MERJA AHONEN

Vitamin D in Human Ovarian and Prostate Cancer

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the big auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on September 20th, 2002, at 13 o'clock.

Acta Universitatis Tamperensis 885
University of Tampere
Tampere 2002

ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital,
Department of Clinical Chemistry
Finland

Localization of 1,25-Dihydroxyvitamin D_3 Receptor (VDR) Expression in Human Prostate
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http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 885
ISBN 951-44-5430-8
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 198
ISBN 951-44-5431-6
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2002

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Kivineva M, Bläuer M, Syvälä H, Tammela T and Tuohimaa P (1998): Localization of 1,25-dihydroxyvitamin D_3 receptor (VDR) expression in human prostate. Journal of Steroid Biochemistry and Molecular Biology 66:121-127.

II Ahonen MH, Zhuang YH, Aine R, Ylikomi T and Tuohimaa P (2000): Androgen receptor and vitamin D receptor in human ovarian cancer: growth stimulation and inhibition by ligands. International Journal of Cancer 86:40-46.

III Miettinen S, Ahonen MH, Lou Y, Manninen T, Tuohimaa P and Ylikomi T: Expression of 1α-hydroxylase does not lead to growth inhibition by 25-hydroxyvitamin D_3 in OVCAR-3 ovarian cancer cells (submitted for publication).

IV Ahonen MH, Tenkanen L, Teppo L, Hakama M and Tuohimaa P (2000): Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes and Control 11:847-852.

ABBREVIATIONS

AF	activation function
AR	androgen receptor
ARC	activator-recruited cofactor
BRCA	breast cancer susceptibility gene
CAR	constitutive androstane receptor
cGMP	cyclic 3',5'-guanosine monophosphate
25(OH)D	25-hydroxyvitamin D (include both $25(OH)D_2$ and $25(OH)D_3$)
$1\alpha,25(OH)_2D$	1α,25-dihydroxyvitamin D
$1\alpha,24,25(OH)_3D$	1α,24,25-trihydroxyvitamin D
$24,25(OH)_2D$	24,25-dihydroxyvitamin D
DHT	dihydrotestosterone (5α-androstan-17β-ol-3-one)
DR	direct repeat
DRIP	vitamin D receptor-interacting protein
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
FXR	farnesoid receptor
HER-2/neu	human epidermal growth factor receptor-2
hVDR	human VDR
IGF	insulin-like growth factor
IgG	immunoglobulin G
LXR	liver X receptor
M_r	relative molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCoR	nuclear receptor corepressor
N-terminal	amino terminal
OR	odds ratio
PBS	phosphate buffered saline
PTH	parathyroid hormone
PTHrP	PTH related protein
PSA	prostate specific antigen
PXR	pregnane X receptor

RAC	receptor associated coactivator
RAR	retinoic acid receptor
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SRC	steroid receptor coactivator
TBS	Tris-HCl buffered saline
TBST	TBS containing 0.05% Tween 20
TGFβ	transforming growth factor β
TIF	transcription intermediary factor
TR	thyroid hormone receptor
DBP	vitamin D binding protein
VDR	vitamin D receptor
VDRE	vitamin D response element

INTRODUCTION

The main function of pleiotropic vitamin D is to preserve calcium and phosphorus homeostasis, together with certain other factors, in order to promote skeletal mineralization and to maintain signal transduction, metabolic activities and neuromuscular function. In addition to the classic target organs involved in mineral homeostasis (intestine, kidney, bone and parathyroid glands), vitamin D regulates cell growth and differentiation in a variety of other tissues, controls other hormonal systems and modulates immunologically mediated processes.

The metabolic activation of vitamin D synthesized in the skin or obtained from the diet is brought about in two stages by the enzymes 25-hydroxylase and 1α-hydroxylase, leading to the hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D ($1\alpha,25(OH)_2D_3$). The action of $1\alpha,25(OH)_2D_3$ is terminated by the enzyme 24-hydroxylase. Two distinct signalling systems are involved in vitamin D-mediated effects. The majority of its actions are mediated through $1\alpha,25(OH)_2D_3$ binding to nuclear vitamin D receptor (VDR), which can directly modulate the transcription of genes possessing a functional binding site for the VDR in their regulatory region. The non-genomic effects of vitamin D involve stimulation of signal transduction systems on the plasma membrane.

