable ductal elongation and branching during the prepubertal period. Stimulation of ductal elongation and branching are directed by estrogen and progesterone, respectively, which are the major proliferative hormones driving mammary gland development. Calcitriol and its receptor, VDR, have been implicated in cell cycle regulation and differentiation of cultured mammary cells, but little is known about their role in the developing mammary gland (Zinser et al., 2002). These data suggest that the vitamin D₃ signaling pathway plays a role in inhibiting the growth by controlling differentiation and proliferation of epithelial cells of the mammary gland (Narvaez et al., 2001; Zinser et al., 2002).

Hematopoietic Differentiation

The differentiation of macrophage precursor cells, or monocytes, by vitamin D is one of the earliest functional responses linking the vitamin D to the immune system (Hewison and O’Riordan, 1997). Several studies on both mouse and human leukemic and monocytic cell lines have shown that calcitriol treatment stimulated monocytic differentiation towards a more mature, macrophage-like phenotype (Hewison and O’Riordan, 1997). These investigations revealed important aspects of hormonal control of hematopoiesis (**Figure 6-18**). Vitamin D has also been shown to induce the

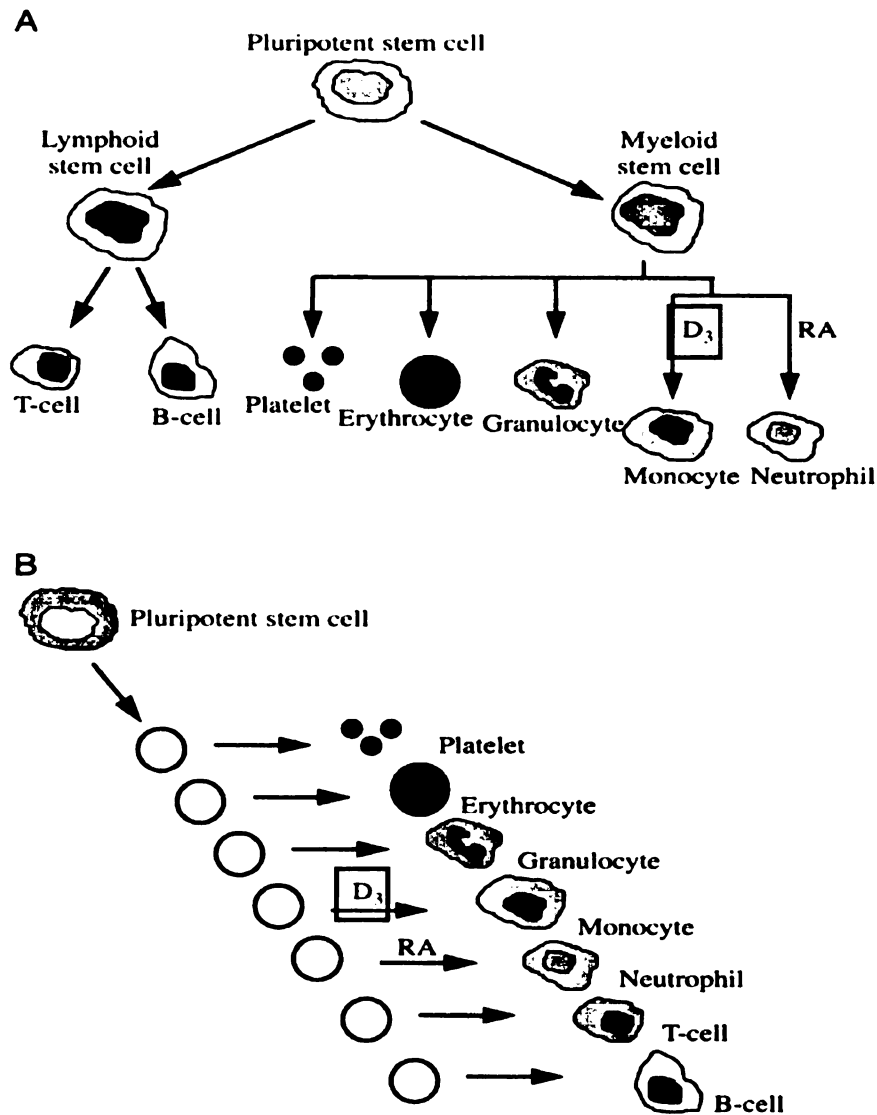


Figure 6-18: Schematic representation of (A) conventional and (B) sequential models of hematopoiesis. The convention model highlights the commitment of stem cells to either lymphoid or myeloid lineage followed by further differentiation to a specific cell type. The sequential model proposes that there is a preferred order to the development of immune cells. Successful maturation toward a particular lineage is dependent on a variety of signals. The influence of calcitriol in differentiation on monocytes is indicated and contrasts with the induction of neutrophils by retinoids. Vitamin D_3 pathways in red boxes (modified from Hewison and O’Riordan, 1997).

differentiation of normal bone marrow cells into monocytes and macrophages (O'Kelly et al., 2002 and Pols et al., 1990).

Differentiation of Other Cell Types

Dental morphogenesis and formation involves a sequence of cell-cell and cell-matrix interactions that is very similar to that of epithelio-mesenchymal organ development. These interactions lead to: 1) morphogenesis of teeth; 2) differentiation of ameloblast, odontoblast, and cementoblast lineages; and 3) matrix secretion and mineralization (Berdal, 1997). Progression of dental morphogenesis and differentiation result from sequential and reciprocal communication between cells of the epithelium and mesenchyme. These cells also follow programmed cell proliferation and differentiation. Vitamin D has also been shown to play a role in the differentiation of the interfollicular epidermis and hair follicles which appear to require the vitamin D receptor (VDR) for normal differentiation. A lack of the VDR results in a reduction of epidermal differentiation (Xie et al., 2002).

Conclusions

Differentiation is the modification of cells that leads to their structural and functional specialization. Differentiation occurs throughout the life of an organism, from embryogenesis to adulthood. Differentiated cell types are found in all animals. Except in very rare cases, once a cell has differentiated it will no longer express additional functions and its transcription patterns are locked down. Since daughter cells contain the same genetic make-up as the parent cell, differentiation is not a result of the loss of genes, but rather from the differential activation and/or suppression of genes.

There are several agents that contribute to the functional modification of the genes in a cell. One that has gained a great deal of interest in recent years is vitamin D. Vitamin D is best known for its role in maintaining calcium and phosphorus homeostasis in the body. But it can also work alone or in concert with other agents, such as, growth factors, cytokines, and components of the extracellular matrix to induce the differentiation of cell types such as prostate cells, keratinocytes, epithelial and epidermal cells, hematopoietic and bone marrow cells, and cells of the mammary glands.

Considerable amount of work is currently in progress to examine the effects of vitamin D on cell differentiation. These and past studies show much promise for the prodifferentiating action of vitamin D and its metabolites in the treatment of hyperproliferative diseases and in the prevention and treatment of cancer. Much more work needs to be done with vitamin D before we can fully understand its mechanisms of action in the process of cell differentiation.

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**CHOLECALCIFEROL (VITAMIN D₃) IN THE PREVENTION AND
TREATMENT OF PROSTATE CANCER: EFFECTS AND MECHANISMS OF
ACTION**

VOLUME II

By

Erik J. Tokar

A DISSERTATION

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Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

2005

Chapter 7

Vitamin D Receptor and Ligand Interactions

Abstract

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Abstract

The vitamin D receptor (VDR) is a member of the steroid hormone nuclear receptor superfamily. Its structure consists of 5 separate regions that play a role in ligand binding, heterodimerization, DNA binding, or transcriptional activation. In order for the VDR to become active it must bind to its ligand, $1,25(\text{OH})_2\text{D}_3$. After it binds to its ligand, the VDR is then able to heterodimerize with the retinoid X receptor (RXR). The heterodimer then binds to DNA and interacts with vitamin D response elements (VDREs) in vitamin D-responsive genes. The heterodimerization process can be blocked by 9-*cis* retinoic acid (9-*cis* RA), a specific retinoid ligand of RXR.

VDR interacts with other members of the nuclear receptor superfamily. There is evidence to suggest that VDR can cross-talk with androgen receptor (AR), a process that is enhanced by 9-*cis* RA. Androgens play a major role in prostate development and growth. Androgen receptor can play a role in growth inhibition of certain prostate cancer cells and also in increasing the levels of prostate specific antigen (PSA) in cells. PSA is the most useful tumor marker for screening and early detection of prostate cancer, which is indicated by rising serum PSA levels. Rising PSA levels are a result of an increase in the total number of cells. $1,25(\text{OH})_2\text{D}_3$ treatment causes an increase in cellular PSA levels, suggesting the ligand may cause certain prostate cells to differentiate thereby inhibiting cellular proliferation and reducing cell numbers, resulting in a decrease in total PSA.

Since its discovery much has been learned about VDR that has helped increase our understanding of this gene, including discoveries concerning vitamin D analogs, ligand binding, changes caused by ligand binding, and its part in the mechanism of

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vitamin D action. But all these new discoveries are just the beginning. Much still needs to be learned before we can fully understand this gene and realize its full potential.

Introduction

The vitamin D receptor is a member of the steroid hormone nuclear receptor superfamily of transcription factors that also includes the thyroid hormone receptors and the retinoic acid receptors (Blutt and Weigel, 1999). Similar to most other members of the nuclear receptor family, VDR is believed to reside mainly in the nucleus prior to activation by $1,25(\text{OH})_2\text{D}_3$, after which VDR binds $1,25(\text{OH})_2\text{D}_3$ with high affinity and high selectivity. A common feature of the nuclear receptors is that they are occupied by small, lipophilic ligands that easily penetrate the plasma membrane and diffuse into the nucleus (Haussler et al., 1997). Although $1,25(\text{OH})_2\text{D}_3$ is derived from cholesterol, vitamin D can also be obtained from the diet, which would better classify it with ligands of nutritional origin like the retinoids. The fact that vitamin D_3 , the precursor to $1,25(\text{OH})_2\text{D}_3$, is also synthesized in the skin by a photosynthetic reaction with ultraviolet light, makes $1,25(\text{OH})_2\text{D}_3$ unique in that it can be obtained through two independent external processes, from the diet and synthesis by the sun.

A second property of the nuclear receptors is their ability to form multimers that facilitate high affinity and specific DNA binding. By binding as dimers to specific hormone response elements, the receptor superfamily is able to control target genes. Based on the interactions between the nuclear receptors and their response elements, the superfamily can be organized into two subfamilies (Haussler et al., 1997a). The first subfamily, the glucocorticoid receptor subfamily, of steroid hormone receptors, which

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includes androgen receptor (AR) and estrogen receptor (ES), homodimerize in response to ligand and bind to inverted repeat elements spaced by 3 base pairs (bp) in DNA. The second subfamily, the VDR subfamily, heterodimerizes with retinoid X receptor (RXR) and binds to direct repeat elements spaced by 1-5 bp (Haussler, 1995). The VDR subfamily includes the thyroid hormone receptor (TR) and retinoic acid receptor (RAR) (Figure 7-1).

It is clear that the VDR, based on the grouping of nuclear receptors into subfamilies, is a member of the heterodimerizing group of nuclear receptors that is occupied by a ligand [1,25(OH)₂D₃] of nutritional origin. It heterodimerizes with RXR in order to bind DNA with high affinity and interacts with a hormone response element, in vitamin D responsive genes, that is a direct repeat spaced by 3 bp (termed DR-3 for direct repeat with three-nucleotide spacer).

The first successful studies to suggest the existence of a receptor to vitamin D were carried out in 1969 by Haussler and Norman (Pike, 1997). These studies demonstrated the preferential uptake of active vitamin into the chromatin fraction of chicken intestines and provided evidence that a specific protein likely mediated the process. Further studies by Brumbaugh and Haussler (1974) and Lawson and Wilson (1974) established the protein nature of the receptor (Pike, 1997). In 1975 Brumbaugh and Haussler first reported the actual existence of a receptor on the chromatin for 1,25(OH)₂D₃ (DeLuca et al., 1990). In their *in vitro* experiments it was discovered that the cytosol-derived vitamin D receptor (VDR) could bind to chromatin fractions in the presence of the hormonal ligand (Pike, 1997).

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Steroid Receptors



GR glucocorticoid
MR Mineralcorticoid
PR Progesterone
AR Androgen
ER Estrogen

RXR Heterodimers



TR α,β
RAR α,β,γ
VDR
PPAR α,β,γ
EcR

thyroid hormone
all-trans RA
1,25-(OH) $_2$ -VD $_3$
elcosinoids
ecdysone

Dimorphic Orphan Receptors



RXR (usp) 9-cis RA
COUP/ARP ?
HNF-4 ?

Monomeric Orphan Receptors



NGFI-B (CEB-1) ?
ELP / SF-1 (Ftz - F1) ?

Figure 7-1: Nuclear receptors can be grouped into four classes. Shown here are the representative receptors for each group (Mangelsdorf et al., 1995).

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The vitamin D receptor has an equilibrium dissociation constant of approximately $1-2 \times 10^{-10}$ M and a molecular weight around 50 kDa (Pike, 1997). Depending on the cell type examined, estimates of receptor abundance range from under 500 to over 25,000 copies of VDR per cell (Pike, 1997). Although this suggests that cells with higher levels of VDR may be more responsive to the $1,25(\text{OH})_2\text{D}_3$ hormone than those with lower levels of VDR, it is important to note that several other factors play a role in the responsiveness of a cell to the hormone. Some of these factors include modulation of VDR activity in a cell- or tissue-specific manner by differential activation events, cellular capacity to internalize and metabolize $1,25(\text{OH})_2\text{D}_3$, the nature, availability, and concentration of numerous partner proteins required for gene activation, and the accessibility and inducibility of specific genes (Pike, 1997).

Vitamin D Receptor

Domains of the VDR

The major functional domains of the VDR participate in nuclear localization, ligand binding, heterodimerization, DNA binding, and transcriptional activation (Haussler et al, 1997a). The functional domain structure of the nuclear receptor superfamily is separated into six parts designated A, B, C, D, and E, F. Segment A/B (**Figure 7-2a**) includes residues on the amino terminal side of the DNA binding domain (DBD). The highly conserved DBD is comprised of segment C. Between segment C and the ligand binding domain lies the hinge region, designated by the letter D. The E/F domain lies on the carboxy-terminal end of the structure and contains the ligand binding domain (LBD) in activated receptors. **Figure 7-2b** shows the mammalian VDR domain

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Figure 7-2: Functional domain structure of the nuclear receptor superfamily.
 (a) Nuclear receptors (NR) are separated into four regions designated A/B, C, D, and E/F. (b) Residue boundaries of corresponding regions within the mammalian VDR (Pike, 1997).

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structure. Compared to the other members of the nuclear receptor family the A/B domain of the VDR is much shorter.

The C region comprises the DBD of the VDR. This is the most conserved domain across all nuclear receptors (McDonnell et al, 1989) and is considered the hallmark of the nuclear receptor family. There are three important subdomains within the DNA binding domains (DBDs) of the nuclear receptors that are required to recognize and bind specific nucleotide sequences of DNA. The first region is referred to as the proximal (P)-box. It is an α -helical region that confers target sequence selectivity for the glucocorticoid (GR) and estrogen receptor (ER) (MacDonald, 1999). The second region, the distal (D)-box, is important in homodimerization of the GR subfamily of receptors (MacDonald, 1999). Residing just C-terminal to the second zinc finger is the region that mediates homodimer and monomer interactions with DNA. This α -helical region is referred to as the T-box. The DBD consists of two similar modules, each comprising a zinc-coordinated finger structure (**Figure 7-3**). The first eight cysteine residues in the DBD coordinate tetrahedrally to form the two zinc fingers, each comprised of four of the cysteine residues. Although these zinc fingers appear to be structurally similar, there are substantial differences in the functions of each of these modules. The function of the amino-terminal module is to direct specific DNA binding in the major groove of the DNA binding site. The carboxy-terminal module serves as a dimerization interface for interaction with a partner protein, such as RXR (Pike, 1997).

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Figure 7.

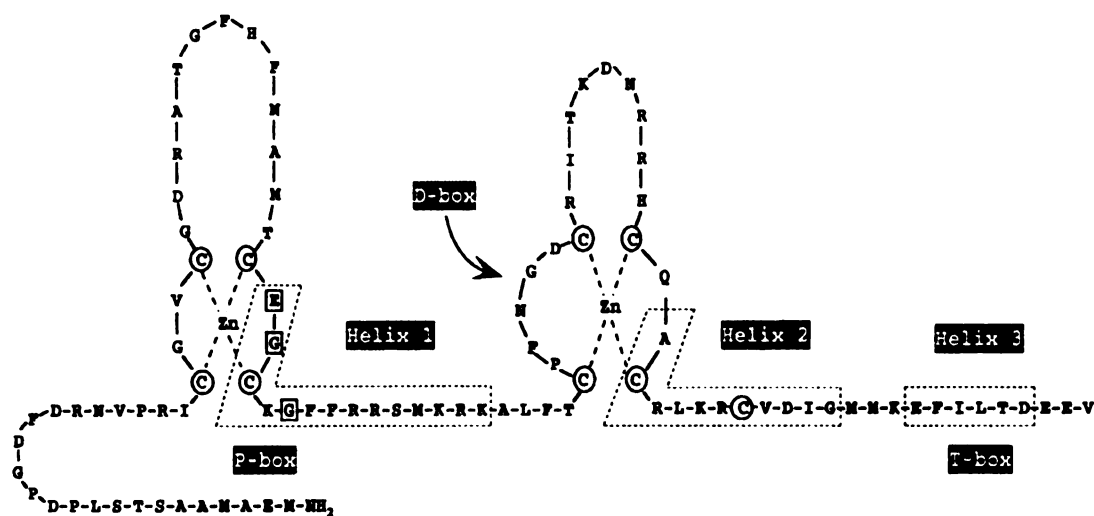


Figure 7-3: The N-terminal, zinc-finger binding motif of the VDR (MacDonald, 1999).

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The D domain of the VDR links the DBD and the E/F domain, also known as the ligand binding domain (LBD). The $1,25(\text{OH})_2\text{D}_3$ binding function of the VDR is located in the E/F domain (McDonnell, 1989). This domain contains several subdomains that exhibit moderate conservation across the entire transcription factor gene family. The E/F domain is responsible for the high-affinity binding of the ligand, $1,25(\text{OH})_2\text{D}_3$. The binding of $1,25(\text{OH})_2\text{D}_3$ is the “switch” that converts the latent transcription factor of the VDR into the active gene regulator. It is hypothesized that the binding of $1,25(\text{OH})_2\text{D}_3$ induces conformational changes in the LBD of the VDR (Pike, 1997).

Localization of VDR

The classic model for steroid hormone action acting only via a nuclear receptor has become somewhat controversial. Recent research has suggested that several important steroid-induced signaling events act independently of gene transcription. These nongenomic biological responses include: 1) estrogen-mediated dilation of blood vessels, 2) estrogen-induced proliferation of breast cell lines, 3) progesterone-induced activation of the acrosomal reaction in sperm (Hammes, 2003). Experiments have been conducted to try to identify the steroid receptors responsible for these nongenomic modulations. Several suggestions have been made, including classical steroid receptors located in the membrane, G-protein coupled receptors, and novel membrane-associated steroid binding proteins (Hammes, 2003).

The nuclear localization of VDR and its role in regulating transcription has been well documented for years. However, increasing evidence has shown calcitriol can exert action on a wide variety of cells via rapid effects on the cell membrane. These effects

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include activation of phospholipase C and phospholipase D, production of diacyl glycerol and inositol triphosphate, activation of protein kinase C and map kinases (Fleet, 1999), activation of voltage-gated ion channels, changes in Ca^{+} ion flux, and alterations in membrane fluidity (Nemere et al., 1998). These effects are specific, which suggests that they are mediated by membrane receptors. Three lines of evidence support the existence of membrane VDR. Utilizing rat cartilage cells and vesicles from these cells Nemere et al. (1998) demonstrated that 1) calcitriol analogs and metabolites that have a very high affinity for the nuclear VDR still stimulate nongenomic actions; 2) rapid effects of calcitriol can be observed in isolated membranes, and 3) rapid effects of calcitriol occur in matrix vesicles. Collectively, these data provide evidence for a membrane VDR that is distinct from the nuclear VDR.

Discovery of the membrane VDR is just the beginning. The gene or genes that encode the membrane VDRs need to be identified in order to determine the hormonal, dietary, and cellular mechanisms regulating this protein and its activity. Determining whether or not the function(s) of the membrane VDR is critical to the function of the nuclear VDR, and determining how its signal is generated are just two more challenges that investigators face.

Although its existence is not fully accepted, there is increasing evidence for the presence of VDR in the cytoplasm. Microscopy in living cells has demonstrated that VDR is distributed between the cytoplasm and the nucleus (Racz and Barsony, 1999; Sunn et al., 2001). Prufer and Barsony (2002) compared VDR partitioning in living and fixed cells and found that the apparent exclusively nuclear localization of VDR is a fixation artifact. In addition, several studies using living cells have shown a calcitriol-

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induced redistribution of VDR from the cytoplasm into the nucleus (Barsony and Prufer, 2002). In a 1997 study, Barsony et al. found a substantial proportion of VDR in the cytoplasm, colocalized in the endoplasmic reticulum, the Golgi complex, and microtubules.

Trafficking of VDR Between the Cytoplasm and Nucleus

For years, the generally accepted model divided nuclear receptors into two categories: 1) receptors that always reside in the nucleus, and 2) receptors that reside in the cytoplasm and translocate into the nucleus only after hormone binding. It is now clear that nuclear receptors are highly mobile. Vitamin D receptor (VDR) constantly shuttles between the cytoplasm and the nucleus. This shuttling is regulated by VDR heterodimerization with RXR; heterodimerization will be discussed in a later section of this chapter. Using fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) experiments, it was demonstrated that VDR and RXR both shuttle across the nuclear membrane with the help of nuclear import and export machinery (Barsony and Prufer, 2002; Prufer and Barsony, 2002). The recovery half-times were 15-30 minutes for the unliganded and 5-15 minutes for the liganded VDR (Prufer and Barsony, 2002).

Nucleocytoplasmic shuttling of nuclear receptors is regulated by several mechanisms, including docking/retention, phosphorylation, and dimerization. Nuclear retention/docking plays an insignificant role and phosphorylation has yet to be explored in the nuclear localization of VDR (Barsony and Prufer, 2002; Prufer and Barsony, 2002).

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Dimerization with RXR has been shown regulate both nuclear import and export of unliganded VDR (Prufer et al., 2000; Prufer and Barsony, 2002). It is well known that ligand binding induces a rapid redistribution of several nuclear receptors, including androgen receptor, from the cytoplasm to the nucleus. Photobleaching and permeabilization experiments showed that liganded VDR accumulated in the nucleus under steady-state conditions (Barsony and Prufer, 2002). Several studies have revealed that VDR and RXR also move rapidly within the nucleus and accumulate in foci upon agonist treatment and that they bind to foci as heterodimers (Prufer et al., 2000; Prufer and Barsony, 2002; and Racz and Barsony, 1999) .

VDR Homodimers

To date, the functional significance of a VDR homodimer is unclear. Studies show that ligand decreases the amount of DNA-bound homodimer and causes dissociation of the homodimer by decreasing the rate of monomer to homodimer formation up to five-fold (Cheskis and Freedman, 1994). These data suggest that calcitriol favors the VDR-RXR heterodimer formation.

Other studies demonstrate conflicting results as to the DNA-binding affinity of VDR homodimers. In vitro experiments suggest the ligand-bound VDR homodimers display lower affinity for DNA response elements than corresponding ligand-induced VDR-RXR heterodimers (Issa et al., 1998). Conversely, using gel mobility shift assays, Kim et al. (1996) demonstrated a VDR homodimer that showed greater affinity for DNA response elements than VDR-RXR heterodimer. To fully understand the functional

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significance of the VDR homodimer, further investigations into homodimeric specific vitamin D responsive genes are required.

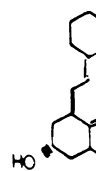
Vitamin D Receptor-Retinoid X Receptor Interaction

In the target cell, a cascade of events that climaxes in alterations in transcription rates of genes is initiated with the binding of the $1,25(\text{OH})_2\text{D}_3$ hormone with the VDR. This binding hyperphosphorylates the VDR (Haussler et al., 1997b), facilitating the recruitment of the RXR into the requisite heterodimeric complex. This heterodimeric complex binds to specific DNA sequences called vitamin D response elements (VDREs) (**Figure 7-4**) in vitamin D-responsive genes and influences the rate of RNA polymerase II mediated transcription (MacDonald, 1999).