Although the physiological effects of vitamin D in the ovary and prostate are in general poorly understood, some experimental studies have shown that $1\alpha,25(OH)_2D_3$ and calcium might be necessary for normal ovarian folliculogenesis. In addition, $1\alpha,25(OH)_2D_3$ has been reported to modulate the growth and induce differentiation in the prostate. Cancers of the ovary, prostate and several other organs are more common in areas where solar UV-B radiation is limited, and it has been hypothesized that vitamin D may mediate the beneficial effects of sunlight. Results of epidemiological studies on the association between prostate cancer and vitamin D have been inconsistent, but experimental findings have supported the hypothesis envisaging $1\alpha,25(OH)_2D_3$-induced growth inhibition in ovarian and prostate cancer cells. Moreover, $1\alpha,25(OH)_2D_3$ has been reported to stimulate cellular differentiation and inhibit the metastatic potential of prostate cancer cells. Various mechanisms have

been shown to be involved in the anti-cancer action of $1\alpha,25(OH)_2D_3$ on prostate cancer cells, including regulation of the cell cycle, induction of programmed cell death (apoptosis) and modulation of growth factor, oncogene and tumor suppressor gene expression. Furthermore, vitamin D metabolizing enzymes have been reported to modulate the action of vitamin D in prostate cells. Little is known, however, as to the mechanisms of $1\alpha,25(OH)_2D_3$-induced growth inhibition in ovarian cancer cells.

This study was undertaken to clarify the controversy raised by epidemiological studies of the role of vitamin D in the etiology of prostate cancer, to reveal the cellular distribution of VDR within normal prostate tissue and to add to knowledge of the actions of vitamin D in ovarian cancer. The results presented provide an insight into the role of vitamin D, VDR and vitamin D metabolizing enzymes in the complicated process of prostate and ovarian carcinogenesis.

REVIEW OF THE LITERATURE

1. VITAMIN D

1.1. Physiological functions of vitamin D

Vitamin D comprises a group of closely related compounds which evince antirachitic activity (Collins and Norman 2001). The two most prominent members of this group are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), together with their various metabolites. In addition, other naturally occurring members of the vitamin D family include vitamin D_4, vitamin D_5, vitamin D_6 and vitamin D_7, which differ from each other in the structure of their side chain (Collins and Norman 2001). In the present discussion, however, vitamin D covers vitamin D_2 and vitamin D_3 and their metabolites.

The main function of vitamin D is to preserve calcium and phosphorus homeostasis by increasing the efficiency of intestinal calcium and phosphorus absorption in order to maintain signal transduction, metabolic activities and neuromuscular function and to promote skeletal mineralization (Holick 2001). In parallel, vitamin D markedly increases the levels of several vitamin D-regulated calcium-binding proteins (calbindin-D) in its classical target tissues (intestine, kidney and bone) (Walters 1992). Hypocalcemia or hypercalcemia is prevented by the joint action of vitamin D, parathyroid hormone (PTH) and probably calcitonin (Fraser 1995).

In addition to the classic target organs involved in mineral homeostasis (intestine, kidney, bone and parathyroid glands), vitamin D has functions in most if not all of other tissues. Vitamin D has been shown to regulate growth and differentiation in, e.g, hematopoietic and reproductive tissues, skin, muscle, heart, cartilage and a variety of cancer cells. Furthermore, it controls other hormonal systems, enhances insulin and inhibits calcitonin synthesis, for example, and modulates the immune system, enhancing control of viral and bacterial infections, and tumor growth. However, it should be noted that part of the vitamin D effects in vivo are achieved at doses which cause hypercalcemia as a side-effect (for reviews see Issa et al. 1998, Brown et al. 1999).

1.2. Vitamin D metabolism

1.2.1. Photobiology of vitamin D

There are two nutritional forms of vitamin D, vitamin D_3 and vitamin D_2. Upon exposure to sunlight, the UV radiation (290-315 nm) penetrate the epidermis of the skin and photolyze 7-dehydrocholesterol (provitamin D_3) to previtamin D_3. In this process, the B-ring of the steroid structure is cleaved, yielding a characteristic secosteroid structure. Once formed, previtamin D_3 undergoes a thermally induced isomerization to vitamin D_3. Continued exposure of previtamin D_3 or vitamin D_3 to sunlight causes isomerization into biologically inactive photoisomers, thus preventing the excess production of vitamin D_3. The amount of vitamin D_3 produced is directly related to the intensity of the exposure and inversely related to the pigmentation of the skin. The amount of 7-dehydrocholesterol in the epidermis decreases with aging, resulting in decreased production of vitamin D_3 (MacLaughlin and Holick 1985).

Vitamin D_2 (ergocalciferol) is produced by UV radiation of the plant steroid ergosterol. Vitamin D_2 is in fact a rare form of vitamin D in nature and is of minor importance in humans (Fraser 1995). It is, however, used as a food additive and in multivitamin preparations, since it has been found able to substitute for vitamin D_3 in humans and to have weaker hypercalcemic side-effects than vitamin D_3 when administered in large and potentially toxic doses (Breslau and Zerwekh 1997, Horst and Reinhardt 1997). Thus, discussion of the metabolism and action of vitamin D_2 is in most cases covered by vitamin D_3.

For humans, there are few natural foods which contain substantial amounts of vitamin D; in general vitamin D status is thus maintained by exposure to sunlight (Fraser 1995). In this sense vitamin D is not actually a vitamin, as vitamins are obtained only from dietary sources. However, people living at latitudes where exposure to sunlight is limited during winter months will easily develop vitamin D insufficiency or deficiency (Lehtonen-Veromaa et al. 1999, Lamberg-Allardt et al. 2001, Outila et al. 2001). The designation “vitamin D insufficient” refers to a subject with reduced levels of vitamin D and “vitamin D deficient” a subject severely lacking in vitamin D. Hence vitamin D supplementation during winter months might be warranted especially for

children and pregnant and lactating women, as their need for vitamin D is increased, and for elderly people, as their production of vitamin D is decreased.

1.2.2. The vitamin D 25-hydroxylase

Both vitamin D_3 and vitamin D_2 are biologically inactive precursors which must undergo successive hydroxylations before they can bind and activate the vitamin D receptor. The metabolism of vitamin D is presented in Figure 1. The first step in its activation is hydroxylation of carbon 25 in the side-chain. Vitamin D produced in the skin (vitamin D_3) or vitamin D from dietary sources (vitamin D_3 or vitamin D_2) is hydroxylated to the prohormone 25-hydroxyvitamin D_3 or 25-hydroxyvitamin D_2, respectively, predominantly in the liver, but also in extra-hepatic tissues such as the kidney, intestine and skin (Gascon-Barre et al. 2001, Schuessler et al. 2001, Theodoropoulos et al. 2001). The hepatic enzyme catalyzing 25-hydroxylation of vitamin D, CYP27A, is located in the mitochondria and belongs to the cytochrome P450 superfamily of mixed-function oxidases (Portale and Miller 2000, Wikvall 2001). The same enzyme may also 26-hydroxylate cholesterol in the liver, which is needed for bile acid biosynthesis (Su et al. 1990). Mitochondrial cytochrome P450 enzymes receive electrons from NADPH (reduced nicotinamide adenine dinucleotide phosphate) via an electron-transfer chain consisting of two proteins, a flavoprotein termed ferredoxin reductase and an iron-sulfur protein termed ferredoxin. Also a microsomal cytochrome P450 enzyme capable of vitamin D 25-hydroxylation have been reported in the pig liver, but its role in humans remains to be determined (Axen et al. 1994). Production of 25-hydroxyvitamin D (25(OH)D) is not significantly regulated, being primarily dependent on substrate concentration. Therefore, serum 25(OH)D concentrations are commonly used as an indicator of vitamin D status. The function of 25(OH)D apart from its role as a vitamin D precursor remains to be determined.