Role for the $1,25(\text{OH})_2\text{D}_3$ Ligand

The $1,25(\text{OH})_2\text{D}_3$ ligand (**Figure 7-5**) dramatically enhances the heterodimer formation of the VDR-RXR complex by both the direct interaction of VDR with RXR in solution and the interaction of the VDR-RXR heterodimer with the VDRE (Sone et al, 1991). Sone *et al.* (1991) demonstrated that in the presence of $1,25(\text{OH})_2\text{D}_3$ there was a 9-fold increase in the affinity of the VDR for RXR in the absence of DNA. Surface plasmon resonance techniques confirmed the binding constants for VDR-RXR interactions and showed a clear $1,25(\text{OH})_2\text{D}_3$ -dependent decrease in VDR interaction with itself (homodimer formation) and a concomitant increase in VDR-RXR heterodimerization (Cheskis et al., 1996). This increase in affinity of VDR for RXR

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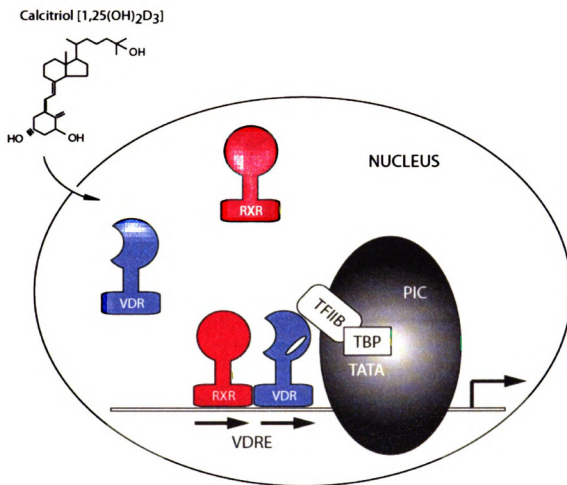


Figure 7-4: Putative molecular communications between VDR and the transcriptional complex (MacDonald, 1999).

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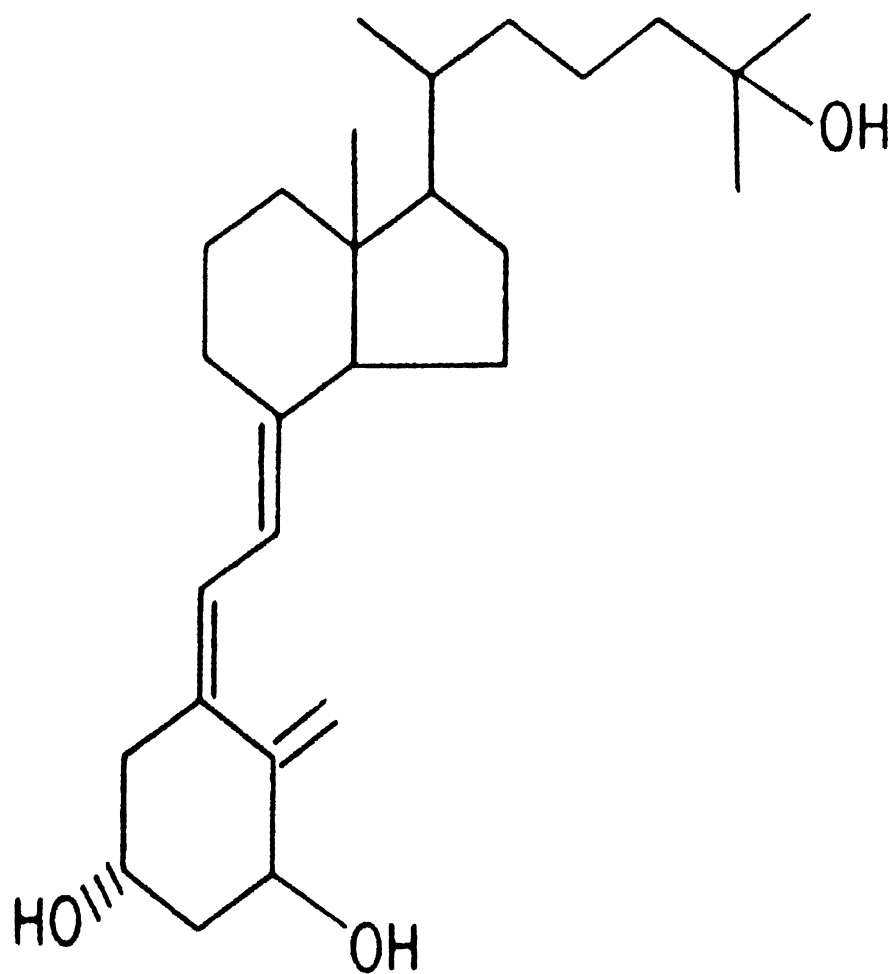


Figure 7-5: Structure of 1,25(OH)₂D₃ (DeLuca, 1997).

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almost certainly reflects a conformational change in the VDR induced by the absence and presence of the $1,25(\text{OH})_2\text{D}_3$ ligand. Therefore, one such role for the $1,25(\text{OH})_2\text{D}_3$ ligand in the VDR-mediated transcription may be to induce a conformational change in VDR that promotes ligand-bound VDR heterodimerization with RXR and disrupts weak homodimers of unliganded VDR (**Figure 7-6**).

Retinoid X Receptors

Retinoid X receptors (RXRs) are nuclear transcription factors that are activated by their retinoid ligand, 9-*cis*-retinoic acid (9-*cis* RA) (Zou et al., 1997). RXRs are thought to form homodimers upon interaction with ligand to activate reputed RXR-selective genes, such as apolipoprotein A1 and cellular retinal-binding protein II through RXR-responsive elements in those genes (Allegretto et al., 1995). RXR is also known to form heterodimers with several other intracellular receptors, including the vitamin D receptor, thyroid hormone receptor (TR), and retinoic acid receptor (RAR). In these interactions, RXR is thought to act as a silent (non-liganded) partner. RXR has also been shown to form homodimers in a complex with DNA upon binding to 9-*cis* RA.

9-*cis* RA acts as a potential inhibitor of $1,25(\text{OH})_2\text{D}_3$ -enhanced VDR-RXR binding to VDREs. Dramatic attenuation of this interaction can occur at concentrations as low as 10^{-7} M (Haussler et al., 1997a). Two possible pathways have been hypothesized to explain the VDR-RXR interaction with VDRE (**Figure 7-7**). In pathway A of **Figure 7-7**, $1,25(\text{OH})_2\text{D}_3$ occupies the monomeric VDR, causing a conformational alteration of the ligand-binding domain. This change in the conformation facilitates the

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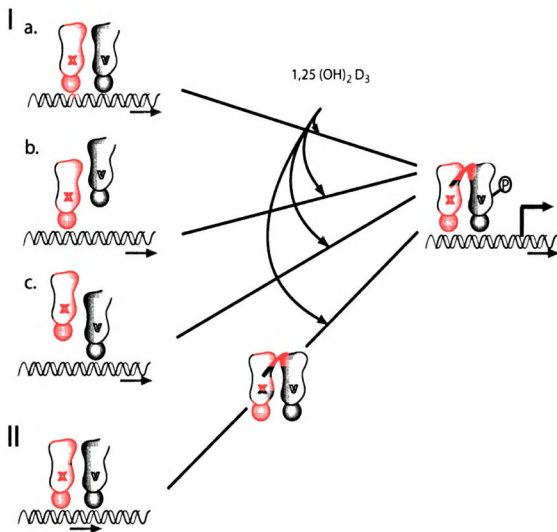


Figure 7-6: Role for $1,25(\text{OH})_2\text{D}_3$ in the formation of functional VDR heterodimer. Monomer of VDR (v) and a partner protein such as RXR (x) are either associated with DNA (I) or free in the nucleus (II) (Pike, 1997).

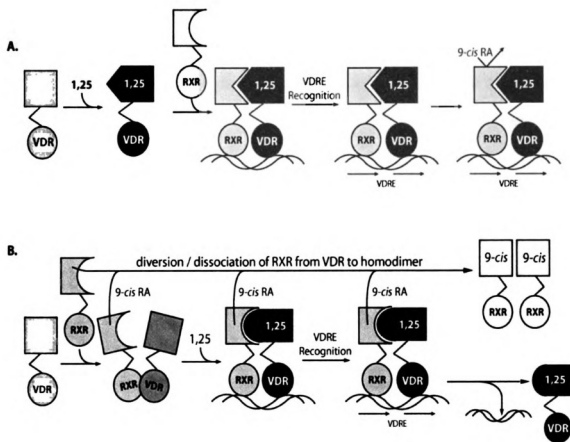


Figure 7-7: Model of two different allosteric pathways for VDR-RXR-1,25(OH)₂D₃ binding to DNA (Haussler et al., 1997b).

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recruitment of RXR for heterodimerization and subsequent VDRE recognition. Haussler et al. (1997a) postulate that VDR conformationally influences RXR in the resulting heterodimer in a way that it is resistant to ligand binding by 9-*cis* RA, thus, preventing 9-*cis* RA from dissociating the RXR-VDR complex in order to divert RXR for retinoid signal transduction. In pathway B of **Figure 7-7** the 9-*cis* RA binding function of the RXR partner has not been suppressed, making this form susceptible to dissociation by 9-*cis* RA, thus favoring the formation of retinoid-occupied RXR homodimers. Therefore, 1,25(OH)₂D₃ must first occupy the VDR monomers in order to prevent 9-*cis* RA from diverting or dissociating RXR blocking the formation of VDR-RXR heterodimer.

VDR-RXR Heterodimerization

Heterodimerization with RXR is essential for VDR action. Because of this, characterization of the regions of the VDR that actually contact the RXR is very important. The ligand binding domain (LBD) of the receptors plays a major role in protein-protein interactions, such as the protein-protein interactions during the heterodimerization of VDR with RXR. There are at least four putative regions in the LBD of VDR that are involved in the mediation of protein-protein contacts with RXR (MacDonald, 1999). The first, a C-terminal heterodimerization domain, is located between 382 and 402 in the VDR sequence (Haussler, 1997a). Complete disruption of the VDR-RXR interaction and elimination of the transcriptional activity of the VDR were observed following mutagenesis in these residues. A second domain that possibly supports RXR heterodimerization resides between residues 318 and 339, corresponding to heptads 9 and 4, respectively (MacDonald, 1999).

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A third region is found in the amino-terminal segment of the LBD and is located between residues 244 and 263, known as E1 (Haussler, 1997a). Point mutations in this region do not alter ligand binding but they do affect the ability of the VDR to heterodimerize with RXR. Located on residues 91 and 92, the fourth region is located in the T-box (see Figure 3) of the VDR (Haussler, 1997a). This region is apparently involved in the mediation of the heterodimerization of the VDR and RXR DNA binding domains (DBDs) on the VDRE scaffold.

Some insight into the structure of the VDR-RXR heterodimer complex can be gained by looking at the crystal structure of the RXR-RXR homodimeric complex. The LBD of the RXR crystal structure consisted of 11 α -helices organized into what was termed a three-layer antiparallel sandwich (MacDonald, 1999). Helix 10 of the RXR dimer, which forms the majority of its interaction surface, corresponds to the C-terminal region of the VDR (Nakajima et al, 1994). This region is crucial for heterodimer formation. The overall structural integrity of the receptor is maintained by the intramolecular contacts with which the other heptad repeats are involved. Therefore, the major interaction surface of the VDR-RXR heterodimer may be comprised of the C-terminal region of the VDR, which includes residues 382-403 (helix 10), making direct contact with helix 10 of the RXR (MacDonald, 1999). This interaction would generate a structurally symmetrical VDR-RXR heterodimer.

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VDR-RXR Heterodimers

The mechanism of VDR binding to VDREs is reflected in the direct repeat nature of the element. Class II receptors of the nuclear receptor superfamily (of which VDR is a member) bind to direct repeat elements as asymmetrical heterodimers (MacDonald, 1999). This asymmetrical heterodimer (RXR in this case) is required for high-affinity binding of VDRs to VDREs.

The asymmetrical direct repeat motif (DR-3) of the VDRE allows the VDR-RXR heterodimer to bind with defined polarity. The 3' half site of the DR-3 element is occupied by VDR and the RXR occupies the 5' half site (MacDonald, 1999). The DNA binding domains (DBDs) of a VDR-RXR heterodimer are arranged in an asymmetrical, head-to-tail assembly with the VDR T-box forming a direct contact with the RXR D-box (MacDonald, 1999). The predominant interaction interface of the C-termini would place the ligand binding domains (LBDs) of VDR and RXR in a symmetrical arrangement when they form the heterodimer (MacDonald, 1999). To explain this difference, Mangelsdorf and Evans (1995) propose that the DBDs of the nuclear receptors have an inherent flexibility. This flexibility, a function of the hinge region of the receptors, permits a high degree of rotational freedom in the DBD that would accommodate such a binding scheme. The next section will discuss how VDR interacts with the androgen receptor, a member of the glucocorticoid subfamily of steroid hormone receptors.

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VDR Interactions with Androgen Receptor

The Androgen Receptor Gene

As previously mentioned, the androgen receptor (AR) belongs to the glucocorticoid receptor subfamily of steroid hormone receptors. These hormone receptors homodimerize in response to ligand and bind to inverted repeat elements spaced by 3 base pairs (bp) in DNA. The gene encoding the AR contains 8 exons and spans approximately 90kb of genomic DNA on the long arm of the X-chromosome (Bentel et al, 1997). The human AR has approximate molecular mass of 110 kDa. Similar to the vitamin D receptor, AR can be divided into four functional domains: 1) an amino terminal transcriptional activation domain; 2) a DNA binding domain (DBD); 3) a hinge region; and 4) a ligand binding domain (LBD) (Evans, 1988). The large amino terminal domain makes up more than half of the AR protein and is required for wild-type activation of the receptor. The DBD contains two zinc binding motifs (zinc fingers) with similar structure and function as those of the vitamin D receptor DBD. Located between the DBD and LBD, the hinge region contains a specific nuclear localization signal (NLS) that is highly conserved among steroid receptors. This NLS directs translocation of the AR protein from the cytoplasm to the nucleus (Bentel et al, 1997). The LBD is responsible for the high-affinity binding of androgens to the AR.

VDR Cross Talk with the AR in Prostate Cancer Cells

A functional AR is necessary for transcriptional regulation of target genes by androgens. Testosterone is metabolized within the prostate to 5 α -dihydrotestosterone (DHT) by the enzyme 5- α reductase. It is now known that it is DHT and not testosterone

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that is the major intracellular androgen that promotes growth within the prostate (Kirby et al, 1996). AR is the key element in the androgen signal transduction cascade and it plays a major role in the regulation of growth and differentiation of the prostate.

LNCaP cells are an androgen-responsive human prostate cancer cell line that express AR and VDR. Using LNCaP cells, Zhao et al., (1999) demonstrated that the cross talk between $1,25(\text{OH})_2\text{D}_3$ and androgens exists and that the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ in these cells are androgen-dependent. It has been shown that 9-*cis* RA acts synergistically with $1,25(\text{OH})_2\text{D}_3$ to inhibit LNCaP cell growth (Blutt et al, 1997). Zhao et al. (1999) found that $1,25(\text{OH})_2\text{D}_3$ up-regulates AR gene expression at both the mRNA and protein levels in the LNCaP cells. 9-*cis* RA, which also up-regulates AR expression in LNCaP cells, enhanced the inductive action of $1,25(\text{OH})_2\text{D}_3$ (Zhao et al., 1999). These data show that growth inhibition induced by $1,25(\text{OH})_2\text{D}_3$ alone or in combination with 9-*cis* RA was accompanied by an increase in AR expression. Also, the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ and 9-*cis* RA were AR-dependent and could be blocked by the AR antagonist, Casodex. These results demonstrate that growth inhibition of LNCaP cells by $1,25(\text{OH})_2\text{D}_3$ and 9-*cis* RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression (Zhao et al, 1999).

It is important to note that the antiproliferative activity of $1,25(\text{OH})_2\text{D}_3$ in LNCaP cells is not the situation in all prostate cancer cells. PC-3, an AR-negative prostate cancer cell line, also demonstrates growth inhibition when exposed to $1,25(\text{OH})_2\text{D}_3$. Zhao et al., (2000) also demonstrated that two new AR-positive prostate cancer cells lines, MDA PCa 2a and MDA PCa 2b, show growth inhibition when exposed to $1,25(\text{OH})_2\text{D}_3$. This suggests that the growth inhibitory effect of $1,25(\text{OH})_2\text{D}_3$ in PC-3 and MDA prostate

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cancer cell lines is AR-independent and mediated by mechanisms other than androgen signaling (Zhao et al., 2000).

Hsieh and Wu (1997) have demonstrated that $1,25(\text{OH})_2\text{D}_3$ increases the expression of AR by facilitating the translocation of AR from the cytosol to the nucleus of LNCaP cells. They suggest that this model would account for the coupled induction of AR and PSA by $1,25(\text{OH})_2\text{D}_3$. Increasing the amount of AR in the nuclear fraction would allow for an efficient and productive interaction with the androgen-response element of PSA gene, thereby increasing transcription of the PSA mRNA leading to an increase in intracellular and secreted PSA (Hsieh and Wu, 1997).

Regulation of Prostate Specific Antigen (PSA) by Vitamin D in Prostate Cells

The androgen receptor regulates the expression of androgen-responsive genes by binding to their androgen-response elements and by interacting with other transcription factors. A notable gene regulated by androgen in the prostate is prostate specific antigen (Crawford et al, 1996). Prostate specific antigen (PSA) (**Figure 7-8**) has emerged as the most useful tumor marker for the screening and early detection of prostate cancer as well as a follow-up tool after therapy. PSA is a 33 kDa glycoprotein that is specific to the prostatic epithelium. It is an important component and one of the most abundant serine proteases in the seminal plasma (Webber et al, 1995).

Differentiated prostate cells secrete PSA. As the total number of cells increases, serum PSA levels increase indicating prostate cancer (Blutt and Weigel, 1999). Cellular

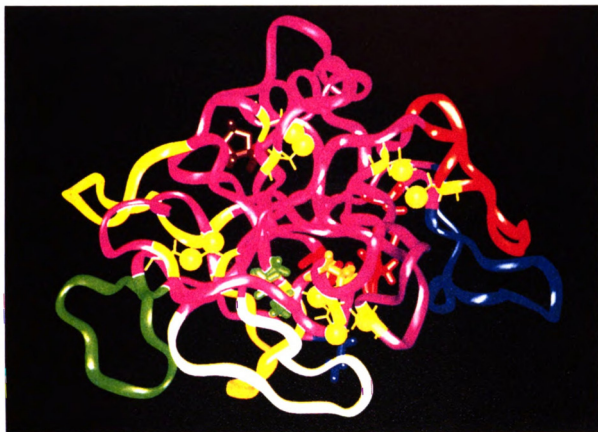


Figure 7-8: Three dimensional model of PSA (Kirby et al., 1996).

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synthesis of PSA increases when prostate cancer cell lines are treated with $1,25(\text{OH})_2\text{D}_3$, suggesting that $1,25(\text{OH})_2\text{D}_3$ may be capable of causing differentiation of prostate cells (Miller et al, 1992; Skowronski et al, 1993). Although this is seemingly inconsistent with the fact that rising serum PSA levels are an indication of prostate cancer, lower levels of PSA will result due to the ability of $1,25(\text{OH})_2\text{D}_3$ to reduce cell number.

Upon treating LNCaP cells with $1,25(\text{OH})_2\text{D}_3$ for 48 hours, Hsieh et al. (1996) observed a two-fold increase in the intracellular and secreted forms of PSA. This increase in PSA levels is most likely due to $1,25(\text{OH})_2\text{D}_3$, which may facilitate the translocation of AR from the cytosol to the nucleus of the cell.

Synthetic vitamin D analogues have also been shown to induce PSA in prostate cancer cells. Hedlund et al. (1997) tested the effects of three vitamin D analogues (Ro 23-7553, Ro 24-5531, and Ro 25-6760) on the secretion of PSA in LNCaP cells. They demonstrated that all three analogues, as well as $1,25(\text{OH})_2\text{D}_3$, induced PSA expression in the LNCaP cells. Since these analogues are less calcemic than the natural hormone $1,25(\text{OH})_2\text{D}_3$, they could possibly be used in place of the hormone to help reduce its hypercalcemic properties.

Mutations in the VDR and AR

Vitamin D Receptor

Genetic polymorphisms have been identified within the VDR gene. The first is located in exon 2 and is represented by a C to T switch in the translation initiation site (Pike, 1997). The presence of a C in this position results in a protein with three additional amino acids at the C-terminus and could well have functional significance.

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Genetic polymorphisms have also been delineated in the introns between exons 7 and 8 as well as the 3' noncoding region of exon 9 (Pike, 1997). These polymorphisms are not located in regions of the gene that might affect structure of the protein, but they appear to be correlated with bone mineral density and it is hypothesized they are predictive for osteoporosis. To date, none of the polymorphisms found are associated with changes in gene function or structure, suggesting it may be an alteration linked to these polymorphisms that alters prostate cancer risk (Blutt and Weigel, 1999).

Racial Differences in Vitamin D Receptor

In 1997, Ingles et al. examined a polymorphism 3' -untranslated region of the VDR in white non-Hispanic men. They designated short alleles (≤ 7 poly-A's) and long alleles (≥ 8 poly A's). The presence of the long allele, whether in the heterozygous or homozygous state, was associated with a 4- to 5-fold increase in prostate cancer risk. In a study done by Taylor et al. (1996), *Taq I* polymorphisms of the VDR were examined in 96 white males and 12 black males, all in the United States. They found that the men who were homozygous or heterozygous for the T allele had a three-fold increase in the risk of developing prostate cancer. Although the low number of black patients does not allow for meaningful analysis of risk, the black patients showed a lower frequency of the tt genotype, which is consistent with higher prostate cancer rates among blacks compared to whites (Taylor et al, 1996). These parallel results of Ingles et al. (1997) and Taylor et al. (1996) greatly strengthen the findings of an association between VDR polymorphisms and prostate cancer risk.

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Ingles et al. (1998) examined the *BsmI*, a restriction length polymorphism, restriction site (B and b alleles) and poly-A microsatellite (L and S alleles) polymorphisms in the VDR gene of African-American men in the United States. The b alleles were associated with a 2-fold decrease in advanced prostate cancer risk, which is in contrast to the 4-fold *increase* in risk previously observed in Caucasians (Ingles et al, 1997). In African-Americans the association was confined to the haplotypes carrying the long (L) allele of the poly-A microsatellite. BL and bL haplotypes showed an increased and decreased risk, respectively, for prostate cancer. Haplotypes carrying the short (S) allele were not associated with prostate cancer risk, regardless of which *BsmI* allele (B or b) they were carrying. Therefore, increased risk of prostate cancer in African-American men seems to be associated with the *BsmI*/poly-A BL haplotype (Ingles et al, 1998).

A study done on Japanese men (Habuchi et al., 2000) examined the association of the *BsmI*, *ApaI*, and *TaqI* polymorphisms of the VDR gene and protection against prostate cancer and benign prostatic hyperplasia (BPH). It was found that the *TaqI* and *ApaI* polymorphisms showed no significant association with either prostate cancer or BPH. However, the results indicate that the *BsmI* polymorphism in the VDR gene plays a significant role in protection against prostate cancer and BPH.

VDR gene polymorphisms may associate with other factors in modulating prostate cancer risk. In a study done on Chinese men, Chokkalingam et al. (2001) demonstrated that the *BsmI* gene polymorphism is not related to prostate cancer, and that the ff genotype of the *FokI* VDR polymorphism may combine with systemic levels of insulin-like growth factor binding protein (IGFBP)-3 and -1 to modulate disease risk. These findings suggest that the insulin-like growth factor (IGF) and the vitamin D

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regulatory systems may interact to affect prostate cancer risk. These findings also bring up the possibility of there being several factors associated with VDR gene polymorphisms that contribute to racial differences in prostate cancer risk.

Androgen Receptor

It is well known that early forms of prostate cancer are androgen-dependent, leading to the belief that the hormone plays an important role in the development of this disease. The androgen receptor (AR) gene has highly variable trinucleotide microsatellite of CAG repeats in exon 1, which results in the incorporation of different numbers of glutamine residues in the N-terminal region of the AR protein. This makes the gene highly polymorphic in human populations. Normal numbers of CAG repeats range between nine and thirty residues and they vary from population to population. There are significant differences in the CAG-repeat polymorphisms between African-American, Caucasian-American, and Asian-American men in the United States (Coetzee and Ross, 1994). African-American men, who have the lowest number of repeats, and Asian-American men, who have the highest number of repeats, have the highest and lowest risk of prostate cancer, respectively. The longer variants of the AR gene have decreased transactivation activity and have been shown to have decreased binding affinity for androgens, whereas the shorter variants show increase responsiveness to androgen and higher transactivation activity (Feldman, 1997). This higher transactivation activity may lead to increased proliferative activity in prostate cancer cells (Ingles et al., 1997). Therefore, this functional difference might be a factor associated with increased risk of prostate cancer.

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Conclusions

Since its discovery in 1975 by Brumbaugh and Haussler (see Pike, 1997), a great deal of insight has been gained into the structure and function of the vitamin D receptor (VDR). VDR is a member of the steroid hormone nuclear receptor superfamily. Its functional domain structure is separated into four regions designated A/B, C, D and E/F. These regions participate in nuclear localization, ligand binding, heterodimerization, DNA binding, and transcriptional activation.

A cascade of events that climaxes in alterations in transcription rates of genes is initiated with the high-affinity, high-selectivity binding of VDR to its ligand, $1,25(\text{OH})_2\text{D}_3$. Once VDR binds to $1,25(\text{OH})_2\text{D}_3$ it undergoes a conformational change that increases its affinity to heterodimerize with retinoid X receptor (RXR). Ligand binding and subsequent heterodimerization play a major role in the subcellular localization of VDR. Heterodimerization is also necessary for the VDR-RXR complex to bind to DNA and interact with a DR-3 hormone response element. Once binding occurs, the 3' half site of the VDRE is occupied by VDR and the RXR occupies the 5' half site. The heterodimerization process can be blocked by 9-*cis* RA, the specific retinoid ligand of RXR. 9-*cis* RA inhibits the VDR-RXR by dissociating RXR from VDR to form an RXR homodimer, which prevents the formation of the VDR-RXR heterodimer.

VDR can also interact with other members of the steroid hormone receptor superfamily. There is evidence to suggest that the androgen receptor (AR) is able to cross talk with VDR in certain prostate epithelial cells. This cross talk can up-regulate the expression of AR gene at both the mRNA and protein level of these cells. This process is enhanced by 9-*cis* RA, which is also able to up-regulate AR. This

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demonstrates that growth inhibition of certain prostate cancer cells by $1,25(\text{OH})_2\text{D}_3$ and 9-*cis* RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. The $1,25(\text{OH})_2\text{D}_3$ can also up-regulate the expression of AR by facilitating the translocation of AR from the cytosol to the nucleus, which also leads to increase in prostate specific antigen (PSA) levels in the cells.

PSA is the most useful tumor marker for the screening and early detection of prostate cancer. Treatment of cells with $1,25(\text{OH})_2\text{D}_3$ increases the cellular synthesis of PSA, which suggests that $1,25(\text{OH})_2\text{D}_3$ may cause certain prostate cancer cells to differentiate. Several studies using $1,25(\text{OH})_2\text{D}_3$ and its analogues have shown this to be true. Other studies have also shown that there are racial differences in the VDR gene which could cause an increase in the risk for prostate cancer.

Much has been learned about the vitamin D receptor in recent years and it is likely that new discoveries will be made that will further increase our understanding of this gene. Discoveries concerning vitamin D analogs and their actions, the nature of the ligand binding site, changes that occur upon ligand binding, the regulation of the VDR at both the transcriptional and post-translational levels, and its contribution to the mechanism of vitamin D action will all contribute to better our understanding of the vitamin D hormone and its receptor. Much has been learned, but much more needs to be understood before we can fully realize the promise that vitamin D and its receptor hold in the treatment and prevention of prostate cancer.

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Part II

Original Research

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Chapter 8

Cholecalciferol (vitamin D₃) Modulates Growth and Invasion, and Up-regulates Nuclear Receptors and 25-hydroxylase (CYP27A1) in Human Prostate Cancer Cells

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Abstract

Prostate cancer is the most common, non-skin, cancer in American men. Epidemiological evidence suggests an inverse relationship between prostate cancer incidence and mortality and serum vitamin D levels. Vitamin D is synthesized in the skin upon exposure to ultraviolet light (UV) and it plays an important role in cell proliferation and differentiation. The objective of this study was to examine the ability of cholecalciferol (vitamin D₃) to inhibit or reverse cellular changes associated with malignant transformation and invasion and to explore its mechanism of action. The RWPE2-W99, human prostate epithelial cell line, which forms slow-growing tumors in nude mice and mimics the majority of the primary human prostate cancers in its behavior, was used for this study. Results show that cholecalciferol, at physiological levels, can: i) inhibit anchorage-dependent and -independent growth; ii) induce differentiation by decreasing vimentin expression and increasing PSA expression; iii) decrease MMP-9 and MMP-2 activity with concomitant decrease in motility and invasion; and iv) exert its effects, by up-regulating nuclear receptors VDR, RXRs, and AR, in a dose-dependent manner. These results show that cholecalciferol has the ability to inhibit changes associated with a malignant phenotype. Furthermore, I demonstrate that human prostate epithelial cells constitutively express 25-hydroxylase protein (CYP27A1), the enzyme known to convert cholecalciferol to 25OHD₃ and that CYP27A1 is up-regulated by cholecalciferol. The presence of 25-hydroxylase in human prostate epithelial cells has not previously been shown. This is the first report of the presence of this enzyme in human prostate epithelial cells. These results demonstrate that human prostate epithelial cells, a target cell type for vitamin D, have the necessary enzymes and rare ability to

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locally convert cholecalciferol to the active hormone calcitriol. A comparison of the effects of the two agents shows that cholecalciferol has effects similar to those of calcitriol on growth, MMP activity, and VDR. Because cholecalciferol can inhibit cellular changes associated with malignant transformation and invasion, I propose that cholecalciferol may be an effective agent for treatment, and for secondary and tertiary prevention of prostate cancer.

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Introduction

Prostate cancer is the most common non-skin cancer and second leading cause of death from cancer in American men. It is expected that approximately 230,000 new cases (33% of all new cancer cases) and 29,500 deaths (10% of all cancer deaths) will occur in 2004 (Cancer Facts and Figures, American Cancer Society, 2004). Despite recent advances in the detection of prostate cancer, its exact cause still remains a mystery. The need for new, effective therapies for the prevention and treatment of prostate cancer is obvious. Because of the 20-30 year latency period before its manifestation, it may be possible to prevent the development of prostate cancer. Prostate cancer incidence rates rise with increasing age, making this disease an even greater problem with the increasing life expectancy. Several factors have been suggested as contributing to prostate cancer risk including age, race, diet, and geography. Interestingly, all of these factors are associated with vitamin D synthesis. Furthermore, there is evidence to suggest a correlation between prostate cancer and vitamin D levels.

First consider the factor of age. Elderly men may receive less exposure to sunlight compared to younger men (Lawson et al., 1979) and their ability to synthesize vitamin D₃ also declines with age (Halloran and Portale, 1997; Holick et al., 1987). Prostate cancer incidence increases with age. A steady rise occurs after the age of 50 but it is rare under the age of 50 (Webber et al., 1988) and 80% of all prostate cancers are found in men over the age of 65 (Schwartz and Hulka, 1990). The second factor is race. African American men have higher incidence of more advanced prostate cancer than their Caucasian American counterparts (Schwartz and Hulka, 1990). Absorption of ultraviolet (UV) light by the skin is essential for vitamin D synthesis. Compared to Caucasian

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American men, African American men have much higher melanin content in their skin, which reduces their efficiency for vitamin D₃ synthesis. African American men tend to have higher levels and greater PSA density than do age-matched white men (Abdalla et al., 1998; Eastham et al., 1998). Considering the strong correlation between elevated PSA serum levels and prostate cancer incidence, this is a good indicator of the possibility that black men may be predisposed to prostate cancer development. Similarly, even with the variation in incidence between specific Asian-American ethnicities, prostate cancer incidence in each of these groups is significantly less than it is in white American men. The incidence rate for Japanese men is 1/20 that of American men (Haenszel and Kurihara, 1968). Genetic polymorphism in the vitamin D receptor (VDR) gene could account for these differences. The effect of VDR polymorphism on prostate cancer risk may be the result of differential activity of the variant VDR alleles in transactivation of vitamin D target genes. Differences in diet could also contribute to these differences. Indigenous Japanese men, who have low clinical prostate cancer rate, have a diet rich in fish and fish oils, which contain high levels of vitamin D. However, Japanese migrants to the U.S., who adopt a Western diet, show a significant increase in prostate cancer incidence (Haenszel and Kurihara, 1968).

Next, consider the factor of geography. Epidemiological studies show a geographic relationship between prostate cancer incidence and mortality rates and UV radiation levels. Ultraviolet radiation is essential for the synthesis of vitamin D in the skin. Ninety to 100% of a human's vitamin D requirement comes from exposure to sunlight (Holick, 2002). White men who live in southern latitudes, where UV radiation levels are higher, have a lesser risk of prostate cancer than those who live in northern

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latitudes. In white men in the United States, there is a strong inverse association between prostate cancer mortality rates and UV radiation levels. Mortality rates are higher in northern tiered states compared to the states in the southern part of the country. Mortality rates are high in North America and northwest Europe but are low in Africa and in Central and South America (Hanchette and Schwartz, 1992; Webber et al., 1988). Native African men have lower prostate cancer rates than African American men (Kovi and Heshmat, 1973). In all these cases, the countries, or parts of countries, located in the southern latitudes show lower prostate cancer incidence and/or mortality rates. All of these geographic locations receive higher levels of UV radiation and, therefore, the people who live in these regions are able to synthesize higher levels of vitamin D₃.

Scandinavian countries (i.e. Finland and Norway) have homogenous populations and are spread over large latitudinal ranges. This makes them good candidates for epidemiological studies involving UV radiation levels. 25-hydroxyvitamin D₃ (25OHD₃) is considered to be the best indicator of vitamin D status in humans. A case-control study of approximately 19,000 middle-aged Finnish men demonstrated that serum levels of 25OHD₃ were inversely related to prostate cancer risk and directly related to age at diagnosis of clinical prostate cancer (Ahonen et al., 2000a). At highest risk were men younger than 52 years who had low 25OHD₃ serum concentration upon entry into the study. It was suggested that the almost disappearance of risk due to low 25OHD₃ levels in older men at entry might be independently due to the decrease in androgen levels during andropause. The appearance of clinical prostate cancer was delayed by almost two years in men with higher levels of 25OHD₃. The men with low 25OHD₃ levels and younger at entry showed an increased risk of non-localized prostate cancer, suggesting

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that vitamin D₃ may be involved in the prevention or inhibition of prostate cancer invasion and metastasis. These results suggest that vitamin D has beneficial effects at all stages (initiation, promotion, and progression) of prostate cancer development. A similar study done in Norway showed a significant variation in case fatality depending on the season of diagnosis. Diagnoses during the seasons with highest levels of vitamin D (summer and fall) were followed by the highest survival rates in prostate, breast, and colon cancers (Robsahm et al., 2004). This would suggest that high levels of vitamin D at the time of diagnosis of these cancers may help improve the prognoses and that vitamin D may be important in preventing the death from these cancers.

African American men have the same incidence of latent, non-invasive prostate cancer as white men. However, the incidence of aggressive, metastasizing cancer is higher in black men than in white men (Breslow et al., 1977). This suggests that vitamin D metabolite levels may be reduced in men with more aggressive prostate cancer. This correlation was substantiated when serum levels of calcitriol were shown to be good predictors of prostate cancer risk (Corder et al., 1993). In men with higher levels of serum calcitriol there was a decrease in prostate cancer risk, a large reduction in the risk for palpable, disseminated, and anaplastic tumors, and fewer anaplastic tumors. These effects were distinct from calcium and phosphate levels and poor kidney function, all of which regulate the levels of calcitriol in the body (Corder et al., 1993). Childhood sunburn, sunbathing score, extent of exposure to UV radiation, and ability of the skin to become pigmented upon UV exposure are all associated with risk for developing prostate cancer. When compared to cases with high UV radiation exposure, low UV exposure resulted in the development of prostate cancer at a younger median age (Bodiwala et al.,

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[1,25(OH)₂D₃]

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suggesting a

Peehl et al., 1990

2003; Luscombe et al., 2001). Although multiple factors for prostate cancer incidence and mortality are likely, each of the factors listed above can be correlated with changes in vitamin D synthesis and vitamin D levels.

Vitamin D₃ is important for normal development, growth, differentiation and maintenance of a healthy skeleton. Vitamin D₃ is best known for its roles in calcium and phosphorus homeostasis and in maintaining skeletal integrity. Synthesis in the skin is the main source of vitamin D in the body but it can also be obtained from the diet (Holick, 1990). Ultraviolet radiation is essential for the *in vivo* synthesis of vitamin D₃. Synthesis begins in the skin with 7-dehydrocholesterol. According to the traditional model for vitamin D₃ synthesis (**Figure 8-1**), upon exposure to ultraviolet (UV) light from the sun, 7-dehydrocholesterol is converted to pre-vitamin D₃. Pre-vitamin D₃ gets converted, through a heat catalyzed reaction, to cholecalciferol [vitamin D₃]. Cholecalciferol is transported to the liver, via the vitamin D binding protein, where it is converted to 25-hydroxyvitamin D₃ [25OHD₃] by the 25-hydroxylase enzyme. 25OHD₃ then gets transported to the kidney where the enzyme 1 α -hydroxylase converts it to calcitriol [1,25(OH)₂D₃]. **Figure 8-2** shows the molecular structures of the vitamin D metabolites and the enzymes involved in the synthesis of calcitriol. Calcitriol is the most biologically active form of vitamin D and it binds to the vitamin D receptor (VDR) in target tissues. VDR then forms heterodimers with retinoid X receptors (RXRs) to exert most of the biological activities of vitamin D. The VDR is expressed in normal prostatic tissue, suggesting a role for vitamin D in the prostate (Gross et al., 1997; Kivineva et al., 1998; Peehl et al., 1994).

7-d

Figure 8


7-dehydrocholesterol

Figure 8-2: A
sy

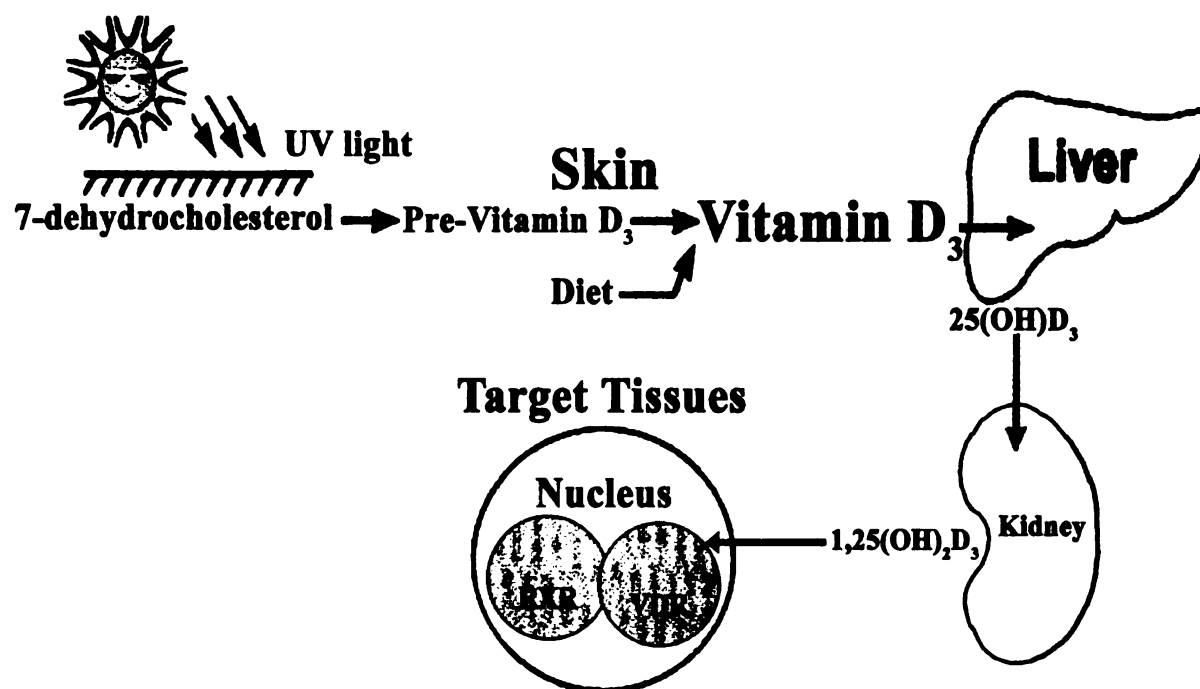


Figure 8-1: Traditional model of the *in vivo* synthesis of vitamin D.

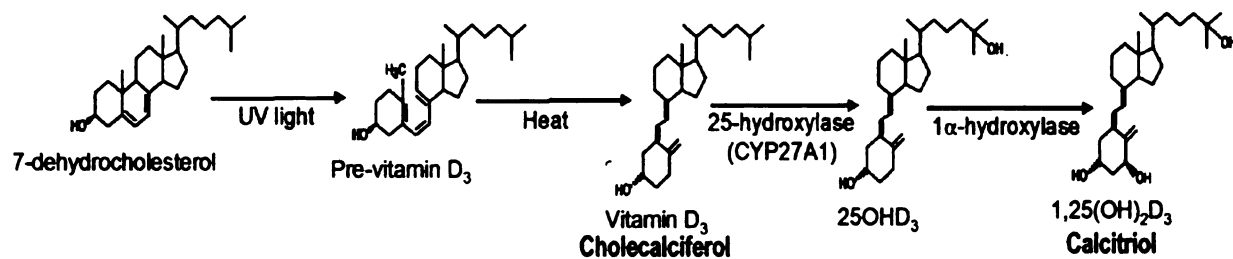


Figure 8-2: Molecular structures of vitamin D metabolites and enzymes involved in the synthesis of calcitriol.

The effects of calcitriol on normal prostate epithelial cells and prostate cancer cells are well documented. Calcitriol has antiproliferative and pro-differentiating effects on normal prostate epithelial cells and prostate cancer cells (Gross et al., 1998; Konety et al., 1996; Krill et al., 1999; Miller et al., 1992; Miller et al., 1995; Peehl et al., 1994; Zhao et al., 2000). Calcitriol also inhibits invasion by prostate cancer cells *in vitro* and *in vivo* (Lokeshwar et al., 1999; Schwartz et al., 1997). Similar effects were seen in several other normal and malignant cell types including breast, endothelial and colon cells, treated with calcitriol (Bernardi et al., 2002; Hansen et al., 1994; Jensen et al., 2001; Shabahang et al., 1993). These data support the use of calcitriol in prostate cancer therapy. However, despite the effectiveness of calcitriol, its clinical use is generally accompanied by elevated serum calcium (hypercalcemia). Therefore, less calcemic alternatives to calcitriol, including natural precursors to calcitriol and vitamin D analogs, are being sought. It has been demonstrated that human prostate epithelial cells possess 1α -hydroxylase activity needed to synthesize calcitriol from 25OHD_3 (Schwartz et al., 1998). Therefore, investigators have used 25OHD_3 , which has a lower calcemic effect, as an alternative to calcitriol (Barreto et al., 2000; Skowronski et al., 1995). 25OHD_3 inhibits the proliferation of primary prostatic epithelial cells in a manner similar to calcitriol (Barreto et al., 2000).

I recently discovered 25-hydroxylase enzyme expression in two human prostatic epithelial cell lines, RWPE-1 and RWPE2-W99. This is the first reported evidence of the presence of this enzyme in any tissue type other than liver, kidney and keratinocytes. The capacity of prostatic epithelial cells to express 25-hydroxylase enzyme suggests that local production of calcitriol can occur in these cells. Local production of calcitriol is

believed to have biological importance so that it may regulate cell growth in a tissue-specific manner. This could potentially decrease the risk of cancer development and progression in tissues possessing 25-hydroxylase and 1α -hydroxylase, two enzymes necessary for the synthesis of calcitriol. This led me to explore the efficacy of cholecalciferol in prostate cancer prevention and treatment, which may be similar to that of 25OHD_3 and calcitriol.

The RWPE2-W99 human prostate cancer cell line, used in this study, was derived in the laboratory of Dr. Mukta M. Webber (Bello et al., 1997; Webber et al., 1997). It belongs to a unique family of cell lines in which each member mimics a different stage involved in the process of prostate carcinogenesis. All members were derived from the parental RWPE-1 human prostate epithelial cells. The RWPE-1 cells, although immortalized, are not tumorigenic and show many characteristics of normal prostatic epithelial cells. However, because they are immortalized, they may also be used as a pre-neoplastic cell model and are ideal for the studies on early stages of prostate carcinogenesis and for investigating new agents for chemoprevention (see chapter 9). On the other hand, the RWPE2-W99 cell line is tumorigenic, forms slow-growing tumors, is invasive and, therefore, mimics the behavior of the majority of prostate cancers in men. Thus, it is a useful model for studying agents that could be used for the treatment of less aggressive prostate cancer.

The present study was conducted to examine the effects of cholecalciferol on the RWPE2-W99 human prostate cancer cells. To the best of my knowledge, the only other study that has examined the effects of cholecalciferol on any normal or cancer cell lines to date examined the effects on growth and binding affinity to VDR in normal human

keratinocytes (Chen et al., 2000). I sought to examine the effects of cholecalciferol on growth, invasion, motility, matrix metalloproteinase activity, and vimentin expression, using separate *in vitro* assays. In order to explore the mechanisms of action of cholecalciferol, I also examined the expression of vitamin D receptor and RXRs, with which VDR forms heterodimers. There is evidence to suggest cross-talk between VDR and androgen receptor (AR). Therefore, the effects of cholecalciferol treatment on the expression of AR and PSA were also examined. In addition, the effects of cholecalciferol were compared to those of calcitriol. My results indicate that treatment of the RWPE2-W99 human prostate carcinoma cells with cholecalciferol inhibits several characteristics associated with malignant transformation. The observed effects were similar to those obtained using calcitriol treated cells.

Materials and Methods

Cells and Cell Culture

The RWPE2-W99 cell line, developed in our laboratory, is a tumorigenic and invasive human prostate carcinoma cell line. The RWPE-2 cell line was derived from RWPE-1 cells after transfection with the *Ki-ras* oncogene (Bello et al., 1997; Webber et al., 1997). RWPE-2 cells were grown in agar culture and several clones were isolated, expanded and screened for *Ki-ras* expression. One of these clones was selected for high *Ki-ras* expression, expanded and was designated as RWPE2-W99 cells (Webber, unpublished data). Cells were maintained in complete keratinocyte serum-free medium (KSFM; Cat. #: 10724-011) containing 50 µg/ml bovine pituitary extract (BPE) and 5 ng/ml epidermal growth factor (EGF) supplements (Cat. #: 37000-015), and 1X antibiotic/antimycotic mixture (Penicillin 100U/ml, Streptomycin 100 µg/ml, and

Fungizone 0.25 µg/ml) (Gibco/Invitrogen, Rockville, MD) . Medium was changed every 48 h. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. When confluent, cells were lifted using trypsin/EDTA (T/E; Sigma, St. Louis, MO) diluted 1:1 with D-PBS (Dulbecco's phosphate buffered saline, Ca²⁺/Mg²⁺ free; Sigma, St. Louis, MO) and passaged and seeded at 1x10⁶ cells/T-25 flask, or 2x10⁶ cells/T-75 flask (BD Falcon, VWR, Batavia, IL).

Dose Response

The effects of cholecalciferol (Cat. #: C-1357, Sigma) and calcitriol (Cat. #: D-1530, Sigma) on anchorage-dependent growth of RWPE2-W99 cells in monolayer culture were examined using a 96-well microplate assay. The drugs were dissolved in 100% ethanol, protected from light and stored at -70°C. The final level of vehicle in the medium was 0.1%. Cells were plated in 100 µl of complete KSFM medium at a density of 10,000 cells/well, six wells per treatment, in 96-well plates (BD Falcon). Concentrations of test agents from 1 nM (10⁻⁹ M) to 10 µM (10⁻⁵ M) were tested. After 48 h, 100 µl of test media containing twice the concentration of cholecalciferol or calcitriol were added to the wells. In subsequent medium changes, every 48 h, all 200 µl of test media were removed and replaced with 200 µl of fresh test media. Effects on growth were measured using the MTT assay (Bello et al., 1997). After 5 days of treatment, 50 µl of MTT (Sigma) were added to each well and incubated (37°C) for 3.5 h. The medium/MTT mixture was slowly aspirated from each well and 150 µl of DMSO were added to each well and triturated several times to extract the stain. Absorbance was read at 540 nm using a Titertek microplate reader where absorbance shows a direct relationship with cell growth.

Absorbance of ethanol treated cells was set at 100% growth and served as the controls to which all other treatments were compared. The dose that inhibits growth and reduces it to 50% of control (ID_{50}) was determined. Initial experiments were conducted using a wide range of cholecalciferol at log dilutions from 10^{-12} M to 10^{-5} M to determine the effective range. Subsequent experiments were then conducted using a narrow range from 10^{-9} M to 10^{-5} M but using small dose increments. Six independent experiments were conducted.