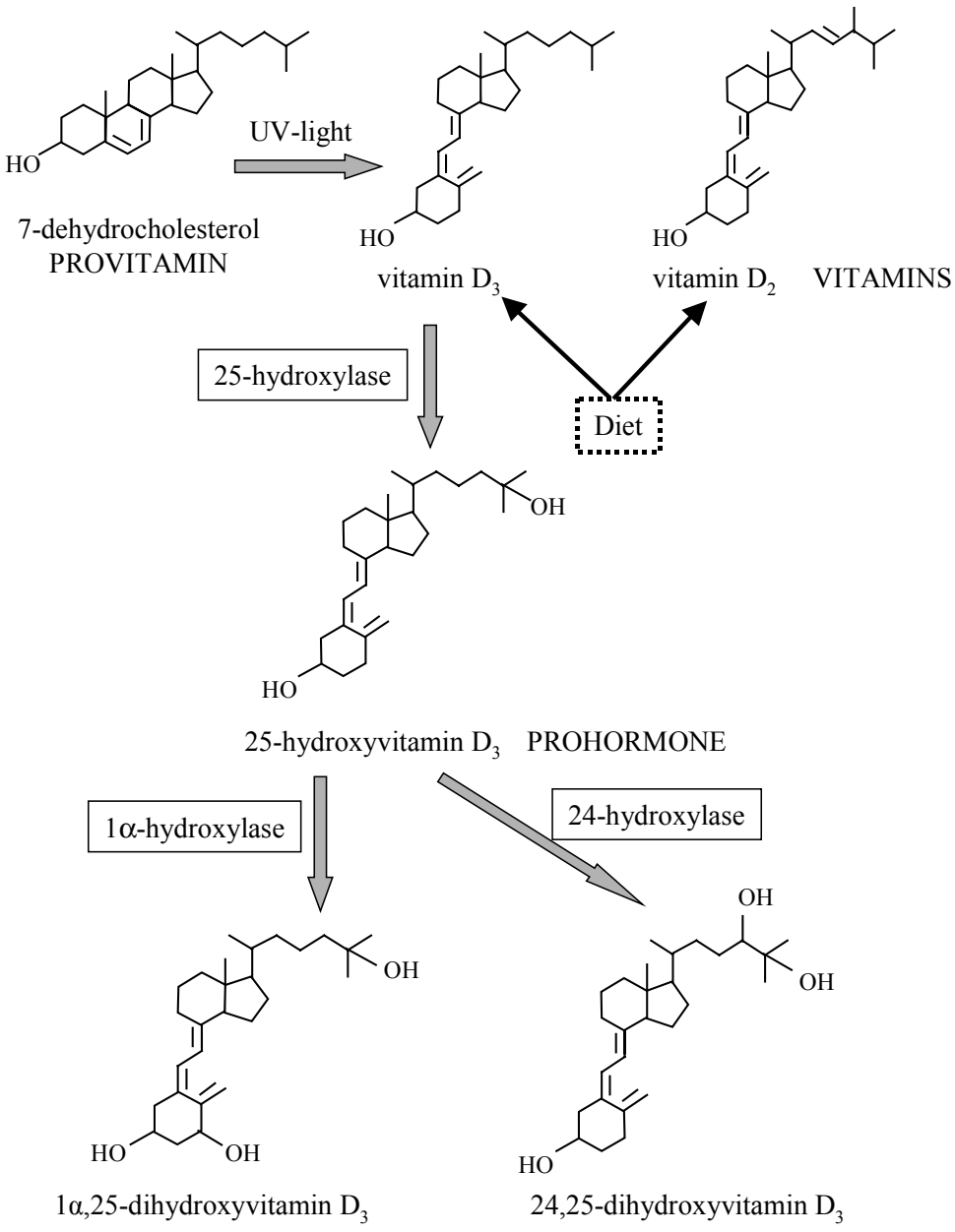


Figure 1. The basic metabolic pathway for the major circulating vitamin D metabolites. The details of the tissue distribution of the key enzymes and the regulation of vitamin D metabolism are discussed in the text. Vitamin D_2 obtained from the diet can be metabolized in a similar fashion to produce several metabolites analogous to vitamin D_3. Further metabolism of 1α,25-dihydroxyvitamin D_3 and 24,25-hydroxyvitamin D_3 involves a number of steps and leads finally to excretion.