Agar assay for anchorage-independent growth

To examine the effects of cholecalciferol on anchorage-independent growth of RWPE2-W99 cells, colony formation in soft agar was performed, in 35 mm plates in triplicate. A 1.25% (w/v) agar solution was prepared by dissolving 1.25 grams of agar in dH₂O over low heat, autoclaved for 25 min, and stored in the refrigerator for up to one month. Dissolving the agar was facilitated by heating it in a microwave oven for ~1 minute, removing it before it began to boil. It was allowed to cool for 3-4 min, and then kept in a 44°C water bath. A fresh solution of 0.5% agar-containing medium was prepared [40% of 1.25% agar, 40% KSFM complete medium, 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), and 10% Dulbecco's phosphate buffered saline Ca²⁺/Mg²⁺-free (DPBS) (Gibco-BRL, Grand Island, NY)] and kept in a 44°C water bath. Two ml of 0.5% agar-containing medium were evenly layered in each 35 mm plate (BD Falcon) and allowed to solidify for 30 min. Cells were subcultured and filtered through a 40- μ m pore size cell strainer to get a single cell suspension. 37,500 cells were added to 1 ml of 10% FBS in complete KSFM containing appropriate cholecalciferol treatment. To this cell suspension 2 ml of 0.5% agar medium was added, giving 0.33% agar in the

medium. The suspension was gently triturated three times to mix and 1 ml was layered on top of the hardened agar in 3 plates/drug treatment, giving 12,500 cells/plate. The plates remained at room temperature for 20 min before being transferred to an incubator. The cells were not fed during the incubation period. After 21 days of incubation at 37°C, the colonies were stained with 1 ml of sterile 1 mg/ml *p*-iodonitrotetrazolium (INT) (Sigma) solution for 12 h at 37°C. The colonies were fixed with 0.5 ml of 10% formalin and immediately counted using a BioTran II automated colony counter. Percent colony forming efficiency (CFE) was then calculated. Ethanol treated dishes were used as controls. The CFE of all treatments was compared to the ethanol control CFE. Results represent the averages of three experiments.

Invasion and motility/chemotaxis assays using Boyden chambers

The effects of test agents on the invasive ability and motility of the tumorigenic RWPE2-W99 cell line were examined by modifying a previously described Boyden chamber invasion assay (Bello et al, 1997). The day prior to running the assay, cells were plated at a density of 3 million cells/T-75 flask. Nucleopore filters (8.0 µm pore size, 13 mm diameter) (Cat. #: 352340, Fisher Scientific, Chicago, IL) were coated with Matrigel (Trevigen Inc., Gaithersburg, MD) diluted 1:20 in cold (4°C), sterile distilled water and allowed to dry overnight at room temperature under sterile conditions. Cells were lifted 24 h after plating by incubation in 1.0 mM EDTA (Gibco-BRL) for ~9 min at 37°C, dislodged by tapping, and recovered by centrifugation. Cells were resuspended at a density of 1×10^6 cells/ml in basal KSFM medium (no BPE or EFG) containing 0.1% bovine serum albumin (BSA) (Pierce Biotechnology, Rockford, IL). To the bottom of the chamber 220 µl of NIH 3T3-conditioned medium, which served as a chemoattractant,

was added. The Matrigel-coated filters were placed on top of the chemoattractant with the Matrigel-coated side facing up. A single cell suspension containing 200,000 cells in 200 μ l was plated in triplicate on top of the filter and allowed to remain for 20 min. The cell suspension was then overlaid with 650 μ l of basal KSFM medium/0.1% BSA solution containing either ethanol (control) or varying concentrations of cholecalciferol. The assay was run for 48 h at 37°C to allow the cells to invade. The filters were then fixed and stained with a HEMA-3 kit (Fisher). The non-invaded cells, remaining on the Matrigel-coated side of the filter, were removed and each filter was transferred to the wells of a 24-well plate (BD Falcon). The stain was extracted from the filters with 300 μ l of 0.1 N HCl for 15 min. 200 μ l from each well was then transferred to a 96-well plate. The absorbance was read at 620 nm using a Titertek microplate reader. In addition, any cells that invaded into the bottom chamber were counted using a Coulter Counter. The percentages from the filters and chamber counts were averaged to obtain total invasion. Ethanol treated chambers were used as controls and set at 100% for invasive ability. The effect of cholecalciferol on the motility/chemotaxis of the RWPE2-W99 cells was investigated by allowing the cells to migrate from the upper to lower chamber for 48 h. The protocol used was the same as described above, except that the filters were not coated with Matrigel for the motility/chemotaxis assay. The results represent the average of at least 3 individual experiments for each assay, and are expressed as $\pm$ SD.

SDS-PAGE Zymography for MMP activity

In order to examine the effects of the test agents on secreted MMP activity, conditioned medium samples were collected. Cells were plated in complete KSFM at a density of one million cells/60 mm dish (BD Falcon), in duplicate dishes for each treatment. After 48 h, the medium was slowly aspirated and each dish was washed twice with 3 ml of DPBS. Next, 2.2 ml basal KSFM (without BPE and EGF) containing the desired concentration of cholecalciferol or calcitriol were added to the dishes. The cells were exposed to the treatments for 48 h at 37°C. Conditioned medium samples from duplicate dishes were pooled, added to sterile 15 ml centrifuge tubes, centrifuged at 2500 rpm for 5 min to remove cellular debris, and the supernatant was collected. To each sample 2% Triton-X 100 was added at a final concentration of 0.1%, the media were gently vortexed to mix, and aliquoted into sterile 200 µl Eppendorf tubes. The protein content of each sample was determined using the Lowry High protein assay (Bio-Rad, Hercules, CA). Samples of conditioned media were stored at -70°C until needed.

To determine the effects of the test agents on MMP-2 and MMP-9 activity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography was conducted. The separating and stacking gels were made using the following protocols and poured into 0.75 mm casting plates. The final gelatin concentration in the gels was 0.1%. The gels were allowed to polymerize overnight at 4°C before use. Conditioned medium samples containing 2 µg/µl of protein and 5 µl of Laemmli Sample Buffer were loaded into each well. Using a Bio-Rad Mini Protean II apparatus, the gels were electrophoresed (4°C) at 157 volts for 1.5 h in approximately 800 ml of 1X running buffer [24 g Tris base, 115.2 g Glycine (Bio-Rad), pH 8.3]. The gels were then treated

Separating Gel

Distilled water	3.91 ml
1.5 Tris-HCl, pH 8.8	2.5 ml
Acylamide/Bis (30% stock)	3.33 ml
Gelatin (10%)	100 µl
Electrophoretic grade 10% (w/v) SDS	100 µl

De-gas for 15 minutes,

10% Ammonium persulfate	50 µl
TEMED	10 µl

Stacking Gel

Distilled water	5.73 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% (w/v) SDS	100 µl
Acrylamide/Bis (30% stock)	1.67 ml

De-gas for 15 minutes

10% ammonium persulfate	100 µl
TEMED	20 µl

with two separate 30 min washes of 2.5% Triton-X 100 on the plate rocker at room temperature to remove the SDS. After the Triton-X treatments, the gels were incubated at 37°C for 18 h in Tris-HCl soaking buffer [50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35,(Bio-Rad)], pH 7.5. The soaking buffer re-natures the proteases, which allows them to lyse the gelatin in the gels. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 for 20 min followed by approximately 20 min of destaining in methanol:acetic acid:dH₂O (4:1:5) solution. Bands of lysis indicating protease activity appear as light bands against a dark purple background. The area of each band was measured densitometrically and compared with the control to determine the effect of the test agents on MMP-2 and MMP-9 activity.

Immunostaining

Immunocytochemistry was conducted to examine the effects of cholecalciferol on the expression of the following: intermediate filament protein vimentin (Sigma, Cat. #: V-6630); the nuclear receptors VDR (Oncogene, San Diego, CA, Cat. #: GR37),

RXR- α (Santa Cruz Biotechnology, Santa Cruz, California, Cat. #: sc-553), RXR- β (Santa Cruz, Cat. #: sc-831), and RXR- γ (Santa Cruz, Cat. #: sc-555); androgen receptor (AR) (Santa Cruz, Cat. #: sc-7305); prostate specific antigen (PSA) (Dako Cytomation, Carpinteria, CA, Cat. #: M-0750) were examined. Monoclonal antibodies for vimentin, PSA, and VDR, and polyclonal antibodies for RXRs and a modified avidin-biotin immunoperoxidase protocol were used (Bello et al., 1997). Cells were plated in 1 ml of complete KSFM on sterile, acid washed glass coverslips in 24-well plates at a density of 7,500 cells/well. The medium was replaced 48 h after plating with 2 ml of medium containing the desired cholecalciferol concentration. The following concentrations of cholecalciferol were used: 1 nM, 10 nM, 100 nM, and 1 μ M. Ethanol treated cells served as the controls. Test media were changed every 48 h. After 5 days of treatment, the test media were slowly aspirated, cells were washed twice with D-PBS, and fixed at room temperature with a warm (25°C) solution of 1:1 methanol (100%)/acetone for 2 min. The coverslips were stored at -20°C until needed. All of the following steps were carried out at room temperature: cells were blocked for 1 h with normal horse serum (Vector Labs, Burlingame, CA), incubated with primary Ab diluted in blocking solution, incubated for 30 min with a biotinylated secondary antibody diluted 1:200 in blocking solution, treated with 3% H₂O₂ to quench endogenous peroxidase activity, incubated with ABC (Vector) for 30 min, and developed with DAB substrate kit for peroxidase (Vector) for 1-3 min. Cells were rinsed 2 X 8 min each between all successive steps after incubation with the primary Ab. Cells not incubated in primary Ab but incubated only in secondary Ab served as controls. Coverslips were dehydrated and mounted on alcohol-washed

microscope slides using Permount. Antibody dilutions and incubation times were: vimentin 1:40 (1 h), VDR 1:100 (2 h), RXR- α , RXR- β , and RXR- γ 1:100 (2 h).

Protein Analysis

To quantify proteins, a Beckman DU Series 530 Spectrophotometer was used for protein assays. Samples were compared to standard concentrations of 0.0, 0.1, 0.5, 1.0, 1.5, and 2.0 $\mu\text{g/ml}$ prepared from the Albumin Standard (bovine serum albumin, 2.0 mg/ml, Pierce). The assays were run using the Lowry High protein assay method.

Western blot analysis

Western blot analysis was performed in order to examine the effects of cholecalciferol on CYP27A1 (Santa Cruz, Cat. #: sc-14835), VDR, RXR- α , β , and γ , AR and vimentin, and the effects of calcitriol on VDR protein expression. RWPE2-W99 cells were plated at a density of 500,000 cells/T-25 flask; two flasks were used for each treatment. 48 h after plating, the medium was removed and replaced with the test media. The cells were treated for 4 days, incubated with 2.0 ml trypsin/EDTA for 7 min, and dislodged by tapping. Cells from duplicate flasks were pooled and centrifuged at 2500 rpm for 5 min and the pellet was resuspended in 1.5 ml of D-PBS and incubated on ice. Nuclear and cytoplasmic extracts were prepared using the NE-PER kit according to the manufacturer protocol (Pierce). Extracts were aliquoted and frozen at -70°C . The Lowry High protein assay method was used to determine protein content of each sample. Samples of cytoplasmic extract containing 10 μg (vimentin) and 40 μg (CYP27A1), and nuclear extract containing 20 μg (VDR) and 25 μg (RXRs and AR) of protein were run in

NuPAGE 10% Bis-Tris pre-cast gels (Invitrogen, Carlsbad, CA) at 175 V for 1.5-1.7 h at 4°C. Proteins were transferred to Immobolin-P transfer membranes (0.45 µm pore size) (Millipore Corp., Bedford, Maryland) at 30 V for 1-1.5 h at 4°C. For immunoblotting, monoclonal antibodies were used for vimentin, AR, and VDR, and polyclonal antibodies were used for RXRs and CYP27A1 (Santa Cruz). All blots were stained using avidin-biotin immunoperoxidase Vector ABC kit (Vector) for 1 h. β -actin (Sigma, Cat. #: A-5441) was used as the loading control. Bands were developed with DAB kit (Vector). Blots were analyzed with the ImageJ analysis program from the National Institutes of Health (NIH). Individual lanes were compared to controls lanes for changes in protein expression. Antibody dilutions and incubation times were as follows: vimentin 1:500 (1.5 h), VDR 1:1000 (2 h), RXR- α (1:100), RXR- β (1:250), and RXR- γ (1:200) (each 2 h), AR 1:500 (2 h), CYP27A1 1:100 (1 h).

Statistical Analysis

All of the results in this study were obtained from three to six independent experiments. Results are expressed as means with standard deviations (SD). Differences between the means were considered significant if $p < 0.05$. Response to individual concentrations was compared to controls using unpaired t-test with Welch correction. GraphPad InStat #3 was used for these analyses.

Results

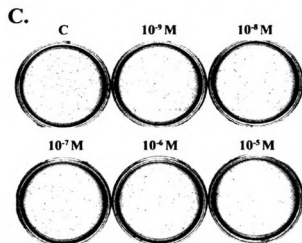
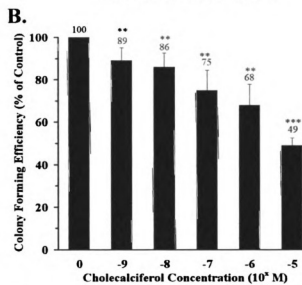
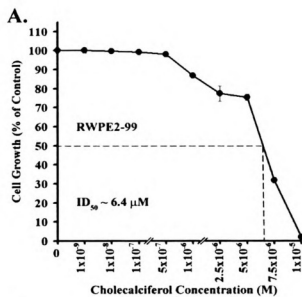
Cholecalciferol inhibits growth of RWPE2-W99 cells:

Dose response experiments were first run to examine the effects of cholecalciferol on anchorage-dependent growth of the RWPE2-W99 cells. Cells were plated in 96-well plates and treated with increasing cholecalciferol concentrations for 5 days as described in Materials and Methods. These results show that cholecalciferol causes a dose-dependent growth inhibition in the RWPE2-W99 cells (**Figure 8-3A**). The ID₅₀ was approximately 6.4 μ M. The results from these experiments were used to determine the cholecalciferol concentrations that would be used in all subsequent assays.

Cholecalciferol reduces anchorage-independent colony forming efficiency (CFE):

A soft agar assay was used to examine the effects of cholecalciferol on the anchorage-independent growth of the tumorigenic RWPE2-W99 cells. This assay is one determinant of the ability of the cells to form tumors *in vivo*. The RWPE2-W99 cells have previously been shown to form colonies in agar and tumors in nude mice (Bello et al., 1997). As seen in **Figure 8-3B**, the treatment of RWPE2-W99 cells with increasing concentrations of cholecalciferol causes a dose-dependent decrease in their colony forming efficiency (CFE). Significant inhibition was seen beginning at 1 nM (10^{-9} M) ($p < 0.01$). Concentrations of 0.1 μ M (10^{-7} M) ($p < 0.01$), 1 μ M (10^{-6} M) ($p < 0.01$), and 10 μ M (10^{-5} M) ($p < 0.001$) inhibited CFE by 25%, 32% and 51%, respectively, when compared to ethanol-treated controls. Representative agar dishes for each cholecalciferol treatment are shown in **Figure 8-3C**.

Figure 8-3: Effect of cholecalciferol on anchorage-dependent (A) and anchorage-independent (B, C) growth of the RWPE2-W99 cells. (A) 48 h after plating 1×10^4 cells/well in 96-well plates, cells were treated with cholecalciferol. After 5 days of treatment, cells were stained with MTT. Absorbance was read at 540 nm, $\pm$ SD, (n = 6). The dashed line shows ID₅₀ of ~ 6.4 μ M. (B) Colony forming efficiency (CFE) of cholecalciferol treated RWPE2-W99 cells was determined using a soft agar assay. Cells were plated at a density of 12,500 cells/30 mm dish. Colonies were stained with INT after 21 days of growth, counted, and results plotted, $\pm$ SD, (n = 3). CFE of ethanol treated control is taken as 100%. (** $p = 0.01$; *** $p < 0.001$). Representative agar dishes for each cholecalciferol concentration are shown in (C).



Cholecalciferol inhibits invasion in a dose-dependent manner:

The effect of cholecalciferol on the invasive ability of RWPE2-W99 cells was tested using an *in vitro* Boyden chamber assay. The use of Matrigel in this assay mimics the basement membrane degraded by invasive cancer cells during the metastatic process *in vivo*. The data in **Figure 8-4A** show that cholecalciferol treatment inhibits the invasive ability of the RWPE2-W99 cells in a dose-dependent manner. Significant inhibition ($p < 0.001$) was observed at all concentrations including as low as 1 nM. Concentrations of 1 μM (10^{-6} M) and 10 μM (10^{-5} M) reduced invasion to 56% and 39% of the ethanol treated controls, respectively.

Cholecalciferol inhibits cell motility/chemotaxis:

To examine the effects of cholecalciferol on the motility/chemotaxis of the RWPE2-W99 cells, the same protocol that was used in the invasion assay was employed, except that the filters were not coated with Matrigel. Similar to the invasion assay, cholecalciferol treatment caused a dose-dependent decrease in the motility of RWPE2-W99 cells. However, higher concentrations were needed in the motility assay in order to see the same inhibitory effects as seen in the invasion assay. Treatment with 10 μM (10^{-5} M) cholecalciferol caused a decrease to 55% of ethanol treated control in the motility assay, whereas an almost identical decrease (56% of control) was seen at 1 μM (10^{-6} M) in the invasion assay. **Figure 8-4B** shows the effects of cholecalciferol treatment on the motility of the RWPE2-W99 cells. Significant inhibition ($p < 0.001$) was observed at all concentrations including as low as 1 nM.

Cholecalciferol inhibits MMP-9 and MMP-2 activity:

To determine potential mechanisms involved in the modulation of invasive ability by cholecalciferol, I examined the secreted levels of type IV collagenases. I used 0.1% gelatin-based SDS-PAGE zymography to determine the gelatinolytic activity of MMP-9 and MMP-2 in conditioned media of cholecalciferol-treated RWPE2-W99 cells. As seen in **Figure 8-4C**, cholecalciferol treatment resulted in a marked decrease in the gelatinolytic activity of both MMP-9 and MMP-2. The densitometric analysis of the zymogram shows a marked dose-dependent decrease in MMP activity with maximum inhibition of 81% and 93% for MMP-9 and MMP-2, respectively, at 10^{-5} M cholecalciferol when compared to the ethanol treated controls.

Cholecalciferol modulates vimentin expression in RWPE2-W99 cells:

Vimentin expression in ethanol-treated control cells was compared with that in cells treated with cholecalciferol for 5 days. Immunostaining results in **Figure 8-5A** suggest that cholecalciferol causes a decrease in vimentin expression. The control cells show strong vimentin staining while the cholecalciferol treated cells show a decrease in vimentin staining. These immunostaining results were confirmed by Western blot analysis (**Figure 8-5B**). Densitometric analysis shows a marked decrease in vimentin expression of approximately 73% at the 1 μ M (10^{-6} M) concentration when compared to ethanol-treated control. These results demonstrate that cholecalciferol causes a dose-dependent decrease in vimentin expression.

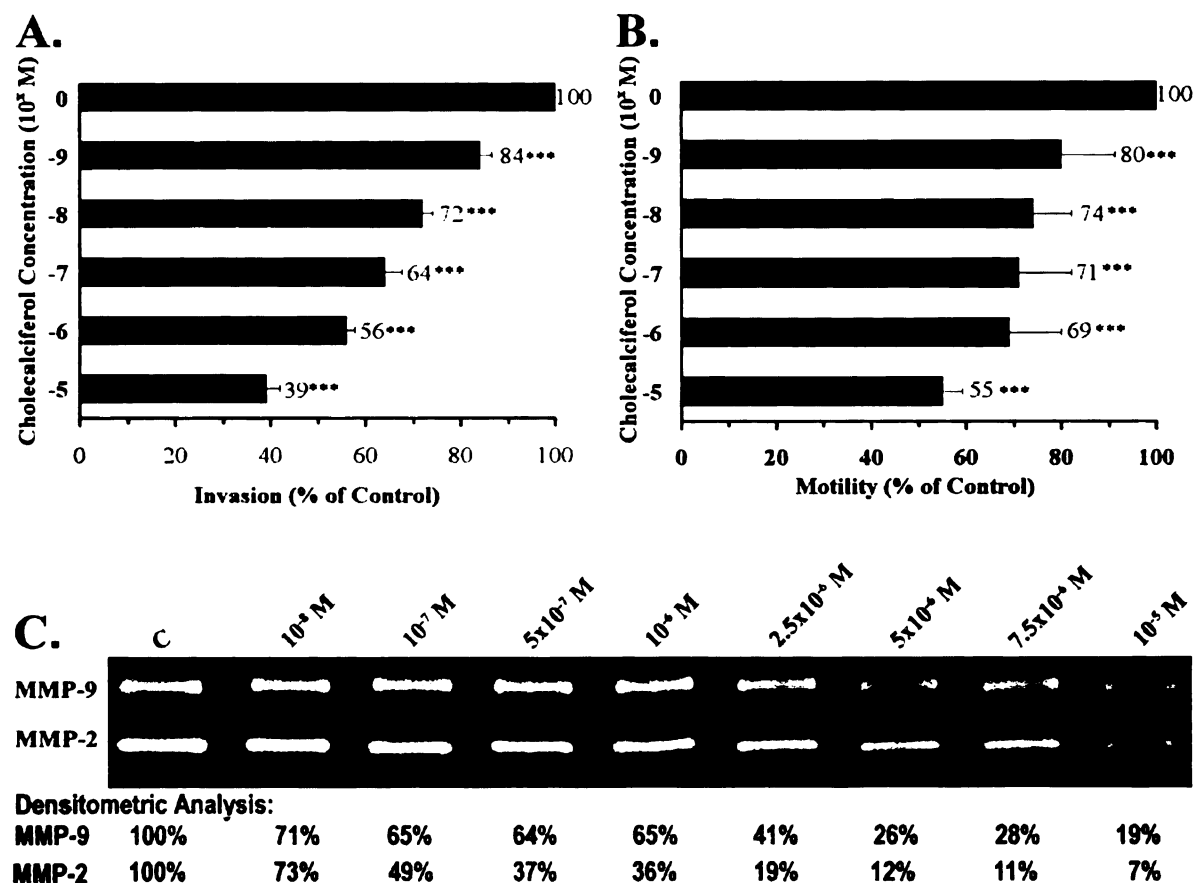


Figure 8-4: The effect of cholecalciferol treatment on the invasive ability (A), motility (B), and MMP activity (C) of RWPE2-W99 cells. (A) Effects of cholecalciferol on invasive ability were examined using the *in vitro* Boyden chamber assay. 2.0×10^5 cells were plated/chamber in triplicate and allowed to invade through Matrigel for 48 h. Invasive ability of ethanol-treated controls was taken as 100%; $\pm$ SD ($n = 3$) (***) = $p < 0.001$. (B) To examine the effects of cholecalciferol on cell motility/chemotaxis, experiments were run as in (A), except that filters were not coated with Matrigel. Motility of ethanol-treated cells was taken as 100%; $\pm$ SD, ($n = 4$) (***) = $p < 0.001$. (C) SDS-PAGE zymography (10% polyacrylamide gel containing 0.1% gelatin) was used to analyze MMP-2 and MMP-9 activity in conditioned medium of RWPE2-W99 cells treated with cholecalciferol for 48 h. $2.0 \mu\text{g}$ of protein were loaded/lane. Densitometric analyses, with ethanol-treated controls taken as 100%, are shown below the zymogram.

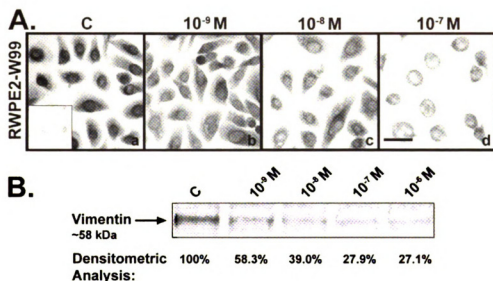


Figure 8-5: Effects of cholecalciferol on the expression of the intermediate filament protein vimentin. (A) Immunostaining results suggest that cholecalciferol treatment causes a dose-dependent decrease in vimentin expression. Treatment concentrations are shown above their respective square. C = ethanol-treated control. Inset in (a) is a negative control lacking primary antibody. Bar = 20 μ m. (B) Western blot analysis of vimentin expression confirms immunostaining results and shows that cholecalciferol treatment decreases vimentin expression in a dose-dependent manner. Vimentin Mr = ~58 kDa. Cholecalciferol concentrations are shown above their respective lane where C = ethanol-treated control. Densitometric analysis of each lane is shown below the blot, where ethanol-treated control is taken as 100%.

Cholecalciferol modulates VDR, RXRs and AR nuclear receptors:

Figure 8-6 shows the immunostaining results for VDR and RXRs after treatment with increasing concentrations of cholecalciferol. Cholecalciferol treatment appears to up-regulate, in a dose-dependent manner, the VDR and RXR- α and RXR- β . RXR- γ did not appear to be significantly up-regulated. The Western blot analyses in **Figure 8-7** confirm the immunostaining results showing that cholecalciferol treatment causes a dose-dependent up-regulation of VDR and RXR- α and, to a lesser extent, that of RXR- γ . Densitometric analysis showed that VDR had the greatest up-regulation (approximately 160% of ethanol-treated control) followed by RXR- α . RXR- γ showed a small dose-dependent increase but appears to be down-regulated at the highest concentration of 10^{-6} M cholecalciferol. The Western blot analysis of the androgen receptor (AR) upon treatment with cholecalciferol (**Figure 8-7**) demonstrates a dose-dependent up-regulation in AR expression.

Cholecalciferol induces the expression of the 25-hydroxylase enzyme, CYP27A1:

Western Blot analysis was conducted to examine the ability of cholecalciferol to induce 25-hydroxylase, CYP27A1, which converts cholecalciferol to 25OHD₃. Using a polyclonal antibody against CYP27A1, a single band at Mr ~54 kDa was detected. The molecular weight of the human CYP27 enzyme has been shown to be 54 kDa (Axen et al., 1994). As seen in **Figure 8-8**, untreated RWPE2-W99 cells (lane c) constitutively express CYP27A1 enzyme. Furthermore, results show that this enzyme is up-regulated, in a dose-dependent manner, upon treatment with cholecalciferol. Compared to ethanol-treated controls, CYP27A1 was up-regulated to approximately 5-7 fold at 10 μ M (10^{-5} M) concentration in different experiments.

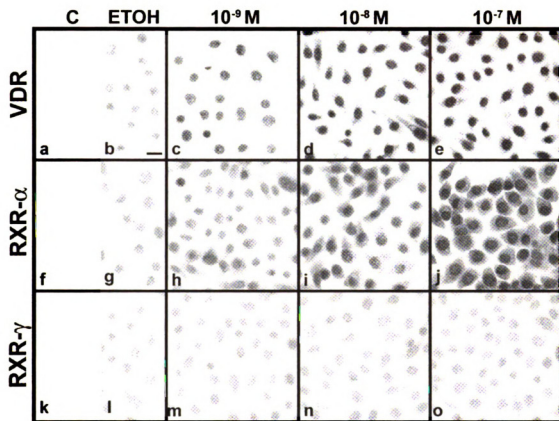


Figure 8-6: Immunostaining for nuclear receptors in RWPE2-W99 cells treated with cholecalciferol. Cells were treated for 5 days. VDR (a-e) and RXR- α (f-j) both demonstrate a marked increase in expression when compared to their respective ethanol-treated control coverslips (b and g). RXR- γ showed a very small increase. Treatment concentrations are 10^{-9} M (c,h,m), 10^{-8} M (d,i,n) and 10^{-7} M (e,j,o) cholecalciferol. (a,f,k) are controls lacking primary antibody. Cells were plated on sterile, glass coverslips at a density of 7,500 cells/coverslip. Bar = 20 μ m.

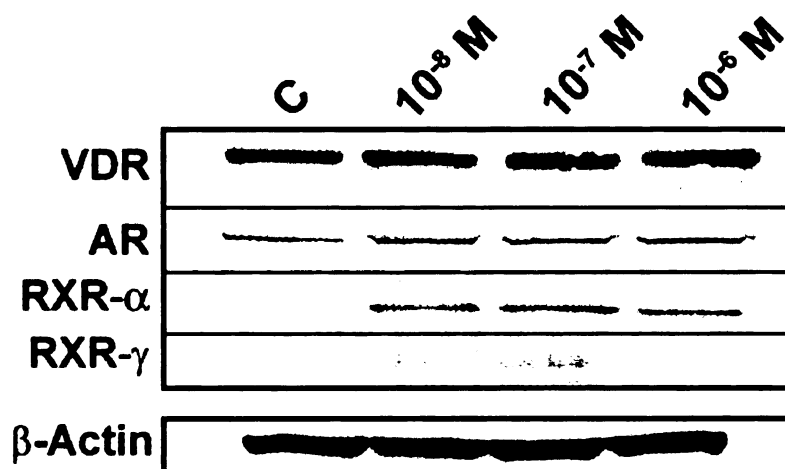


Figure 8-7: Western blot analysis showing the effects of cholecalciferol on nuclear receptors. Results show that cholecalciferol treatment caused a dose-dependent up-regulation of VDR, RXR- α , and AR. RXR- γ expression is also up-regulated but at a lower level. RXR- γ is then down-regulated at the highest concentration of 10^{-6} M cholecalciferol. For VDR 20 μ g and for RXRs and AR 25 μ g of protein was loaded/lane. β -actin was used as the loading control.

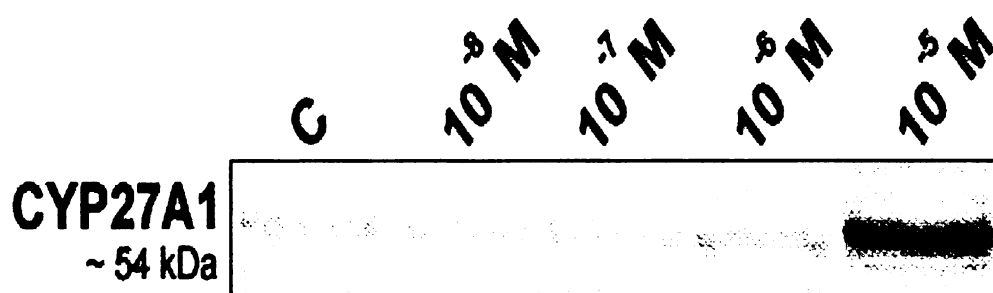


Figure 8-8: Western blot for 25-hydroxylase enzyme, CYP27A1, in RWPE2-W99 cells shows that while the enzyme is present in untreated cells it is also up-regulated upon treatment with cholecalciferol. Cholecalciferol concentrations are shown for the respective lanes. 40 μ g protein was loaded/lane. Molecular weight of CYP27A1 is shown to be ~54kDa.

Effects of calcitriol:

In order to compare the efficacy of cholecalciferol, several of the assays, mentioned above, were also conducted using calcitriol-treated cells. **Figure 8-9A** shows the results of the calcitriol dose response assay. The cells were more sensitive to calcitriol treatment, with an approximate ID_{50} of 1.05 μM , compared to $\sim 6.4 \mu M$ for the cholecalciferol treated cells. Results from calcitriol zymograms are shown in **Figure 8-9B**. Similar to the cholecalciferol zymograms, calcitriol treatment caused a dose-dependent decrease in MMP-9 and MMP-2 activity. Western blot analysis of VDR expression (**Figure 8-9C**) in calcitriol-treated cells shows that the dose-dependent up-regulation pattern is similar to that observed for cholecalciferol treated cells, however, calcitriol is approximately 6 to 10 fold more effective in the different assays conducted, when compared to cholecalciferol.

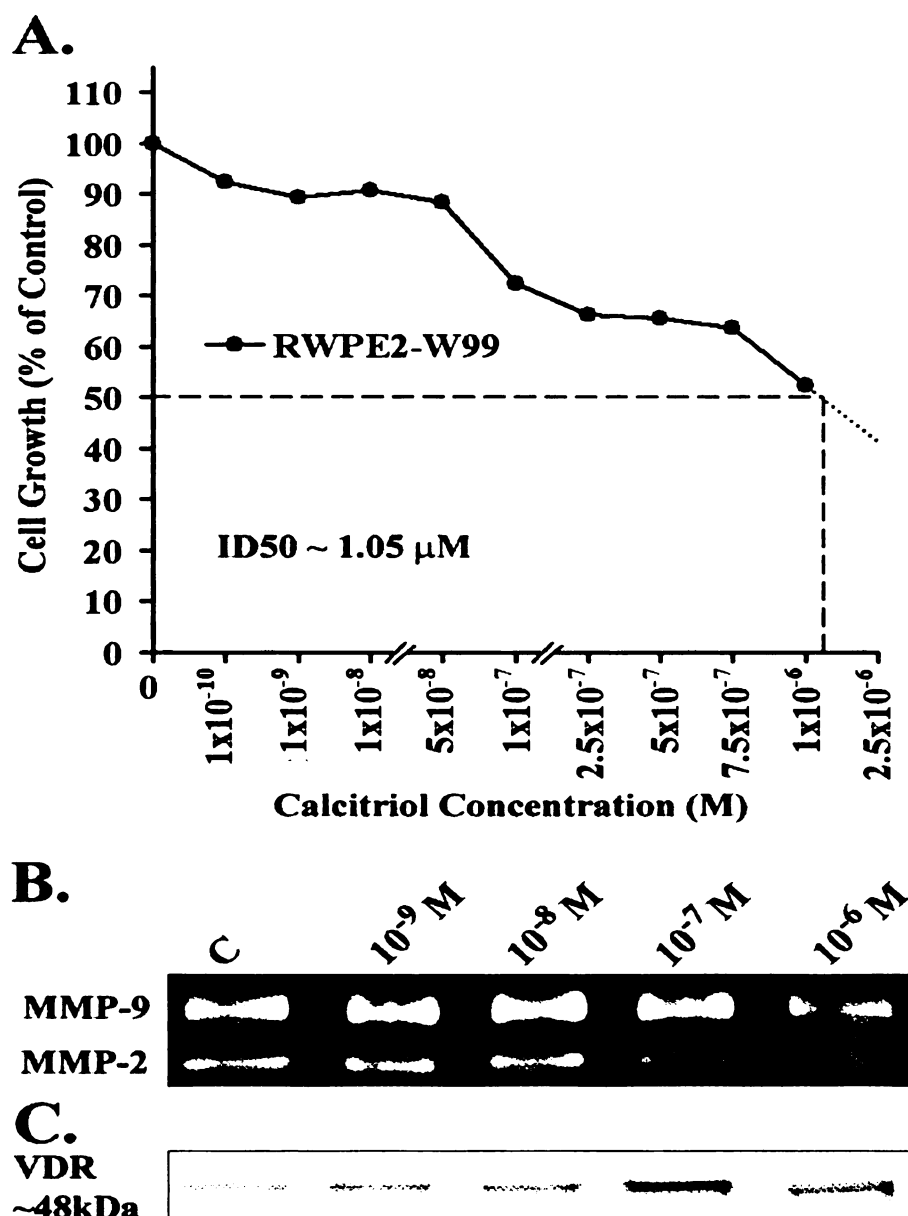


Figure 8-9: Effects of calcitriol on (A) anchorage-dependent growth, (B) MMP activity, and (C) VDR expression in RWPE2-W99 cells. (A) Cells were plated at a density of 1.0×10^4 cells/well in 96-well plates. 48 h after plating, cells were treated with calcitriol. Plates were stained with MTT after 5 days of treatment and absorbance was read at 540 nm. The dashed line shows ID₅₀ of ~1.05 μ M. (B) The activity of MMP-9 and MMP-2 was analyzed by SDS-PAGE zymography of condition medium from RWPE2-W99 cells treated with calcitriol for 48 h. 2.0 μ g of protein was loaded/lane. (C) Western blot for VDR in calcitriol-treated RWPE2-W99 cells shows a dose-dependent up-regulation of VDR expression with increasing calcitriol concentrations but is then down-regulated at the highest concentration of 10^{-6} M cholecalciferol. 20 μ g protein was loaded/lane.

Discussion

In addition to its crucial role in mineral homeostasis and bone regulation, vitamin D is also involved in the regulation of proliferation and differentiation of several normal and cancer cell types (Bernardi et al., 2002; Gross et al., 1998; Jensen et al., 2001; Konety et al., 1996; Krill et al., 1999; Miller et al., 1992; Miller et al., 1995; Peehl et al., 1994; Shabahang et al., 1993; Young et al., 1995; Zhao et al., 2000). Calcitriol [$1,25(\text{OH})_2\text{D}_3$] has also been shown to inhibit invasive ability and tumorigenicity of cancer cells (Hansen et al., 1994; Lokeshwar et al., 1999; Schwartz et al., 1997). The vitamin D receptor (VDR) has been found in many normal and malignant cells. Several epidemiological studies suggest an inverse relation between vitamin D levels and cancer risk (Ahonen et al., 2000a; Bodiwala et al., 2003; Corder et al., 1993; Luscombe et al., 2001; Hanchette and Schwartz, 1992). Based on these facts, much work has been done to test the efficacy of calcitriol as a chemopreventative and chemotherapeutic agent. However, due to the adverse calcemic effects caused by calcitriol treatment, alternative forms of vitamin D, including calcitriol precursors and vitamin D analogs, are being studied.

UV light converts 7-dehydrocholesterol to pre-vitamin D_3 , which is then converted to vitamin D_3 (cholecalciferol). Cholecalciferol is converted to 25-hydroxyvitamin D_3 (25OHD_3) and subsequently to 1,25-dihydroxyvitamin D_3 (calcitriol), the most biologically active form of the hormone (see **Figure 8-1**). Given the significant biological effects of vitamin D, the conversion of vitamin D_3 to calcitriol is obviously very important in the human body. Two enzymes are necessary for the conversion of cholecalciferol to calcitriol. The 1α -hydroxylase enzyme converts

25OHD₃ to calcitriol. Several tissue types in the body have the capacity to express 1 α -hydroxylase, including colon, keratinocytes, pancreas, brain, lymph nodes, and osteoblasts (Holick 2003; Osborne and Hutchinson, 2002) and, therefore, have the ability to synthesize calcitriol from 25OHD₃. Recently, it was shown that prostatic epithelial cells also possess the 1 α -hydroxylase enzyme (Schwartz et al., 1998). Interestingly, all of these tissues express vitamin D receptor, suggesting a role for vitamin D in these target tissues.

The conversion of cholecalciferol to 25OHD₃ requires the 25-hydroxylase enzyme, CYP27A1. Liver expresses 25-hydroxylase and is generally considered to be the major site of synthesis of 25OHD₃. However, the kidney and keratinocytes are other cell types that have been shown to express the 25-hydroxylase enzyme in humans (Bikle et al., 1986; Lehmann et al., 1999). Therefore, it stands to reason that in order to regulate cell proliferation and differentiation in a tissue-specific manner, vitamin D target cells that express VDR, may have the capacity to locally metabolize some vitamin D precursors to its active forms. To the best of my knowledge, I demonstrate, for the first time, the presence of 25-hydroxylase in prostatic epithelial cells as detected by a polyclonal antibody to CYP27A1. I have shown that untreated RWPE2-W99 cells constitutively express the enzyme, and cholecalciferol treatment results in a dose-dependent increase in its expression (**Figure 8-8**). These data, coupled with the presence of 1 α -hydroxylase activity (Schwartz et al., 1998), demonstrate that prostatic epithelial cells appear to possess the unique ability to locally produce calcitriol from cholecalciferol. This would suggest that cholecalciferol could have effects similar to those of calcitriol and 25OHD₃, on prostate cells, but at higher dose levels.

Local production of calcitriol in the prostate is presumed to be of biological importance, perhaps for the purpose of regulating cell growth in a tissue-specific manner. This would decrease the risk of cancer development and progression in tissues possessing either one or both of the enzymes necessary for the synthesis of calcitriol. 25OHD₃ is considered to be the best indicator of vitamin D status. Low serum levels of 25OHD₃ correlate with increased risk of prostate cancer (Ahonen et al., 2000a), and, compared to calcitriol, 25OHD₃ exerts similar antiproliferative effects, with similar potency, on normal prostatic epithelial cells (Barreto et al., 2000). Therefore, circulating levels of 25OHD₃ may be more relevant to the control of prostate cancer than circulating levels of calcitriol. If local production of calcitriol is more important than systemic levels, then the availability of circulating levels of calcitriol precursors (e.g. 25OHD₃ and cholecalciferol) may be more significant than circulating levels of calcitriol. For these reasons, I believe that cholecalciferol, a natural precursor to calcitriol and immediate precursor to 25OHD₃, may have chemopreventive and chemotherapeutic potential. I report, for the first time, the effects and mechanisms of action of cholecalciferol on human prostate epithelial cells. I also provide, for the first time, evidence indicating that prostatic epithelial cell lines, RWPE-1 and RWPE2-W99, possess the 25-hydroxylase enzyme needed to convert cholecalciferol to 25OHD₃.

The RWPE-2 cell line was derived from RWPE-1 cells by transformation with the *Ki-ras* oncogene (Bello et al., 1997; Webber et al., 1997). The malignant RWPE2-W99 cell line was cloned from RWPE-2, and chosen for its high *Ki-ras* expression. The majority of studies have used cell lines (i.e. DU-145, PC-3, and LNCaP) isolated from metastatic tumors which represent late stage, advanced disease. However, the majority of

prostate cancers are slow-growing primary tumors. RWPE2-W99 forms slow growing tumors, thus, mimicking the majority of prostate cancers. Therefore, RWPE2-W99 is a more appropriate model for studying the effects and mechanisms of action of agents for reversing the disease once it has been initiated (secondary prevention) and preventing invasion and metastasis of disease (tertiary prevention). The RWPE2-W99 cells form tumors in nude mice, form colonies in agar, and are invasive. I examined the effects of cholecalciferol on several malignant characteristics of the RWPE2-W99 cells. To the best of my knowledge, these are the first investigations to test the effects of the cholecalciferol form of vitamin D on prostate, or any other, cell types.

In the present study, cholecalciferol was shown to inhibit the anchorage-dependent growth of RWPE2-W99 human prostate carcinoma cells (**Figure 8-3A**) in a dose-dependent manner. RWPE2-W99 cells treated with calcitriol also showed a dose-dependent inhibition of anchorage-dependent growth (**Figure 8-9**). These cells were ~6-fold more sensitive to calcitriol with an ID_{50} of ~1.05 μM as compared to ~6.4 μM for cholecalciferol. Similar results were seen with keratinocytes treated with cholecalciferol and calcitriol (Chen et al., 2000). This is not surprising given that both prostate epithelial cells and keratinocytes express the 25-hydroxylase and 1α -hydroxylase enzymes needed to convert cholecalciferol to calcitriol (Bikle et al., 1986; Lehmann et al., 1999; Schwartz et al., 1998). Keratinocytes use these two enzymes to locally produce calcitriol. Calcitriol regulates the differentiation of keratinocytes and is also used to treat psoriasis, a hyperproliferative skin disease. These facts, and these similarities shared between prostatic epithelial cells and keratinocytes, suggest that cholecalciferol has the potential to be an effective agent for prevention and treatment not only of prostate cancer but also

other cancers. This also raises the possibility that cholecalciferol treatment could have similar growth inhibitory effects in other tissues that express VDR and the 1α -hydroxylase enzyme, such as the colon and pancreas. It is interesting to point out that these tissues, like the prostate, are sites of common epithelial malignancies.

To date, studies show that calcitriol modulates the proliferation and differentiation of several cell types that express VDR through a number of mechanisms, including modulation of genes involved in cell cycle regulation, alterations in growth factors, and regulation of hormones and their receptors, for example, androgen in the prostate gland. Aberrations in the G1/S checkpoint of the cell cycle have been observed in many cancers. Calcitriol exerts most of its antiproliferative effects, and may produce complete growth arrest, at this checkpoint. Exposure of cancer cells to calcitriol results in fewer cells entering the S phase of the cell cycle.

Calcitriol treatment causes a down-regulation of several cyclins and cdks and up-regulation of p21 and p27 (Jensen et al., 2001; Verlinden et al., 1998; Zhuang et al., 1998). Cyclins are proteins that govern transition through distinct phases of the cell cycle by forming complexes with and regulating the activity of the cyclin-dependent kinases (cdk). Once formed, these complexes help cells overcome inherent blocks in the cell cycle. Cdks are inhibited by cdk inhibitors (cdki), including p21 and p27. It has been suggested that the effect of calcitriol on p21 is secondary to the effects of calcitriol on TGF- β . Calcitriol inhibits cell proliferation and promotes differentiation of prostate epithelial cells by elevating TGF- β production and signaling (Danielpour, 1996; Murthy and Weigel, 2004). We have previously shown that TGF- β inhibits the growth of RWPE2-W99 cells (Bello et al., 1997). However, calcitriol could also act directly on p21

because the promoter region of p21 contains a vitamin D response element (VDRE) (Verlinden et al., 1998). Thus, vitamin D could inhibit cell proliferation by two independent mechanisms.

Malignant cells, such as RWPE2-W99, show anchorage independence which is related to tumorigenicity. In addition to inhibiting anchorage-dependent growth, cholecalciferol was also shown to inhibit, in a dose-dependent manner, the anchorage-independent growth, i.e colony forming efficiency, of RWPE2-W99 cells (**Figure 8-3B**). In a similar agar assay, calcitriol and several vitamin D analogs inhibited the clonal proliferation of several prostate cancer cell lines (de Vos et al., 1997). Calcitriol reduces the size and number of metastases derived from Dunning rat prostate tumors *in vivo* (Getzenberg et al., 1997). Also, Copenhagen rats injected with MAT LyLu prostate cancer cells showed a significant decrease in the size and number of lung metastases in the presence of calcitriol (Lokeshwar et al., 1999). Results from the present study show that cholecalciferol is effective in inhibiting anchorage-independent growth of RWPE2-W99 cells. Therefore, its efficacy in inhibiting tumor growth should be investigated.

The ability of cancer cells to invade depends on multiple factors including loss of cell:cell and cell:matrix adhesion, loss of cell polarization, expression of vimentin and secretion of proteases, and cell motility. In order for cancer cells to invade, the basement membrane is degraded by several proteases resulting in a loss of cell adhesion to the basement membrane and neighboring cells. Calcitriol inhibits invasion and metastasis in several malignant cell types, both *in vitro* and *in vivo* (Getzenberg et al., 1997; Hansen et al., 1994; Koli and Keski-Oja, 2000; Konety et al., 2001; Schwartz et al., 1997). In

relation to mechanisms for these effects, calcitriol was shown to inhibit the activity of serine proteases and metalloproteinases that are important determinants of invasion. Calcitriol decreased activity of components of the plasminogen activator (PA) system, including urokinase PA (uPA), tissue-type PA (tPA) and increased the expression of PA inhibitor. It is interesting to note that the uPA promoter region contains a VDRE (Koli and Keski-Oja, 2000). Prostatic epithelial cells have been shown to intrinsically secrete tPA and uPA, which was proposed to play a key role in tumor progression (Waghray and Webber, 1995).

The $\alpha 6$ and $\beta 4$ integrins are associated with increased migration and invasion of prostate cancer cells. Calcitriol reduces migration and invasion by down-regulating both of these integrins (Sung and Feldman, 2000). The $\beta 3$ integrin gene contains a functional VDRE (Osborne and Hutchinson, 2002), suggesting that calcitriol could inhibit migration and invasion by modulating other members of the integrin family. Loss of fibronectin (FN), an extracellular matrix protein, results in the loss of cellular adhesiveness and the acquisition of metastatic phenotype in malignant cells. The FN gene also contains a VDRE and calcitriol treatment induces FN transcription (Polly et al., 1996). I have shown that cholecalciferol treatment also results in the inhibition of invasion (**Figure 8-4A**) and motility (**Figure 8-4B**) of the RWPE2-W99 prostate cancer cells. Metastatic spread continues to be the greatest barrier to cancer cure. Cells must exhibit an invasive and motile phenotype in order to produce metastases. Therefore, the ability of cholecalciferol and calcitriol to inhibit invasion and motility, and, thus, reduce the formation of metastatic tumors would be extremely beneficial in the treatment of prostate cancer.

To investigate the mechanisms of action of cholecalciferol that are involved in inhibition of invasion, I tested its effects on the activity of MMP-9 and MMP-2. Cancer invasion and metastasis are the result of proteolytic degradation of the extracellular matrix. Matrix metalloproteinases (MMPs), particularly MMP-9 and MMP-2, have been suggested to play a major role in this degradation (Liotta and Stetler-Stevenson, 1991). MMP-9 and MMP-2 are expressed in prostate cancer cells and their increased expression is associated with prostate cancer progression (Stearns and Stearns, 1996a; Stearns and Stearns, 1996b). Calcitriol has been shown to decrease the activity of both MMP-9 and MMP-2 (Koli and Keska-Oja, 2000; Schwartz et al., 1997). I observed a similar, dose-dependent, decrease in the activity of both MMP-9 and MMP-2 in RWPE2-W99 prostate cancer cells treated with cholecalciferol. This inhibition of MMP activity would cause a marked reduction of proteolytic degradation of the extracellular matrix, resulting in a decrease in invasive ability of RWPE2-W99 cells. Cholecalciferol may inhibit invasion and metastasis not only by modulating MMP activity but also by modulating the activity of members of the plasminogen activator system directly or indirectly through their inhibitors. These mechanisms remain to be elucidated.