1.2.3. The 25-hydroxyvitamin D 1α-hydroxylase

The hydroxylation of circulating 25-hydroxyvitamin D to the biologically active secosteroid form, 1α,25-dihydroxyvitamin D (1α,25$(OH)_2$D), takes place mainly in the kidney. The mitochondrial cytochrome P450 enzyme CYP27B is generally considered to be the most important 1α-hydroxylase in humans, catalyzing hydroxylation of carbon 1 in the A-ring of 25(OH)D (Wikvall 2001). In addition, mitochondrial 25-hydroxylase might have a role in 1α-hydroxylation in humans (Pikuleva et al. 1997, Sawada et al. 2000). In contrast to vitamin D 25-hydroxylase, renal vitamin D 1α-hydroxylase is tightly regulated to maintain calcium homeostasis. The most important agencies of regulation here are 1α,25$(OH)_2D_3$ itself suppressing the activity of the enzyme that produces it, and parathyroid hormone up-regulating this activity (Breslau 1988, Kain and Henry 1989, Murayama et al. 1998). The other reported inducers of 1α-hydroxylase activity are low serum calcium and phosphorus levels, calcitonin, insulin-like growth factor, estrogen and possibly prolactin, while high serum calcium and phosphorus levels are reported to suppress the enzymatic activity (Breslau 1988, Verhaeghe and Bouillon 1992, Hewison et al. 2000, Portale and Miller 2000). The promoter of the human 1α-hydroxylase gene has been found to contain a positively regulatory region for parathyroid hormone and calcitonin and a negatively regulatory region for 1α,25$(OH)_2D_3$, indicating that the regulation of 1α-hydroxylase may take place at transcriptional level (Murayama et al. 1998).

Although circulating concentrations of 1α,25$(OH)_2D_3$ primarily reflect its renal synthesis, 1α-hydroxylase activity is also found in human skin, lymph nodes, macrophages, colon, pancreas, adrenal medulla, brain and placenta and in human prostate, lung and colon cancer cells (Mawer et al. 1994, Schwartz et al. 1998, Jones et al. 1999, Monkawa et al. 2000, Tangpricha et al. 2001, Zehnder et al. 2001). Although renal and extra-renal 1α-hydroxylase activity is reported to be due to a single gene product, the expression and regulation of 1α-hydroxylase in extra-renal tissues is evidently different from that observed with the kidney enzyme (Jones et al. 1999, Hewison et al. 2000). For example in human colon adenocarcinoma and lung carcinoma cells and in primary human keratinocytes, 1α-hydroxylase expression is constitutive and unaffected by 25$(OH)D_3$ or 1α,25$(OH)_2D_3$ (Cross et al. 1997, Jones et al. 1999, Schuessler et al. 2001). The co-expression of 1α-hydroxylase and a specific

receptor for 1α,25(OH)$_2$D$_3$ in several tissues suggest a role for 1α-hydroxylase as an intracrine modulator of vitamin D function in peripheral tissues.

1.2.4. The 25-hydroxyvitamin D 24-hydroxylase

The 25-hydroxyvitamin D 24-hydroxylase, CYP24, is a mitochondrial cytochrome P450 enzyme able to oxidize the side-chain of 25(OH)D or 1α,25(OH)$_2$D to 24,25-dihydroxyvitamin D (24,25(OH)$_2$D) or 1α,24,25-trihydroxyvitamin D (1α,24,25(OH)$_3$D), respectively (Portale and Miller 2000). It has been reported that 24-hydroxylase might also catalyze further hydroxylation steps of vitamin D metabolites, leading to calcitroic acid and excretion in the bile (Sakaki et al. 1999). Although 24-hydroxylase is regarded as a catabolic enzyme leading to inactivation of 1α,25(OH)$_2$D$_3$, some 24-hydroxylated metabolites of vitamin D may have biological activities. 24,25(OH)$_2$D$_3$ has been found to play a role in the suppression of PTH secretion, in the mineralization of bone and in fracture healing (Collins and Norman 2001). Recent reports suggest that 1α,24,25(OH)$_3$D$_3$ and the further oxidized product, 1α,25(OH)$_2$-24-oxo-vitamin D$_3$, might exert growth-modulating effects (Campbell et al. 1999, Rashid et al. 2001). Like 1α-hydroxylase, 24-hydroxylase is predominantly expressed in the kidney and regulated to maintain calcium homeostasis. These two renal enzyme activities are frequently regulated in reciprocal manner. For example, 1α-hydroxylase activity is up-regulated during high PTH/low calcium states, whereas 24-hydroxylase activity is down-regulated. In contrast, a high 1α,25(OH)$_2$D$_3$/high calcium concentration results in low 1α-hydroxylase and high 24-hydroxylase activity.