The primary function of MMP inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), is to inhibit the degradative actions of MMPs during tissue remodeling (Nagase and Woessner, 1999). A subset of MMPs, known as membrane-type MMPs (MT-MMPs), are able to activate secreted MMPs, particularly MMP-2 (Corcoran et al., 1996; Strongin et al., 1995). MT-MMPs can also cleave proteins in the basement membrane, which enhances the migration of prostate cancer cells (Udayakumar et al, 2003). The observed decrease in MMP activity in my experiments may, in part, be the

result of a cholecalciferol-related increase in TIMP levels, a decrease in MMP and/or MT-MMP levels. Increased levels of activated MMP-2a have been observed with increasing Gleason scores of prostate cancer (Stearns and Stearns, 1996b), suggesting an inverse relationship between differentiation and MMP-2a levels. Levels of MMP-2 increase progressively from the normal prostate, through prostatic intraepithelial neoplasia, to invasive cancer (Montironi et al., 1996). The effects of vitamin D on TIMPs and MT-MMPs on prostate cancer cells have not been studied and the suggestion that cholecalciferol works to decrease MMP activity through one of these mechanisms needs further investigation.

To further examine factors that contribute to inhibition of invasion by cholecalciferol, I examined the effects of cholecalciferol on the expression of the intermediated filament protein vimentin. Vimentin is normally expressed in mesenchymal cells. Vimentin is not normally expressed in differentiated, secretory epithelial cells, but may be expressed by rapidly proliferating epithelial cells in culture, and undifferentiated epithelial cells, such as prostate cancer cells. Vimentin expression increases with increasing motility of prostate cancer cells (Fraga et al., 2002; Lang et al., 2002). Several *in vivo* studies suggest that vimentin expression in epithelial cells may be related to tumor progression (Gilles et al., 1996). My results show (**Figure 8-5**) that cholecalciferol causes a dose-dependent decrease in the expression of the intermediate filament protein vimentin. These results suggest that cholecalciferol may induce differentiation by decreasing vimentin expression and, thus, inhibit motility and invasion of prostate cancer cells.

In addition to growth and tumor invasion and metastasis, cholecalciferol may modulate other characteristics associated with a malignant phenotype. Loss of differentiation is an important characteristic associated with malignant transformation in epithelial cells. Epithelial cells normally express the intermediate filaments, cytokeratins. The epithelial origin of the RWPE2-W99 cells has been established previously (Bello et al., 1997) and substantiated by my Western blot analyses (data not shown). Cholecalciferol treatment had no effect on the expression of cytokeratin 18 (data not shown), which is in agreement with previous studies using calcitriol (Peehl et al., 1994). So far I have demonstrated that cholecalciferol treatment inhibits both anchorage-dependent and -independent growth, induces differentiation, and inhibits MMP-9 and MMP-2 activity and expression of intermediate filament protein vimentin, with concomitant decrease in motility and invasion.

The biological effects of vitamin D are mediated via its nuclear receptor, VDR. To explore possible mechanisms of action, I examined the expression of several nuclear receptors and their modulation by cholecalciferol. These receptors include the vitamin D receptor (VDR), retinoid X receptors (RXR- α , - β , and - γ), and the androgen receptor (AR). The discovery that VDRs are a common feature of normal, benign and malignant prostatic epithelium (Miller et al., 1995) suggests that these receptors could modulate the malignant phenotype of prostate cancer *in vivo*. Prostatic epithelial cells appear to be the primary target of vitamin D (Miller et al., 1995). VDR expression is also the highest in the peripheral zone of the prostate (Krill et al., 2001). This is particularly important given that most prostate cancers develop in the peripheral zone and are of epithelial origin. Upon binding to its ligand, the VDR forms heterodimers with retinoid X

receptors (α , β , and γ) to carry out its transcriptional effects. Calcitriol enhances the formation of VDR:RXR heterodimers and promotes their binding to vitamin D response elements (VDRE). The binding of calcitriol and RXR to VDR is required to induce a VDR conformation that is competent to activate transcription. The dimerization with RXRs promotes nuclear localization of unliganded VDRs. RXRs promote nuclear accumulation of VDR and inhibit export of VDR from the nucleus. Degradation of RXRs reduces the sensitivity of cells to antiproliferative effects of calcitriol. Elevated levels of RXR- α increase the antiproliferative effects of calcitriol by increasing calcitriol-induced expression of p21, a cyclin-dependent kinase inhibitor (Prufer et al., 2000; Prufer and Barsony, 2002), while disruption of RXR- α results in the development of dysplasia in prostates of mice (Huang et al., 2002). This illustrates the importance of RXRs in treatment of prostate cancer with vitamin D.

My results show that treatment with cholecalciferol caused a marked increase in the expression of VDR. Furthermore, amongst the RXRs, the expression of RXR- α was also markedly increased. These results are consistent with the observed growth inhibition induced by cholecalciferol in RWPE2-W99 cells. In addition, these results are also consistent with reports indicating that increased RXR- α levels increase the antiproliferative effects of calcitriol (Prufer and Barsony, 2002; Prufer et al., 2000). Increasing VDR and RXR expression could increase the sensitivity of cells to both cholecalciferol and retinoids, suggesting a role for combination treatments with these two agents. Several studies have examined the effects of combination therapies using calcitriol and retinoids and found these treatments to have a synergistic effect on cell growth. We have found that combined treatment with cholecalciferol and

N-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid, resulted in a synergistic inhibition of growth of RWPE-1 cells (manuscript in preparation). These results suggest that combined treatment with cholecalciferol and 4-HPR may have enhanced chemopreventive and chemotherapeutic ability. It will be interesting to examine combination treatments with cholecalciferol and other retinoids to determine if they will have similar effects. I have not yet explored the effects of cholecalciferol on retinoic acid receptors (RARs), but this will be an important area of future study using cholecalciferol.

It is well known that early forms of prostate cancer are androgen-dependent, leading to the belief that the androgen plays an important role in the development of this disease. The androgen receptor regulates the expression of androgen-responsive genes by binding to their androgen-response elements and by interacting with other transcription factors. A notable gene regulated by androgen in the prostate is prostate specific antigen (PSA) (Crawford et al, 1996). Prostate carcinogenesis is driven by androgen, therefore, the androgen receptor is a prime target for cancer treatment and prevention. It is interesting to note that not only does calcitriol up-regulate AR expression but that the androgen 5 α -dihydrotestosterone (5 α -DHT) up-regulates VDR, indicating a possible cross-talk between VDR and AR (Ahonen et al., 2000b). My results demonstrate that cholecalciferol treatment caused an up-regulation of androgen receptor expression in RWPE2-W99 cells (Figure 8-8). The induction of AR by cholecalciferol could potentially restore androgen responsiveness to prostate cancer patients who are not responsive to current therapies. If cholecalciferol functions as a differentiating agent, perhaps the increased levels of AR represent a more differentiated phenotype. Induction of VDR and AR by cholecalciferol may explain the growth inhibitory effects of

cholecalciferol. This induction of differentiation is a positive step in using cholecalciferol in chemoprevention.

Differentiation of prostate epithelial cells to luminal, secretory cells, is associated with synthesis and secretion of prostate specific antigen (PSA). PSA has emerged as the most useful tumor marker for the screening and early detection of prostate cancer as well as a follow-up tool after therapy. PSA is a 33 kDa glycoprotein that is specific to the prostatic epithelium. It is an important component and one of the most abundant serine proteases in the seminal plasma (Webber et al, 1995). Differentiated prostate epithelial cells secrete PSA. As the total number of cells increases, serum PSA levels increase, indicating the possibility of prostate cancer (Blutt and Weigel, 1999). Cellular synthesis of PSA increases when prostate cancer cell lines are treated with $1,25(\text{OH})_2\text{D}_3$, suggesting $1,25(\text{OH})_2\text{D}_3$ may be capable of causing differentiation of prostate cells (Miller et al, 1992). Although this is seemingly inconsistent with the fact that rising serum PSA levels may be an indication of prostate cancer, lower levels of PSA will result due to the ability of $1,25(\text{OH})_2\text{D}_3$ to reduce cell number by inhibiting growth. Cholecalciferol treatment caused an up-regulation of PSA in the RWPE2-W99 and RWPE-1 cells (see Chapter 9), providing further proof that cholecalciferol induces differentiation in this cell line.

To compare the efficacy of cholecalciferol with that of calcitriol, I performed several of the same assays described above using calcitriol as the test agent. Interestingly, but not surprisingly, compared to calcitriol, cholecalciferol showed very similar results (Figures 8-3 and 8-9). The results for each agent were within normal physiological concentrations. In all assays studied, the effects of calcitriol appear to

occur at approximately a 6- to 10-fold lower concentration than cholecalciferol. Normal physiological levels of calcitriol are 0.1 nM (10^{-10} M) to 1 nM (10^{-9} M) and pharmacological levels are approximately 0.1 μ M (10^{-7} M) (Friedrich 1988; Schwartz et al., 1997). In comparison, physiological levels of cholecalciferol fall between 0.01 μ M (10^{-8} M) and 0.1 μ M (10^{-7} M) (Friedrich 1988). I am not aware of any other experimental or clinical studies using cholecalciferol, therefore, pharmacological doses may not yet have been determined. However, there has never been a report of hypercalcemia due to overexposure to sunlight, which is the main source of cholecalciferol in the body. Based on known normal physiological levels for calcitriol and cholecalciferol, and pharmacological doses for calcitriol, the concentrations used in my experiments appear to fall within the normal physiological and possibly pharmacological ranges for cholecalciferol.

The ratio of serum levels of cholecalciferol to 25OHD₃ to calcitriol is estimated to be 80:800:1 (Berginer et al., 1993), meaning 80 times the amount of cholecalciferol is needed to reach equivalent calcitriol levels. The need for higher cholecalciferol concentrations to see the same results as calcitriol in my studies could be explained simply by the conversion of cholecalciferol to calcitriol. Only a portion of 25OHD₃ gets converted to calcitriol by the 1 α -hydroxylase enzyme. The same could be possible for the conversion of cholecalciferol to 25OHD₃ by the 25-hydroxylase enzyme CYP27A1. In this study, I have demonstrated that human prostate epithelial cells constitutively express CYP27A1. Prostate epithelial cells also possess the 24-hydroxylase enzyme, the activity and production of which is increased in the presence of calcitriol (Miller et al., 1995). The 24-hydroxylase enzyme converts 25OHD₃ to 24,25-hydroxyvitamin D₃ and

also catalyzes the conversion of calcitriol to 1,24,25-hydroxyvitamin D₃, a less active metabolite than calcitriol. Therefore, some of the cholecalciferol that was converted to 25OHD₃ could be subsequently converted to 24,25-dihydroxyvitamin D₃ or 1,24,25-hydroxyvitamin D₃, instead of calcitriol. Cholecalciferol can bind directly to the VDR. However, its affinity for VDR is much lower than that of calcitriol (Chen et al., 2000). Thus, to reach the same calcitriol levels, and therefore, the same potency, one would require higher concentrations of cholecalciferol. It is also possible that cholecalciferol works through completely different mechanisms than calcitriol. My results would suggest that since treatment with cholecalciferol resulted in up-regulation of VDR, cholecalciferol works via the VDR. This finding is supported by the fact that cholecalciferol can indeed bind to the VDR (Chen et al, 2000). However, cholecalciferol could exert some of its effects also by VDR-independent mechanisms.

Based on epidemiological studies and experimental data from *in vitro* studies and *in vivo* studies with mice, clinical trials, using calcitriol, are being conducted to test its efficacy in the treatment of different cancers. Compared to other available alternatives calcitriol is a relatively safe form of cancer treatment, with the main impediment in the clinical setting being hypercalcemia and hypercalciuria. A small pilot study was carried out on patients with early recurrent prostate cancer (Gross et al., 1998). The subjects in this study exhibited no evidence of metastasis and had previously received primary therapy with radiation or surgery. The rate of PSA rise during calcitriol treatment versus before treatment was significantly decreased in 6/7 patients and one showed a decrease in serum PSA. All patients showed at least a 2-fold decrease in their PSA doubling time, while in some it was prolonged 5 to 6 fold. Some patients had stabilization of PSA levels

for greater than one year. No subject became hypercalcemic, however all subjects became hypercalciuric with two developing asymptotic renal stones (Gross et al., 1998).

An early phase II trial involving patients with advanced metastatic, hormone-refractory prostate cancer failed to see significant improvements in patients (Osborn et al., 1995). Only 2/13 of the men showed decreases in PSA levels of 25% and 45% for 30 and 45 days, respectively. The large tumor burden present before the initiation of treatment may have mitigated the effect of calcitriol. More recently, two phase I trials studied calcitriol effects on advanced, hormone refractory malignancies. While neither study saw significant anti-tumor effects, both helped determine maximum tolerable doses of calcitriol without showing hypercalcemic side effects. Subcutaneous (s.c.) injection of calcitriol every other day allowed for a maximum tolerated dose of 8 µg; none of the patients at this dose level developed hypercalcemia (Smith et al., 1999). Weekly pulse dosing of calcitriol allows substantial dose escalation, approximately 25-fold above the upper limit of physiological range, with minimal toxicity. These levels were well within the therapeutic range of calcitriol suggested by *in vitro* experiments (Beer et al., 2001a). Further studies using calcitriol need to be carried out to determine if s.c. injection or pulse dosing could have positive effects on the treatment of prostate cancer.

To ameliorate the hypercalcemic effects often caused by calcitriol, investigators have begun using vitamin D analogs and calcitriol in combination with other agents in clinical studies. One Phase I and one Phase II trial were carried out in prostate cancer patients using 1α-hydroxyvitamin D₂ (Lui et al., 2002; Lui et al., 2003). In the Phase II trial, 30% of the patients showed disease stability of greater than 6 months and the observed median survival was 630 days (21 months), which was higher than predicted by

the survival nomogram. Increases in TGF- β and PSA levels were also observed in several of the patients. TGF- β inhibits the growth prostatic epithelial cells and PSA is secreted by differentiated prostatic epithelial cells. Increases in both suggest anti-proliferative and pro-differentiating effects.

Clinical trials have been executed using calcitriol in combination with chemotherapeutic agents, such as docetaxel and paclitaxel, that attenuate the hypercalcemic effect (Beer et al., 2001b; Muindi et al., 2002). The combination treatments allowed for the use of higher doses of calcitriol, and treatments were well tolerated with no problems with hypercalcemia. In addition, PSA levels were decreased by at least 50% in all patients receiving calcitriol/docetaxel treatments (Beer et al., 2001b). Clinical studies using calcitriol or vitamin D analogs are in the early stages of development, and, therefore, only a small number of studies have been reported. Additional studies involving vitamin D and vitamin D analogs, alone and in combination, are needed. To the best of my knowledge, to date there are no clinical trials using 25OHD₃ or cholecalciferol, alone or in combination.

Epidemiological evidence strongly suggests that prostate cancer risk, development, and progression are linked with low serum vitamin D levels. Numerous studies have shown calcitriol [1,25(OH)₂D₃] and 25-hydroxyvitamin D₃ (25OHD₃) work through multiple mechanisms and pathways to reverse or inhibit the malignant characteristics of several cancer types. However, the effects of cholecalciferol, the natural precursor to 25OHD₃ and calcitriol, have not previously been examined. I present, for the first time, the effects of cholecalciferol on a tumorigenic, invasive human prostate cancer cell line, RWPE2-W99. Furthermore, I also provided evidence, for the

first time, demonstrating that prostatic epithelial cells possess the 25-hydroxylase enzyme (CYP27A1) necessary for the conversion of cholecalciferol to 25OHD₃. This indicates that prostatic epithelial cells can locally produce calcitriol and that the traditional model of calcitriol metabolism may not necessarily apply to special target cells, such as, prostate epithelial cells.

Conclusions

In conclusion, I have demonstrated that human prostate epithelial cells constitutively express 25-hydroxylase protein (CYP27A1), the enzyme known to convert cholecalciferol to 25OHD₃. This is the first time that the presence of CYP27A1 has been reported in human prostate epithelial cells. Further work will need to be done to show enzyme activity. The presence of 1 α -hydroxylase, which converts 25OHD₃ to calcitriol, has been previously shown. Taken together, these results show that human prostate epithelial cells, a target cell type for vitamin D, have the necessary enzymes and the rare ability to locally convert cholecalciferol to calcitriol. My results also show that physiological levels of cholecalciferol: i) inhibit both anchorage-dependent and —independent growth; ii) induce differentiation by increasing PSA expression and decreasing vimentin expression; iii) decrease MMP-9 and MMP-2 activity and vimentin expression with concomitant decrease in invasion and motility; and iv) cause a dose-dependent up-regulation of VDR and RXR- α , suggesting that the observed effects are receptor-mediated.

Based on similarities between results obtained from cholecalciferol and calcitriol, my results show that several of the mechanisms are potentially the same. Whether

cholecalciferol and calcitriol share any other effects or mechanisms of action in the treatment of prostate cancer cells remains to be discovered. Furthermore, the effects of cholecalciferol appear to be receptor-mediated, which has been proven to be the case with several of the effects of calcitriol. Based on the findings in this study, cholecalciferol appears to reverse or inhibit several characteristics involved in the malignant transformation of human prostate cancer cells. Therefore, cholecalciferol could hold much potential in the future for prostate cancer prevention and treatment. Further *in vitro* and *in vivo* studies will help to fully elucidate all the mechanisms of action and determine the full potential of cholecalciferol, as well as determine similarities between cholecalciferol and calcitriol in the treatment and prevention of prostate cancer. This promises to be an extremely fertile area in the field of cancer research.

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Chapter 9

Cholecalciferol (vitamin D₃) and Chemoprevention of Prostate Cancer

Abstract

Mortality from prostate cancer remains unacceptably high. Chemoprevention may be the ultimate answer to this problem. Prostate carcinogenesis is a multi-step process which appears to occur over a period of 20-30 years. This long latency period, before its manifestation, allows for several points of intervention to prevent disease development. The objective of this study was to examine the chemopreventive ability of cholecalciferol (vitamin D₃) to block or reverse changes associated with malignant transformation in human prostate epithelial cells. Immortalization is an early step in carcinogenesis. Therefore, I used the RWPE-1 human prostate epithelial cell line, which is an immortalized but a non-tumorigenic cell line. This makes it a useful model for studies on chemoprevention of prostate cancer. Unlike the tumorigenic RWPE2-W99 cells, RWPE-1 cells do not grow in an anchorage-independent manner and are not invasive. Results show that physiological levels of cholecalciferol: i) inhibit anchorage-dependent growth; ii) decrease MMP-9 and MMP-2 activity; iii) induce differentiation by decreasing vimentin expression and increasing PSA expression; iv) up-regulate VDR, RXRs, and AR, suggesting that its effects are receptor-mediated. These results demonstrate that, based on *in vitro* studies, cholecalciferol is an effective chemopreventive agent. Furthermore, I have shown that RWPE-1 cells constitutively express 25-hydroxylase (CPYP27A1) protein, and that this expression is up-regulated in response to treatment with cholecalciferol. The presence of 25-hydroxylase in human prostate epithelial cells has not previously been shown. This suggests that prostate epithelial cells possess the ability to locally produce the biologically active calcitriol from

cholecalciferol. Based on these data, I propose that cholecalciferol could be an effective **agent** for the chemoprevention of prostate cancer.

Introduction

Prostate cancer is the most common non-skin cancer in American men. Its incidence increases sharply with age from about the age of 60 onwards and more than 80% of prostate cancer is found in men over the age of 65 (Schwartz and Hulka, 1990). Approximately 8% of American men are diagnosed with prostate cancer during their lifetime and about 2-3% of all deaths in men are the results of this disease (O'Shaughnessy et al., 2002). Although prostate cancer grows more slowly than most other cancers, and even with early detection by PSA screening, mortality rates due to this disease remain high. As a result, prostate cancer has become a major health concern among men in recent years.

Carcinogenesis in the prostate is a multi-step process which takes several years before its culmination to invasive carcinoma. This multi-year, multi-step process provides a large window of opportunity for chemoprevention because it permits intervention at several steps to block or reverse the development of the disease. Pre-cancerous lesions, known as prostatic intraepithelial neoplasia (PIN), may develop over a period of 20 years and may precede cancer by at least 10 years (O'Shaughnessy et al., 2002; Sakr et al., 1993). The presence of PIN is an indicator for increased risk of prostate cancer and PIN lesions are considered to be precursors of prostate cancers. Studies have shown that patients with high-grade PIN (HGPIN), but no detectable cancer, progressed to a 40% incidence of cancer in three years and to approximately 80% incidence in 10 years (Sakr et al., 2000). Preventing or reversing these pre-cancerous lesions reduces the risk of invasive and metastatic prostate cancer, the primary cause of

death from this disease. Because of the 20-30 year latency period before its manifestation, it may be possible to prevent the development of prostate cancer.

Chemoprevention is the use of drugs or natural agents to inhibit, delay, or reverse the process of carcinogenesis before invasion and metastasis (Hong and Sporn, 1997; O'Shaughnessy et al., 2002). Chemoprevention is recognized as a promising and important area in cancer research and may be the ultimate answer to solving the prostate cancer problem (Hong and Sporn, 1997; Lippman et al., 1998; Quader et al., 2001; Webber et al., 1999). Recent advances in understanding the mechanisms involved in carcinogenesis have led to the discovery and development of new chemopreventive agents. Dietary compounds are of particular interest because of their low cost and expected safety for long-term administration to healthy people for chemoprevention. These agents, including vitamin D and retinoids, are effective due to their effects on several cancer cell characteristics, which include inhibition of cell proliferation, induction of differentiation and apoptosis, modulation of tumor suppressor and intermediate filament protein expression, inhibition of anchorage-dependent and -independent growth, decrease in protease activity, and inhibition of motility and invasion.

Calcitriol [1,25-dihydroxyvitamin D₃], the most biologically active form of vitamin D₃, has been shown to have chemopreventive activity on prostate cancer cells through a variety of effects on different cancer cell characteristics. Some of these effects include the inhibition of cell proliferation, induction of differentiation and apoptosis, and inhibition of protease activity and invasion (Guzey et al., 2002; Miller et al., 1992; Schwartz et al., 1997; Skowronski et al., 1993; Zhuang and Burnstein, 1998). However, calcitriol treatment is generally accompanied by hypercalcemia. Chemopreventive agents

should have low toxicity in order to be clinically useful. Therefore, alternative vitamin D treatments are being tested, including calcitriol precursors and vitamin D analogs. These less calcemic alternatives have proven to be just as, and sometime more, effective as calcitriol.

Vitamin D exerts its effects through genomic and non-genomic mechanisms. The activity of vitamin D is thought to be mediated primarily through its binding to the vitamin D receptor (VDR). VDR is a ligand-activated transcription factor and a member of the steroid hormone-receptor superfamily (Mangelsdorf et al., 1995). VDR is expressed in approximately 30 tissues, including prostate, breast, colon, and skin (Minghetti and Normal, 1988). This wide distribution of VDR is the underlying reason behind the ability of vitamin D to exert its pleiotropic effects throughout the body. The genomic effects are mediated via the nuclear vitamin D receptor (VDR). The ligand-VDR complex forms heterodimers with retinoid X receptors (RXRs). The formation of VDR:RXR heterodimers and their binding to VDREs are enhanced by the presence of calcitriol. The heterodimers bind to the vitamin D response elements (VDREs) in the target genes to carry out transcriptional activity. Functional VDREs are found in the promoter region of several genes. These include several bone-related genes (e.g. osteocalcin, osteopontin, calbindin-D28k and D9k), cell cycle regulator p21, 25OHD₃ 24-hydroxylase, urokinase plasminogen activator, transforming growth factor β 2 and fibronectin, just to name a few (Beer and Myrthue, 2004).

In addition to the classic genomic effects of VDR, the presence of a non-genomic pathway has been demonstrated in a number of different tissues. Modulation of several cytoplasmic pathways by vitamin D results in rapid biological responses, including

inhibition of cell proliferation, stimulation of differentiation, and apoptosis (Beer and Myrthue, 2004). These pathways may interact with the classic genomic pathways through the nuclear VDR. There is evidence of a membrane-bound VDR, which may mediate the generation of non-genomic effects (Nemere et al., 1993). However, the existence of a membrane-bound VDR is still controversial. Possible alternatives to a specific membrane-bound VDR include membrane-associated annexin II that would serve as a receptor for vitamin D or extracellular binding sites for the VDR-ligand complex (Baran et al., 2000; Kim et al., 1996).