In addition to the kidney, 24-hydroxylase activity has been documented in a wide variety of other human tissues, for example lymphocytes, fibroblasts, bone, keratinocytes, macrophages and human colon adenocarcinoma, lung and prostatic carcinoma cells (Armbrecht et al. 1992, Miller et al. 1995). The main stimulator of 24-hydroxylase mRNA in both renal and extra-renal tissues is 1α,25(OH)$_2$D$_3$, and regulation is most likely transcriptional, as two or even three vitamin D response elements (VDREs) have been reported in the promoter region of the human and rat 24-hydroxylase gene, respectively (Chen and DeLuca 1995, Kerry et al. 1996, Ohyama et al. 1996). Estrogen and testosterone may modulate the expression of renal

24-hydroxylase in birds (Tanaka et al. 1976, Trechsel et al. 1979). The regulation of the enzyme by PTH or serum calcium is different in renal and extra-renal tissues. The stimulation of 24-hydroxylase mRNA in response to 1α,25$(OH)_2D_3$ is inhibited by PTH in the kidney, whereas in the intestine PTH has no effect and in bone PTH enhances stimulation (Shinki et al. 1992, Armbrecht and Hodam 1994, Armbrecht et al. 1996, Armbrecht et al. 1998). Low serum calcium is reported to reduce renal and increase bone 24-hydroxylase (Shinki et al. 1992, Nishimura et al. 1994, Matkovits and Christakos 1995).

The widespread distribution of 24-hydroxylase and its induction by 1α,25$(OH)_2D_3$ supports the contention that a major role for the enzyme involves regulating the local concentration and action of 1α,25$(OH)_2D_3$ (Cross et al. 1997, Jones et al. 1999, Schuessler et al. 2001). The simultaneous expression of VDR, constitutive 1α-hydroxylase, and inducible 24-hydroxylase activity in the same cell or in cells of the immediate environment suggests that they constitute a means for in situ regulation of the response to vitamin D (Dusso et al. 1994).

1.3. Transport and storage of vitamin D

Vitamin D and its hydroxylated metabolites 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1α,25-dihydroxyvitamin D are lipophilic molecules. In the circulation, vitamin D compounds are therefore transported bound to plasma proteins. The most important of the carrier proteins is the vitamin D-binding protein (DBP). Other carrier proteins for vitamin D metabolites are albumin and lipoproteins. Under normal physiological conditions, the majority of circulating vitamin D compounds are bound to protein, this prolonging their half-life in circulation (Safadi et al. 1999). The concentration of the free vitamin D fraction in the circulation is tightly regulated and is considered to be the active fraction, as it has been hypothesized that only unbound sterol can diffuse through the plasma membrane into the target cell (Bikle and Gee 1989). The validity of this diffusion hypothesis has however recently been challenged. Results on DBP null mice have suggested that DBP may have an additional active role in modulating the rates of bioavailability, activation and end-organ responsiveness of vitamin D (Safadi et al. 1999). It has also been reported that renal uptake of DBP-bound 25(OH)D and activation of 25(OH)D might involve the

endocytic receptor megalin (Nykjaer et al. 1999). It is not known, however, whether a similar endocytic mechanism exists in other tissues.

After intramuscular injection of high doses of vitamin D or prolonged oral administration of vitamin D, serum 25(OH)D concentration remains at a fairly high level for several months, although the half-life of 25(OH)D is about three weeks (Heikinheimo et al. 1991, Breslau and Zerwekh 1997). The means to keep serum 25(OH)D at a suitable level is to store vitamin D in tissues which provide for its sustained release. In human, adipose tissue and muscle are reported to be the major storage sites for vitamin D (Collins and Norman 2001).

2. VITAMIN D RECEPTOR

2.1. The nuclear receptor superfamily

The majority of the actions of $1\alpha,25(OH)_2D_3$ are mediated via the VDR, which belongs to the superfamily of nuclear receptors. These receptors form the largest known family of eukaryotic transcription factors, which is reported to contain 48 members in humans (Maglich et al. 2001). The receptors bind to DNA and regulate the transcription of specific genes in response to their cognate ligands, and thus control numerous physiological events in development, growth, reproduction and cell differentiation, proliferation and apoptosis, and in the maintenance of homeostasis. In addition to vitamin D, known natural ligands for nuclear receptors include steroid and thyroid hormones, retinoic acid and dietary lipids (Renaud and Moras 2000, Chawla et al. 2001). On the basis of amino acid sequence homology and mode of action, the VDR forms a subfamily with the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), the liver X receptor (LXR) and the farnesoid receptor (FXR) (Maglich et al. 2001). Structurally, nuclear receptors show significant homology in amino acid sequences and have a modular structure composed of five to six domains designated A-F. The most conserved domains in the nuclear receptor family are C, the DNA binding domain, and E, the ligand-binding domain (Renaud and Moras 2000).