The majority of established human prostate cancer cell lines currently being used (i.e. LNCaP, PC-3, DU-145) are from biopsies of metastatic prostate cancer. These cell lines are more appropriate for examining agents that could be used for the treatment of advanced prostate cancer. The non-tumorigenic human prostate epithelial cell line, RWPE-1, was derived by immortalization of prostatic epithelial cells isolated from the peripheral zone of a normal human prostate (Bello et al., 1997; Webber et al., 1997). This cell line shares many characteristics with normal prostate epithelial cells, but also mimics an early pre-neoplastic step in prostate carcinogenesis because it is an immortalized cell line. RWPE-1 is, therefore, an appropriate cell model for the study of prostate cancer prevention.

In the present study, I have elected to examine the effects of cholecalciferol (vitamin D₃), a natural calcitriol precursor, on the RWPE-1 human prostate epithelial cell line. My long term objective is to determine the efficacy of cholecalciferol as a potential agent in the chemoprevention of prostate cancer. In this study, I have examined the ability of cholecalciferol to block or reverse changes that occur in pre-neoplastic prostate

epithelial cells. The effects of cholecalciferol on anchorage-dependent growth, vimentin expression, matrix metalloproteinase (MMP) activity, expression of vitamin D receptor (VDR), retinoid X receptors (RXRs), androgen receptor (AR), and prostate specific antigen (PSA), and the presence and increase in the 25-hydroxylase enzyme (CYP27A1) activity were examined to determine potential mechanisms of action. I also provide the first reported evidence for the ability of prostatic epithelial cells (RWPE-1) to express the 25-hydroxylase enzyme, CYP27A1, necessary to convert cholecalciferol to calcitriol. A comparison between the efficacy of cholecalciferol and calcitriol was also made in several of the assays. These experiments will help to determine the effectiveness and mechanisms of action of cholecalciferol in prostate cancer chemoprevention.

Materials and Methods

All the material and methods used in this chapter are identical to those used in Chapter 8. Therefore, only a brief summary of materials and methods is given in this chapter. For more detail on buffers, reagents, protocols, catalog numbers and suppliers, please refer to Chapter 8.

Cells and Cell Culture

The RWPE-1 cell line is a non-tumorigenic, non-invasive human prostate epithelial cell line. It was derived from the peripheral zone of a normal human prostate and immortalized with a single copy of the human papilloma virus-18 DNA (HPV-18) (Bello et al., 1997; Webber et al., 1997). Cells were maintained in complete keratinocyte serum free medium (KSFM; Cat. #: 10724-011) containing 50 µg/ml BPE and 5 ng/ml

EGF (Cat. #: 37000-015), and 1X antibiotic/antimycotic mixture (Penicillin 100U/ml, Streptomycin 100 µg/ml, and Fungizone 0.25 µg/ml) (Gibco/Invitrogen, Rockville, MD). Medium was changed every 48 h. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. When confluent, cells were passaged and seeded at 1x10⁶ cells/T-25 flask, or 2x10⁶ cells/T-75 flask.

Dose Response

The effects of cholecalciferol (Sigma, St Louis, MO, Cat. #: C-1357) or calcitriol (Sigma, Cat. #: D-1530) on the growth of RWPE-1 and RWPE2-W99 cells were examined using a 96-well microplate assay. Cells were plated at a density of 10,000 cells/well, 6 wells/treatment, in a 96-well plate (BD Falcon). 48 h after plating, cells were treated with cholecalciferol or calcitriol. Test media were changed every 48 h. After 5 days of treatment, cells were stained with MTT for 3.5 h at 37°C. Absorbance was read at 540 nm. Ethanol-treated wells were set to 100% growth and served as the controls to which all other treatments were compared. Six independent experiments were run for each cell line.

SDS-PAGE Zymography for MMP activity

To examine the effects of cholecalciferol on MMP-9 and MMP-2 activity in RWPE-1 cells, conditioned medium was collected and analyzed. Cells were plated in complete KSFM medium at a density of 1 X 10⁶ cells/60 mm dish (BD Falcon), in duplicate dishes for each treatment. 48 h after plating, cells were washed twice with D-PBS. Next 2.2 ml of basal KSFM (no EGF or BPE), containing desired cholecalciferol

or calcitriol treatments were added to the dishes. Cells were treated for 48 h at 37°C. Conditioned medium from duplicate dishes was pooled and collected and aliquoted. The protein content of each sample was determined using the Lowry High protein assay. Samples of conditioned media were stored at -70°C until needed.

To determine the effects of the test agents on MMP-2 and MMP-9 activity, gelatin-based SDS-PAGE zymography was performed. The separating and stacking gels were made using the established protocol (see Chapter 8, pg. 241) and poured into 0.75 mm casting plates. The final gelatin concentration in the gels was 0.1 %. The gels were allowed to polymerize overnight at 4°C before use. 2.0 µg of protein were loaded/lane. Gels were electrophoresed (4°C) at 157 volts for 1.5 h. The gels were then treated with 2.5% Triton-X 100 for 1h to remove the SDS. The gels were then incubated (37°C) for 18 h in soaking buffer in order to re-nature the enzymes and allow them to lyse the gelatin. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 and de-stained in methanol:acetic acid:dH₂O (4:1:5) solution. Bands of lysis, indicating protease activity, appear as light bands against a dark purple background. The area of each band was measured densitometrically and compared with the control to determine the effect of the test agents on MMP-9 and MMP-2 activity.

Immunostaining

Immunocytochemistry was conducted to examine the effects of cholecalciferol on the expression of the following: intermediate filament protein vimentin (Sigma, Cat. #: V-6630); the nuclear receptors VDR (Oncogene, San Diego, CA, Cat. #: GR37), RXR-α (Santa Cruz Biotechnology, Santa Cruz, California, Cat. #: sc-553), RXR-β

(Santa Cruz, Cat. #: sc-831), and RXR- γ (Santa Cruz, Cat. #: sc-555). Monoclonal antibodies for vimentin and VDR and polyclonal antibodies for the RXRs and a modified avidin-biotin immunoperoxidase protocol were used (Bello et al., 1997). Cells were plated in 1 ml of complete KSFM on sterile, acid washed glass coverslips in 24-well plates at a density of 7,500 cells/well. 48 h after plating, the medium was replaced with medium containing the desired cholecalciferol concentration. The following concentrations of cholecalciferol were used: 1 nM (10^{-9} M), 10 nM (10^{-8} M), 100 nM (10^{-7} M), and 1 μ M (10^{-6} M). Ethanol treated cells were used as controls. Test media were changed every 48 h. After 5 days of treatment the cells were washed with D-PBS and fixed with a solution of 1:1 methanol (100%)/acetone for 2 min. The coverslips were stored at -20°C until needed. All of the following steps were carried out at room temperature: cells were blocked for 1 h with normal horse serum (Vector Labs, Burlingame, CA), incubated with primary Ab diluted in blocking solution, incubated for 30 min with a biotinylated secondary antibody diluted 1:200 in blocking solution, treated with 3% H_2O_2 to quench endogenous peroxidase activity, incubated with ABC (Vector) for 30 min, and developed with DAB substrate kit for peroxidase (Vector) for 1-3 min. Cells were washed 2X, 8 min each, between all successive steps after incubation with the primary Ab. Cells not incubated in primary Ab but incubated in secondary Ab served as controls. Coverslips were dehydrated and mounted on alcohol-washed microscope slides using Permount. Antibody dilutions and incubation times were as follows: vimentin 1:40 (1 h), VDR 1:100 (2 h), RXR- α , - β , and - γ 1:100 (2 h).

Protein Analysis

To quantify proteins, a Beckman DU Series 530 Spectrophotometer was used for protein assays. Samples were compared to standard concentrations of 0.0, 0.1, 0.5, 1.0, 1.5, and 2.0 $\mu\text{g/ml}$ prepared from the Albumin Standard (bovine serum albumin, 2.0 mg/ml, Pierce). The assays were run using the Lowry High protein assay method.

Western blot analysis

Western blot analysis was performed in order to examine the effects of cholecalciferol on VDR, RXR- α , β , and γ , AR and PSA, and CYP27A1 and the effects of calcitriol on VDR protein expression in RWPE-1 cells. Cells were plated at a density of 500,000 cells/T-25 flask in duplicate flasks. 48 h after plating, the medium was removed and replaced with the test media. The cells were treated for 4 days, incubated with 2.0 ml trypsin/EDTA (diluted 1:1 with D-PBS) for 7 min, and dislodged by tapping. Cells from duplicate flasks were pooled and collected. Nuclear and cytoplasmic extracts were prepared using the NE-PER kit according to the manufacturer's protocol (Pierce). Extracts were aliquoted and frozen at -70°C . The Lowry High protein assay method was used to determine protein content of each sample. Samples containing 10 μg (vimentin), 20 μg (VDR), 25 μg (RXRs and AR), and 40 μg (CYP27A1 and PSA) of protein were run in NuPAGE 10% Bis-Tris pre-cast gels (Invitrogen, Carlsbad, CA) at 175 V for 1.5-1.7 h at 4°C . Proteins were transferred to Immobolin-P transfer membranes (0.45 μm pore size) (Millipore Corp., Bedford, Maryland) at 30 V for 1-1.5 h at 4°C . For immunoblotting, monoclonal antibodies were used for vimentin, VDR, AR (Santa Cruz, Cat. #: sc-7305), and prostate specific antigen (PSA) (Dako Cytomation, Carpinteria, CA,

Cat. #: M-0750), and polyclonal antibodies were used for RXRs and CYP27A1 (Santa Cruz, Cat. #: sc-14835). All blots were stained using avidin-biotin immunoperoxidase ABC kit (Vector) and developed using DAB kit (Vector) as described in Chapter 8. Blots were analyzed with the ImageJ analysis program from the National Institutes of Health (NIH). Individual lanes were compared to control lane for changes in protein expression. Antibody dilutions and incubation times were as follows: VDR 1:1000 (2 h), RXR- α (1:100), RXR- β (1:250), and RXR- γ (1:200) (each 2 h), AR 1:500 (2 h), CYP27A1 1:100 (1 h), PSA 1:100 (24 h).

Statistical Analysis

Results in this study were obtained from three to six independent experiments. Results are expressed as means with standard deviations (SD). Differences between the means were considered significant if $p < 0.05$. One-way analysis of variance (ANOVA) was used to compare cholecalciferol effects on the growth of RWPE-1 and RWPE2-W99 cell lines. Response to individual concentrations was compared to controls using unpaired t-test with Welch correction. GraphPad InStat #3 was used for these analyses.

Results

Cholecalciferol inhibits the growth of RWPE-1 and RWPE2-W99 cells:

Dose response experiments were first run to determine the effects of cholecalciferol on anchorage-dependent growth of RWPE-1 and RWPE2-W99 cells. Cells were plated in 96-well plates and treated for 5 days with cholecalciferol as described in Materials and Methods. Results show that cholecalciferol causes a dose-dependent growth inhibition of both RWPE-1 and RWPE2-W99 cells (**Figure 9-1**). The

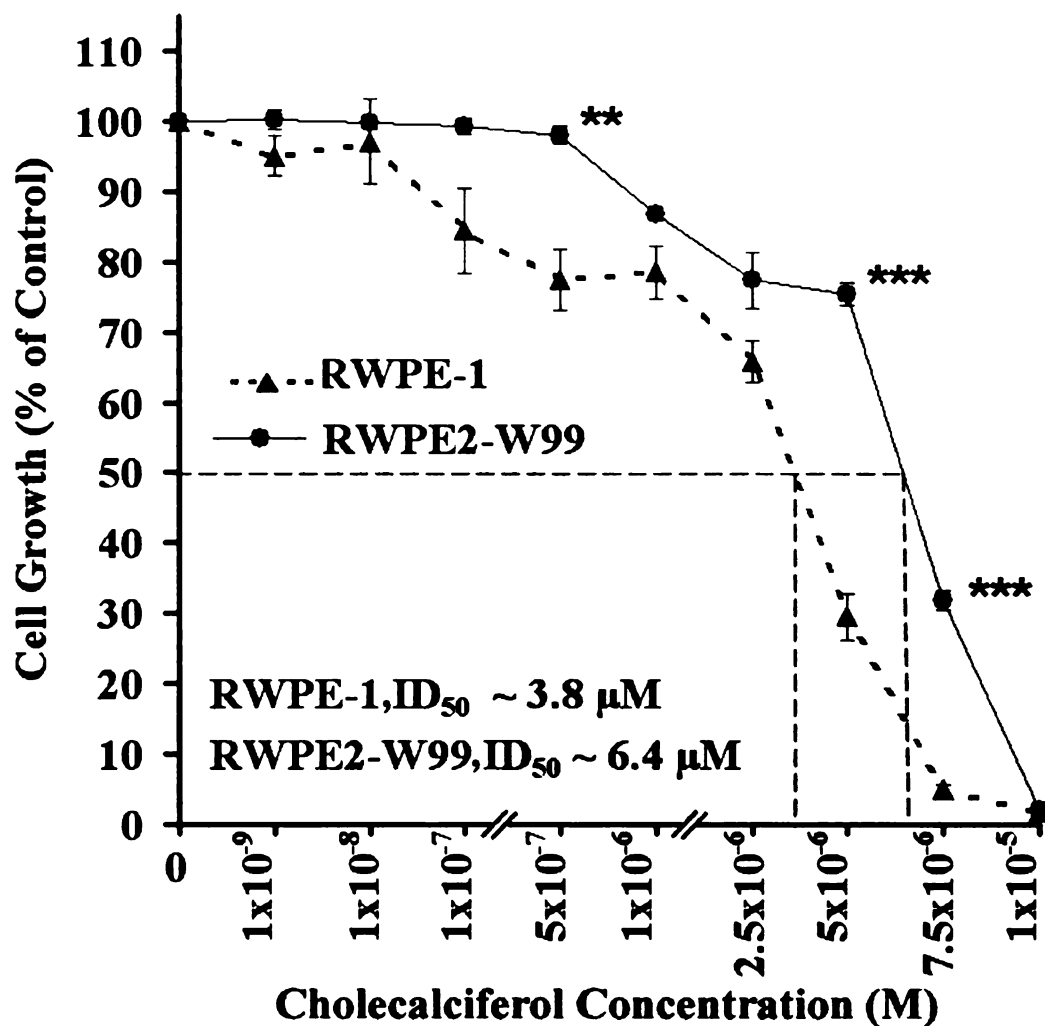
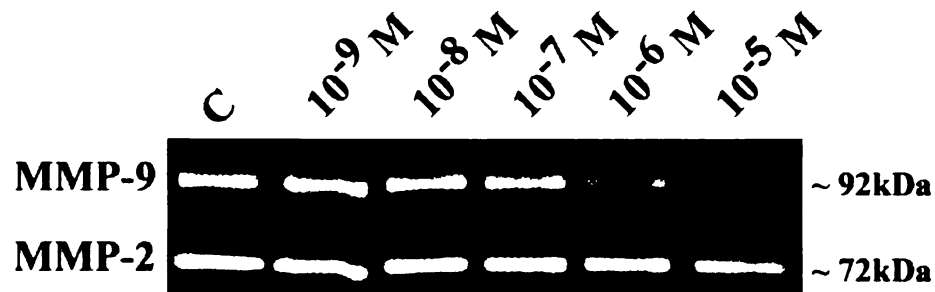


Figure 9-1: The effects of cholecalciferol on anchorage-dependent growth of the non-tumorigenic RWPE-1 and the malignant RWPE2-W99 cells lines. 48 h after plating 1×10^4 cells/well in 96-well plates, cells were treated with cholecalciferol. After 5 days of treatment, cells were stained with MTT. Absorbance was read at 540 nm, $\pm$ SD, (n = 6). The dashed line shows ID₅₀ of ~3.8 μM for RWPE-1 and ~6.4 μM for RWPE2-W99 cells. ** $p = 0.01$; *** $p < 0.001$

non-tumorigenic RWPE-1 cells are more sensitive to cholecalciferol treatment than RWPE2-W99. A significant difference (** $p = 0.01$; *** $p < 0.001$) in response between the two cell lines can be seen starting at concentrations as low as $0.5 \mu\text{M}$ ($5 \times 10^{-7} \text{ M}$). The ID_{50} concentrations were $\sim 3.8 \mu\text{M}$ for RWPE-1 and $\sim 6.4 \mu\text{M}$ for RWPE2-W99. These results show that the non-tumorigenic RWPE-1 cells are more responsive, and show greater growth inhibition upon exposure to cholecalciferol treatment, than the malignant RWPE2-W99 cells when grown in an anchorage-dependent manner. These results were used to determine the cholecalciferol concentrations used in all subsequent assays.

Cholecalciferol inhibits MMP-9 and MMP-2 activity:

Gelatin-based (0.1%) SDS-PAGE zymography was performed to examine the effects of cholecalciferol on the gelatinolytic activity of MMP-9 and MMP-2. Results in **Figure 9-2** show that cholecalciferol markedly decreases the activity of both MMP-9 and MMP-2 in conditioned medium of RWPE-1 cells. The densitometric analysis of the zymogram shows a marked dose-dependent decrease in MMP activity with maximum inhibition of 70% and 44% for MMP-9 and MMP-2, respectively, at 10^{-5} M concentration, when compared to ethanol-treated controls.



Densitometric Analysis:

MMP-9	100%	106%	94%	72%	40%	30%
MMP-2	100%	95%	98%	98%	80%	56%

Figure 9-2: SDS-PAGE zymography (10% polyacrylamide gel containing 0.1% gelatin) was used to analyze MMP-9 and MMP-2 activity in conditioned medium of RWPE-1 cells treated for 48 h with cholecalciferol. 2.0 µg of protein were loaded/lane. Densitometric analyses, with ethanol-treated controls taken as 100%, are shown below the zymogram.

Cholecalciferol decreases vimentin expression in RWPE-1 cells:

The effect of cholecalciferol on the expression of vimentin was examined by immunostaining. Cells were treated for 5 days. The results in **Figure 9-3** show that cholecalciferol causes a dose-dependent decrease in vimentin expression. Ethanol-treated control cells show strong staining for vimentin while the cholecalciferol treated cells show a decrease in staining for vimentin.

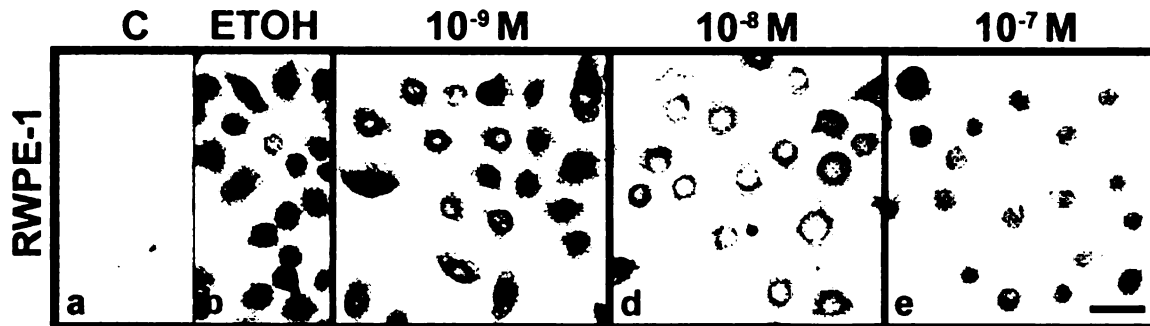


Figure 9-3: Effect of cholecalciferol on the expression of intermediate filament protein vimentin. Immunostaining results show that cholecalciferol treatment causes a dose-dependent decrease in vimentin expression. Treatment concentrations are shown above their respective square. C = ethanol-treated control; (a) is a negative control lacking primary antibody, ETOH = ethanol-treated control. Bar = 20 μ m.

Cholecalciferol up-regulates VDR, RXRs, and AR nuclear receptors:

Immunostaining results for VDR and RXRs after cholecalciferol treatment can be seen in **Figure 9-4**. Cholecalciferol treatment appears to up-regulate the expression of VDR, RXR- α , RXR- β , and RXR- γ , in a dose-dependent manner. Western blot analyses in **Figure 9-5** confirm the immunostaining results. Cholecalciferol treatment causes a marked dose-dependent up-regulation of VDR and RXR- α and RXR- γ . RXR- β showed barely detectable levels of expression in the control and at lower cholecalciferol concentrations but was markedly up-regulated at 10^{-6} M cholecalciferol. An up-regulation in the expression of androgen receptor (AR) can also be seen in **Figure 9-5** at 10^{-8} M and 10^{-7} M level.

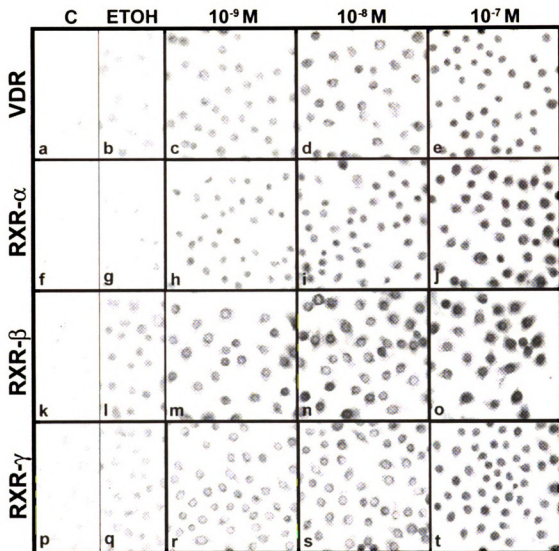


Figure 9-4: Immunostaining for nuclear receptors in RWPE-1 cells treated with cholecalciferol. Cells were treated for 5 days. VDR (a-e), RXR- α (f-j), RXR- β (k-o), and RXR- γ (p-t) all demonstrate a marked increase in expression when compared to their respective ethanol-treated control cells (b,g,l and q). Treatment concentrations used are 10^{-9} M (c,h,m,r), 10^{-8} M (d,i,n,s) and 10^{-7} M (e,j,o,t). (a,f,k,p) are controls lacking primary antibody. Cells were plated on sterile, glass coverslips at density of 7,500 cells/coverslip. Bar = 20 μ m.

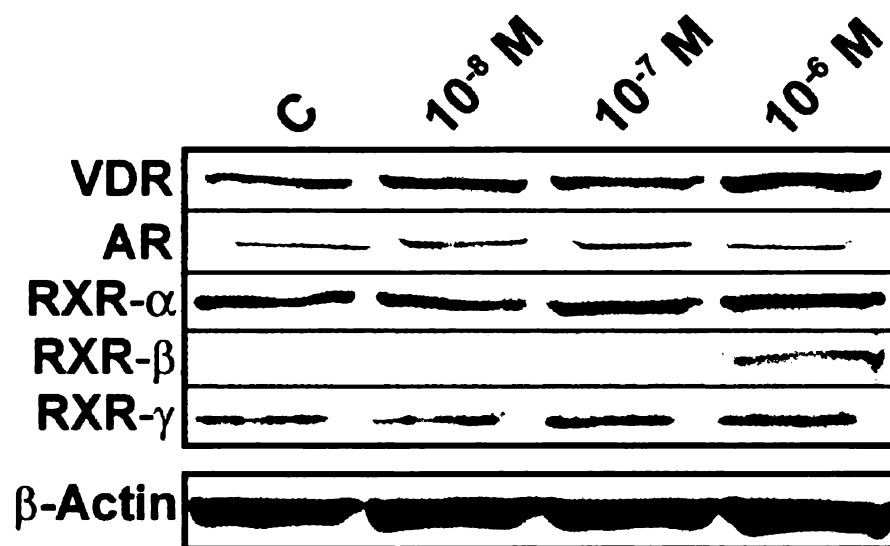


Figure 9-5: Western blot analysis showing the effects of cholecalciferol on nuclear receptors. Lane 'C' represents the control. Cholecalciferol concentrations are shown above their respective lanes. Results show that cholecalciferol treatment causes a dose-dependent up-regulation of VDR, RXR- α , and RXR- γ . Very low levels of RXR- β were detected until a marked up-regulation was seen at the 10^{-6} M concentration. For VDR 20 μg and RXRs and AR 25 μg of protein were loaded/lane. β -actin was used as the loading control.

Cholecalciferol induces expression of PSA in RWPE-1 cells:

Western blot analysis was conducted to examine the effects of cholecalciferol on the expression of PSA. Results in **Figure 9-6** show that PSA expression is up-regulated in a dose-dependent manner in cells treated with cholecalciferol.