2.2. Structure of VDR

Structure-function studies of VDR by deletion mutation analysis and amino acid sequence comparison with other nuclear receptors has led to the identification of four domains in VDR, designated A/B, C, D and E (Issa et al. 1998). The domain structure of human VDR and several functional activities within these domains are presented in Figure 2.

The aminoterminal A/B domain of the human VDR is short (21 amino acids) compared to 88-421 amino acids in other human nuclear receptors (Baker et al. 1988, Issa et al. 1998). The A/B domain of VDR in other species studied is about the size of that in the human VDR, but the avian VDR has an N-terminal extension of 20 amino acids (Elaroussi et al. 1994, Suzuki et al. 2000). In the majority of nuclear receptors, the ligand-independent transactivation function (AF-1) is located in the A/B domain (Renaud and Moras 2000). However, VDR has no known AF-1 (Sone et al. 1991).

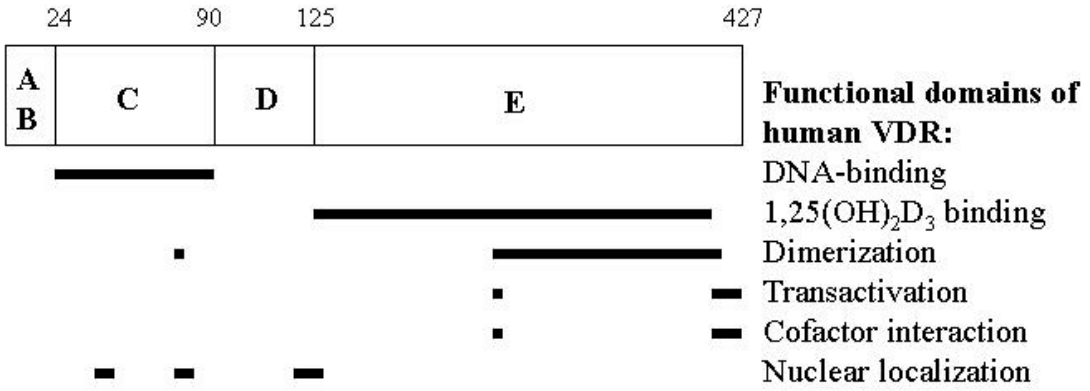


Figure 2. Domain structure of human vitamin D receptor (VDR) and the functional activities of the domains.

The DNA-binding domain (domain C) of VDR is located in amino acid residues 24-90 of the human VDR (McDonnell et al. 1989, Sone et al. 1991). A defining feature of the DNA-binding domain of nuclear hormone receptors is eight positionally conserved cysteine residues which tetrahedrally coordinate two zinc atoms to form zinc finger DNA-binding motifs (Issa et al. 1998). The two zinc modules of the

nuclear receptors appear to serve different functions. The amino-terminal zinc module makes specific contact with DNA response elements, whereas the carboxy-terminal zinc module forms a dimerization interface for other nuclear receptor DBDs (Freedman 1992). The amino acid residues 92 and 93 of VDR in the T-box may be involved in heterodimerization with the retinoid X receptor (RXR) (Freedman 1992, Hsieh et al. 1995, Rastinejad et al. 1995). In addition, the amino acids 49-58 of VDR correspond to a region between the two zinc fingers and may represent a nuclear localization signal (Hsieh et al. 1998). Also basic residues at both ends of VDR sequence 79-105 might be equally necessary for nuclear accumulation (Luo et al. 1994). However, it is not known whether these nuclear localization regions function together or independently.