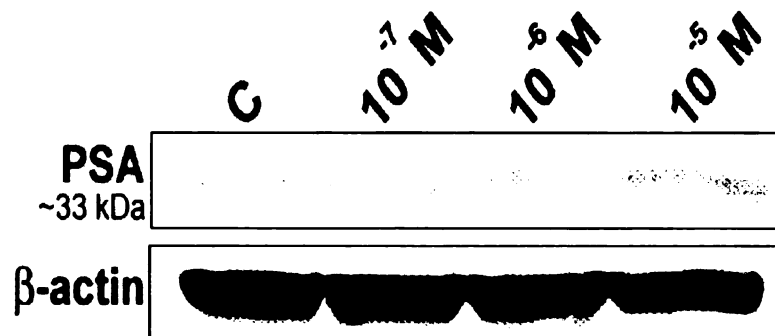


Figure 9-6: Western blot for PSA expression in RWPE-1 cells treated with cholecalciferol. Lane 'C' represents the control. Cholecalciferol concentrations are shown above their respective lanes. Results show that cholecalciferol causes an up-regulation in the expression of PSA. 40 μ g of protein was loaded/lane. β -actin was used as the loading control.

Cholecalciferol induces the expression of 25-hydroxylase enzyme, CYP27A1:

Western blot analysis was performed to examine the ability of cholecalciferol to induce 25-hydroxylase, CYP27A1, which converts cholecalciferol to 25OHD₃. Using a polyclonal antibody against CYP27A1, a single band at MR ~54 kDa was detected. The molecular weight of the human CYP27 enzyme has been shown to be ~54 kDa. As seen in **Figure 9-7**, CYP27A1 is constitutively expressed in the control RWPE-1 cells (lane C). In addition, CYP27A1 enzyme expression is up-regulated, in a dose-dependent manner, to over 200% of ethanol-treated control at the 10⁻⁵ M concentration.

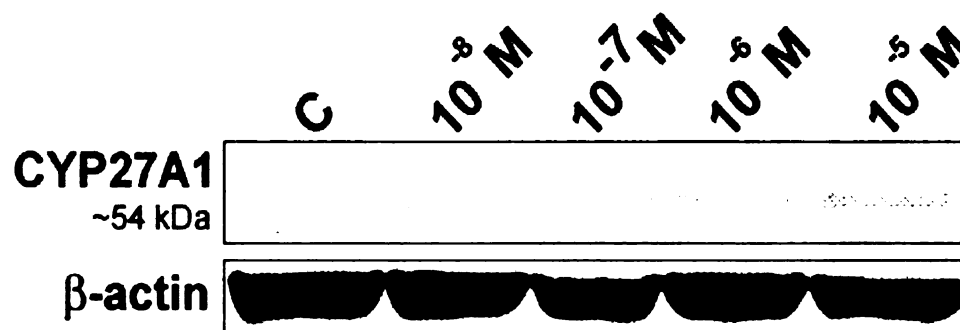


Figure 9-7: Western blot for 25-hydroxylase enzyme, CYP27A1, in RWPE-1 cells treated with cholecalciferol. Lane 'C' represents the control. Cholecalciferol concentrations are shown above their respective lanes. Results show that while the enzyme is present in untreated cells it is also up-regulated upon cholecalciferol treatment. 40 µg protein were loaded/lane. Molecular weight of CYP27A1 is shown to be ~54kDa. β-actin was used as the loading control.

Effects of calcitriol:

In order to compare the efficacy of cholecalciferol, several of the assays were also conducted using calcitriol-treated cells. **Figure 9-8A** shows the results of the calcitriol dose response assay. The RWPE-1 cells were more sensitive to calcitriol treatment with an ID₅₀ of ~1.25 µM. This is compared to the ID₅₀ of ~3.8 µM for cholecalciferol-treated cells (see **Figure 9-1**). Results from calcitriol zymograms are shown in **Figure 9-8B**. Calcitriol treatment caused a dose-dependent decrease in MMP-9 and MMP-2 activity, similar to cholecalciferol zymograms. Western blot analysis of VDR expression is seen in **Figure 9-8C**. The results show that calcitriol caused a dose-dependent up-regulation of VDR, similar to that seen in cholecalciferol-treated cells. However, when compared to cholecalciferol, calcitriol is approximately 3-10 fold more effective in the different assays performed. VDR was up-regulated at cholecalciferol concentrations up to 10⁻⁷ M,

however, with further increase in concentration to 10^{-6} M, VDR was then down-regulated. Over-stimulation of receptors often leads to a down-regulation.

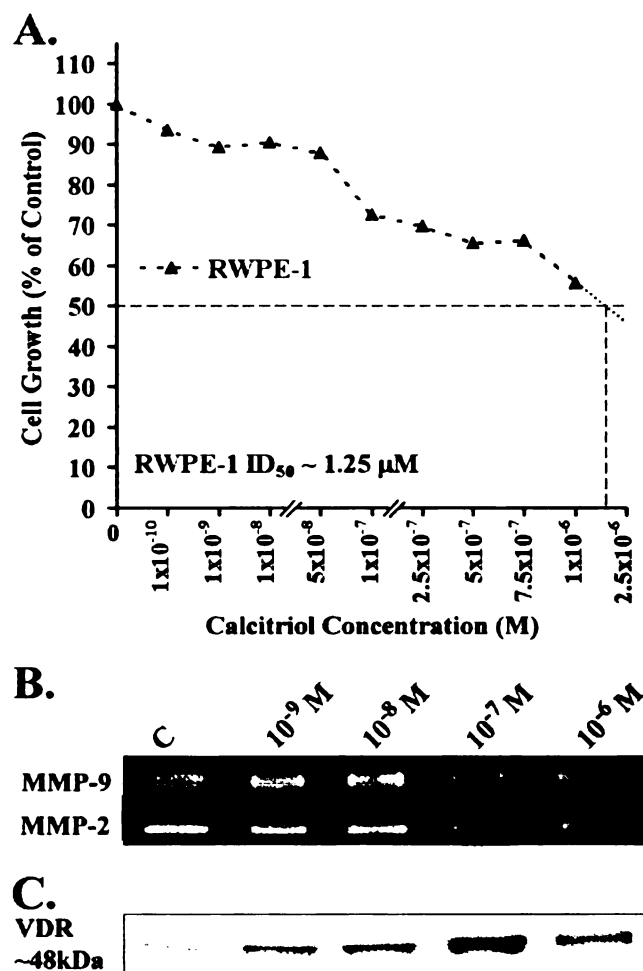


Figure 9-8: Effects of calcitriol on (A) anchorage-dependent growth, (B) MMP activity, and (C) VDR expression in RWPE-1 cells. (A) Cells were plated at a density of 1×10^4 cells/well in 96-well plates. 48 h after plating cells were treated with cholecalciferol. Plates were stained with MTT after 5 days of treatment. Absorbance was read at 540 nm. The dashed line shows ID_{50} of $\sim 1.25 \mu M$. (B) The activity of MMP-9 and MMP-2 was analyzed by SDS-PAGE zymography of conditioned medium from RWPE-1 cells treated for 48 h with cholecalciferol. $2.0 \mu g$ of protein were loaded/lane. (C) Western blot for VDR in calcitriol-treated RWPE-1 cells shows a dose-dependent up-regulation of VDR expression with increasing calcitriol concentrations up to 10^{-7} M. $20 \mu g$ protein were loaded/lane.

Discussion

Chemoprevention is the prevention of cancer by either preventing or treating pre-malignant lesions. Because of the 20 or more years between initiation and the manifestation of prostate cancer, chemoprevention is, perhaps, the most practical approach to the prostate cancer problem. A great deal of interest in examining the efficacy of vitamin D as a chemopreventive and chemotherapeutic agent has been shown since it was first hypothesized that vitamin D levels are associated with prostate cancer (Schwartz and Hulka, 1990). A wealth of data has been accumulated and through these numerous studies it has been demonstrated that there are several mechanisms underlying the chemopreventive activity of calcitriol [$1,25(\text{OH})_2 \text{D}_3$] (for reviews see Krishnan et al., 2003; Osborne and Hutchinson, 2002; Polek and Weigel, 2002). Despite the effectiveness of calcitriol, its clinical use is limited because it causes elevation in serum calcium levels (hypercalcemia). As a result, the effects of vitamin D analogs and 25-hydroxyvitamin D_3 (25OHD_3), the immediate precursor to calcitriol, have been examined. These less calcemic, alternative agents exert their effects through many of the same mechanisms and have similar, sometimes better, potency than calcitriol (Barreto et al., 2000; Polek and Weigel, 2002).

The majority of established human prostate cancer cell lines currently being used (i.e. LNCaP, PC-3, DU-145) were developed from biopsies of metastatic prostate cancer. Therefore, they are not appropriate models to study the effects of chemopreventive agents on prostate cancer. These cell lines are more appropriate for examining agents that may be used for the treatment of advanced prostate cancer. The human papillomas virus-18 (HPV-18) immortalized cell line, RWPE-1, was derived from prostatic epithelial cells

isolated from the peripheral zone of a normal human prostate (Bello et al., 1997; Webber et al., 1997). This cell line shares many characteristics with normal prostate epithelial cells, but also mimics early stages of prostate carcinogenesis. Therefore, RWPE-1 is an appropriate cell model for the studying the effects of agents with chemopreventive potential.

Ninety to 100% of the vitamin D in the human body comes from the *in vivo* synthesis in the skin (Holick 2002). According to the traditional model of vitamin D synthesis, vitamin D₃ in the skin is transported to the liver and converted to 25OHD₃ by the 25-hydroxylase enzyme, CYP27. The liver is the major site of 25OHD₃ synthesis. From the liver, 25OHD₃ is transported to the kidney where it is converted to 1,25(OH)₂D₃ by the 1 α -hydroxylase enzyme. My results show (**Figure 9-7**) that the RWPE-1 human prostatic epithelial cells constitutively express 25-hydroxylase. To the best of my knowledge, this is the first reported evidence of 25-hydroxylase in cells other than kidney, liver, and keratinocytes (Bikle et al., 1986; Lehmann et al., 1999). Prostatic epithelial cells possess 1 α -hydroxylase activity (Schwartz et al., 1998). Therefore, possessing two enzymes, the 25-hydroxylase and 1 α -hydroxylase, involved in the synthesis of calcitriol, would allow prostatic epithelial cells to locally produce calcitriol from cholecalciferol. Given the antiproliferative and differentiating effects of calcitriol, local production of calcitriol in pre-neoplastic prostate cells could prevent carcinogenesis at an early stage, thus, indicating that cholecalciferol has chemopreventive potential.

In the present study, I have demonstrated that cholecalciferol inhibits, in a dose-dependent manner, the growth of the non-tumorigenic RWPE-1 and the malignant RWPE2-W99 human prostate cancer cell lines (**Figure 9-1**). Interestingly, the non-

tumorigenic RWPE-1 cells are more sensitive to cholecalciferol treatment. Transforming growth factor- β (TGF- β) regulates several cellular processes, including cell proliferation and differentiation of prostate epithelial cells (Culig et al., 1996). We have previously shown that TGF- β inhibits the growth of RWPE-1 cells, and to a lesser degree, the growth of RWPE2-W99 cells (Bello et al., 1997). It has been shown that calcitriol induces TGF- β (Danielpour, 1996; Murthy and Weigel, 2004). Elevation of TGF- β production and signaling by calcitriol is thought to be involved in the inhibition of proliferation and induction differentiation in prostatic epithelial cells (Danielpour, 1996; Murthy and Weigel, 2004). It is possible that induction of TGF- β is also involved in the inhibition of growth of RWPE-1 and RWPE2-W99 cells by cholecalciferol.

The 1α -hydroxylase enzyme is involved in the synthesis of calcitriol and its expression in human prostate epithelial cells has been shown (Schwartz et al., 1998). Compared to normal prostate epithelial cells, BPH and prostate cancer cells and cancer cell lines demonstrate a marked decline in the activity of this enzyme (Hsu et al., 2001; Whitlach et al., 2002). This suggests that, compared to the malignant RWPE2-W99 cells, the non-tumorigenic RWPE-1 cells may have a higher capacity to convert cholecalciferol to calcitriol and, thus, would be more growth inhibited by cholecalciferol treatment. The difference between the two cell lines in sensitivity to cholecalciferol may also be explained by the expression and up-regulation of certain nuclear receptors, which will be discussed in more detail.

Matrix metalloproteinases (MMPs) have repeatedly been implicated in the degradation of extracellular matrix during cancer cell invasion and metastasis. But recent data suggest that MMPs may play a more complex role than was originally thought, and

they may also make important contributions at other steps in tumor development. MMPs can influence tumor development and maintain an environment that supports the maintenance of growth of benign and malignant tumors. Blocking or inhibiting MMPs (MMP-3, MMP-7 and MMP-11) led to significant reductions in the onset and numbers of tumors (Noel et al., 1996; Sternlicht et al., 1999; Wilson et al., 1997; Witty et al., 1994). The mechanisms by which MMPs are able to promote tumor development are not fully understood. MMPs may stimulate cell proliferation by releasing growth factors during extracellular matrix degradation or by altering cell:matrix interactions. It was suggested that MMPs may contribute to tumor metastasis subsequent to the growth of the primary tumor (Witty et al., 1994) and that MMPs may promote the effects of carcinogens and pre-existing gene defects (Sternlicht et al., 1999). This would suggest that agents that can block or inhibit the action of MMPs may be effective in inhibiting early stages of carcinogenesis and could have potential chemopreventive ability. RWPE-1 cells are non-invasive (Bello et al., 1997), however, they express latent MMP-9 and MMP-2. My results show that cholecalciferol treatment decreases the activity of both of these proteases (**Figure 9-2**) and, therefore, may have the ability to inhibit or block the development of primary tumors. Thus, the decrease in activity of MMP-9 and MMP-2 upon cholecalciferol treatment (**Figure 9-2**) warrants further investigation into this agent in a chemopreventive setting. Whether cholecalciferol inhibits MMP activity by increasing TIMP levels, which inhibit MMP activity, or by decreasing levels of MT-MMPs, which can activate MMPs (Corcoran et al., 1996; Strongin et al., 1995) would need further investigation.

The intermediate filament profile of a prostate cell is a good indicator of its differentiation status. Cytokeratins are the standard markers for epithelial cell origin. The epithelial origin of the RWPE-1 cells was confirmed previously by expression of cytokeratins 8 and 18, using immunostaining (Bello et al., 1997), which I substantiated with Western blot analysis (data not shown). Calcitriol treatment does not affect the expression of cytokeratin 18 in primary cultures of normal, BPH, and prostate cancer tissues (Peehl et al., 1994). I observed similar results when RWPE-1 cells were treated with cholecalciferol (data not shown).

Vimentin is normally expressed in cells of mesenchymal origin but epithelial cells in culture and rapidly proliferating epithelial cells *in vivo* may also express vimentin. In human prostate cancer cells, expression of vimentin shows an inverse relationship with differentiation status. Prostate cells expressing vimentin are more likely to be associated with poorly differentiated tumors (Lang et al., 2002). When compared to cells in the adjacent normal epithelium, cells in benign prostatic hyperplasia (BPH) express significantly higher levels of vimentin. In normal prostatic epithelium, ~2% of cells expressed vimentin compared to ~71% of cells in BPH (Fraga et al., 2002). It was suggested that the difference in proliferation between BPH and normal tissues causes the difference in vimentin expression, with vimentin expression being directly proportional to proliferative activity. PIN, which are precursors to malignant prostate cancer, show increased cell proliferation (O'Shaughnessy et al., 2002). Vimentin expression in neoplastic epithelial cells may be related to tumor progression (Gilles et al., 1996). From this, it is reasonable to suggest that agents that cause a decrease in vimentin expression may have chemopreventive potential because of their anti-proliferative and

pro-differentiating effects (Quader et al., 2001; Webber et al., 1999). My results show (Figure 9-3) that cholecalciferol causes a dose-dependent decrease in the expression of the intermediate filament protein vimentin in RWPE-1 cells. These data suggest that cholecalciferol has the ability to inhibit proliferation and induce differentiation of pre-neoplastic prostate cells by decreasing vimentin expression, which may, in part, account for the observed growth inhibition of the RWPE-1 cells.

Most of the biological effects of vitamin D are mediated via the vitamin D receptor (VDR). The level of VDR expression may be associated with the degree of differentiation of human cancer. The loss of VDR expression correlates with loss of differentiation and progression of prostate cancer (Shabahang et al., 1993). Most prostate cancers develop in the peripheral zone and are of epithelial origin. The expression of VDR, which is the highest in the peripheral zone, suggests that prostatic epithelial cells are target cells for vitamin D (Krill et al., 2001; Miller et al., 1995). This would suggest that vitamin D and its receptor might be important in the chemoprevention of prostate cancer. In addition, RXRs are also likely to play an important role in the chemopreventive effects vitamin D for several reasons. First, RXRs form heterodimers with VDR to carry out the transcriptional effects of vitamin D. Calcitriol enhances heterodimer formation and their binding to VDREs. Second, RXRs promote nuclear accumulation, localization and retention of unbound VDR (Prufer and Barsony, 2002). Third, degradation of RXRs reduces the sensitivity of cells to antiproliferative effects of calcitriol (Prufer et al., 2000).

So far I have demonstrated that cholecalciferol treatment inhibits anchorage-dependent growth, induces differentiation and inhibits proliferation by down-regulating

vimentin expression, and decreases the activity of MMP-9 and MMP-2, all in a dose-dependent manner. To explore possible mechanisms of action, I examined the expression of several nuclear receptors and their modulation in response to treatment with cholecalciferol. Immunostaining results (**Figure 9-4**), confirmed by Western blot analysis (**Figure 9-5**), demonstrate that cholecalciferol treatment causes a dose-dependent up-regulation of vitamin D receptor (VDR), and all three isoforms of retinoid X receptor (RXR- α , - β , and γ).

Although VDR forms heterodimers with all three RXRs, it has been suggested that it preferentially associates with RXR- α and RXR- γ to stimulate transcription (Jin et al., 1996; Kephart et al., 1996). Treatment with cholecalciferol caused a marked increase in the expression of RXR- α and RXR- γ in the non-tumorigenic RWPE-1 cells, while the malignant RWPE2-W99 cells appear to show lower expression of both RXR- α and RXR- γ , and no obvious up-regulation RXR- γ (see **Figures 8-6 & 8-7, pg. 254, 255**). This is consistent with studies which show that elevated levels of RXR- α increase the sensitivity to growth inhibition by calcitriol (Prufer et al., 2002), and disruption of RXR- α results in the development of pre-neoplastic lesions in the prostates of mice (Huang et al., 2002). Furthermore, these results are consistent with an observed 2 to 9 fold decrease and loss of nuclear expression of RXR- α in prostate cancer cells compared to normal prostate cells (Zhong et al., 2003). RXR- β was also up-regulated in the RWPE-1 cells. However, there was no detectable expression or up-regulation of RXR- β in the malignant RWPE2-W99 cells. These results may, in part, help to explain why the growth of RWPE-1 cells is more inhibited by cholecalciferol than that of RWPE2-W99 (**Figure 9-1**). These results also suggest that a loss of expression of certain RXRs, or a decrease

in their expression levels, may be a marker of prostate cancer progression and restoration of their expression could prove to be an effective chemopreventive strategy.

Increasing VDR and RXR expression could increase the sensitivity of cells to both cholecalciferol and retinoids, suggesting a role for combination treatments with these two agents. Numerous studies have examined the effects of combination therapies using calcitriol and retinoids. We have found that combined treatment with cholecalciferol and *N*-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid, resulted in a synergistic inhibition of growth of RWPE-1 cells (manuscript in preparation), suggesting that such a combination treatment may have chemopreventive ability. It will be interesting to study and compare the effects of cholecalciferol on the expression of retinoic acid receptors (RARs), in both the RWPE-1 and RWPE2-W99 cell lines.

Prostate carcinogenesis is driven by androgen, therefore the androgen receptor is a prime target for chemoprevention. Studies have shown a possible cross-talk between VDR and AR (Ahonen et al., 2000b). My results demonstrate that cholecalciferol treatment caused an up-regulation in androgen receptor expression in RWPE-1 cells (**Figure 9-5**). It is possible that the increased levels of AR that result from cholecalciferol treatment represent a more differentiated phenotype. This would suggest that cholecalciferol may exert some of its chemopreventive effects indirectly by the induction of differentiation, as indicated by up-regulation of AR. PSA is a useful tool for prostate cancer detection. It is secreted by differentiated luminal, secretory cells of the prostatic epithelium. My results show (**Figure 9-6**) that cholecalciferol treatment causes

a dose-dependent increase in PSA expression in RWPE-1 cells. These data further demonstrate that cholecalciferol induces differentiation in the RWPE-1 cell line.

In order to compare the efficacies of cholecalciferol with that of calcitriol, several assays were also conducted using calcitriol. Cholecalciferol showed very similar results to those obtained using calcitriol treated cells (**Figures 9-1, 9-2, 9-5 and 9-8**). In all assays studied, the effects of calcitriol appear to occur at approximately a 3 to 10 fold lower concentration than cholecalciferol. Based on the results on VDR, which is similarly up-regulated by both agents, the effects of cholecalciferol and calcitriol appear to be receptor-mediated. The concentrations used for both agents were within their respective normal physiological levels. The normal physiological range of calcitriol is 0.1 nM (10^{-10} M) to 1 nM (10^{-9} M) compared to 0.01 μ M (10^{-8} M) to 0.1 μ M (10^{-7} M) for cholecalciferol (Friedrich 1988; Schwartz et al., 1997) (**Table 9-1**).

Table 9-1: Normal physiological and pharmacological levels and half lives of the three major vitamin D metabolites.

	Physiological Levels	Pharmacological Levels	Half Life
Cholecalciferol	10^{-8} M – 10^{-7} M	(10^{-5} M)?	4-7 days
25OHD₃	10^{-8} M – 10^{-7} M	(10^{-5} M)?	3-4 weeks
Calcitriol	10^{-10} M – 10^{-9} M	10^{-7} M	1-8 hours

The “?” indicates estimated level based on known physiological and pharmacological levels for calcitriol.

Several reasons could explain why 3 to 10 fold higher concentrations were needed for cholecalciferol to obtain similar results as calcitriol. First, if the observed cholecalciferol effects are due to its conversion to calcitriol, then it must be first metabolized to 25OHD₃ and then to calcitriol. Second, the 24-hydroxylase enzyme could convert 25OHD₃ to 24,25-dihydroxyvitamin D₃ or calcitriol [1,25(OH)₂D₃] to 1,24,25-hydroxyvitamin D₃. Third, some of the cholecalciferol is converted to 25OHD₃, which binds to VDR to carry out its effects, while the remaining cholecalciferol goes through the subsequent hydroxylation to form calcitriol. 25OHD₃, like cholecalciferol, has a much lower binding affinity for VDR compared to that of calcitriol (Barreto et al., 2000). And finally, compared to calcitriol, cholecalciferol has a much lower binding affinity for VDR, therefore, higher concentrations of cholecalciferol would be needed to see effects similar to those of calcitriol. Other possible explanations, that would need further investigation, are how RXRs and/or RARs affect the mechanisms of action of cholecalciferol, and that cholecalciferol may work through mechanisms other than VDR (non-genomic pathways).

Conclusions

In conclusion, I have shown that the non-tumorigenic RWPE-1 human prostate epithelial cells express the 25-hydroxylase (CYP27A1) protein necessary for the conversion of cholecalciferol to 25OHD₃. This is the first reported evidence of the presence of this enzyme in human prostatic epithelial cells. Coupled with previous evidence that prostate epithelial cells also express 1 α -hydroxylase enzyme, this shows that prostate epithelial cells have the ability to locally produce the biologically active

calcitriol from cholecalciferol. Therefore, cholecalciferol could be an effective chemopreventive agent for prostate cancer. In support of this, my results show that cholecalciferol, in the physiological range, can: i) inhibit anchorage-dependent growth; ii) induce differentiation by decreasing vimentin expression and increasing PSA synthesis; and iii) up-regulate VDR, RXR- α , RXR- β , RXR- γ , and AR, in a dose-dependent manner. Whether cholecalciferol exerts these effects by acting directly or indirectly with the VDR, and through the same mechanisms as calcitriol, remains to be elucidated. However, the similarities between the effects of these two forms of vitamin D, in this and the previous chapter, suggest that cholecalciferol and calcitriol exert their effects through the same, or similar, mechanisms. Based on these results, cholecalciferol appears to have much potential as a chemopreventive and chemotherapeutic agent. Dietary compounds, such as cholecalciferol (vitamin D₃), are of particular interest because they are readily available to the public at low cost and they are expected to be safe for long-term administration to healthy people for chemoprevention. One gram of cholecalciferol costs \$36, compared to approximately \$56,000 and \$4,000,000, respectively, for 1 gram of 25OHD₃ and calcitriol (Sigma Catalog, St. Louis, MO). Further investigations are needed into the pathways and mechanisms shared by calcitriol and cholecalciferol in the prevention and treatment of prostate cancer.

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