Domain D (hinge domain) of VDR is located in amino acids 90-125 and links the DNA-binding and ligand binding domains in a highly flexible fashion to allow for simultaneous receptor dimerization and DNA binding (McDonnell et al. 1989, Chawla et al. 2001). Domain D of VDR might also be involved in specific DNA contact (Miyamoto et al. 2001). In addition, the hinge domain contains a nuclear localization signal (Michigami et al. 1999).

The ligand-binding domain (domain E) is located in residues 125-427 of the human VDR. A crystalization study has revealed that domain E is composed of 12 α-helical structures and a three-stranded β sheet (Rochel et al. 2000). The last of these helixes, helix 12 at the extreme C-terminus of VDR, is held to function like a lid closing a hydrophobic ligand-binding pocket of nuclear receptor upon ligand binding and exposing an interface for potential interaction with coactivators (Moras and Gronemeyer 1998). Residues 246 and 420 together serve as a charge clamp for coactivator interaction, although the direct interaction is mediated by hydrophobic amino acids on the surface of VDR (Väisänen et al. 2002). Activation function 2 (AF-2) involved in transcriptional control has been located in helix 12 of the VDR (Jurutka et al. 1997, Issa et al. 1998). However, Whitfield and coworkers (1995) have suggested that residue 246 might also be involved in transactivation. In domain E of VDR there are also regions needed for heterodimerization with RXR (Nakajima et al. 1994, Haussler et al. 1995, Jin et al. 1996, Gampe et al. 2000).

2.3. Regulation of VDR abundance

The regulation of VDR abundance plays an important role in determining the magnitude of the target cell response to $1\alpha,25(OH)_2D_3$. The amount of intracellular VDR in a target cell is regulated by $1\alpha,25(OH)_2D_3$ or other VDR ligands (homologous regulation) and by other hormonal and physiological signals (heterologous regulation) (Krishnan and Feldman 1997). The regulation is species-, tissue- and cell-type-specific and is altered during development and aging and in disease states (Christakos et al. 1996, Issa et al. 1998). The state of differentiation of the cells, which is closely related to the rate of proliferation, may also be involved in the regulation of VDR abundance (Krishnan and Feldman 1997). The mechanism of regulation may involve transcriptional, post-transcriptional (alteration of mRNA stability), or post-translational effects (alteration in receptor half-life).

Ligands of VDR have been reported to up-regulate VDR abundance in many models, e.g. in pig kidney cells and human skin fibroblasts (Costa et al. 1985, Li et al. 1999), rat and human osteosarcoma cells (Arbour et al. 1993, Mahonen and Mäenpää 1994) and human colon cancer cells (Zhao and Feldman 1993). However, some reports suggest that VDR is not regulated or even down-regulated by its ligands (Kizaki et al. 1991, Hulla et al. 1995, Song 1996). The mechanism of homologous up-regulation involves stabilization of VDR protein with no change (Wiese et al. 1992, Arbour et al. 1993, van den Bemd et al. 1996) or increase in VDR mRNA abundance (Costa and Feldman 1986, Mahonen and Mäenpää 1994), depending on the model system used. A recent report suggests that $1\alpha,25(OH)_2D_3$ may up-regulate VDR in human skin by blocking ubiquitin/proteasome-mediated degradation without altering VDR mRNA levels (Li et al. 1999).

The heterologous regulators of VDR abundance include hormones such as glucocorticoids, estrogens, androgens, retinoids, growth factors, activators of specific intracellular second messenger systems (protein kinase A, protein kinase C and intracellular calcium), and other growth and developmental signals (Escaleira et al. 1993, Krishnan and Feldman 1997). The effect of glucocorticoids on intestinal VDR abundance appears to be species-specific; up-regulation occurs in the rat and dog, down-regulation in the mouse and no change in chickens (Krishnan and Feldman 1997)). Androgen (dihydrotestosterone) has been reported to induce an increase in

VDR levels in T47D breast cancer cells (Escaleira et al. 1993). The capacity of parathyroid hormone to activate multiple second messenger systems might provide a mechanism for tissue-, cell- and differentiation state-specific variations in VDR regulation (Krishnan and Feldman 1997). The mechanisms of heterologous regulation are complex, but the outcome is to sensitize (up-regulation) or de-sensitize (down-regulation) the target cells to the actions of vitamin D.

3. THE MECHANISMS OF VITAMIN D RECEPTOR ACTION

In general, binding of the ligand to the nuclear receptor initiates a series of events which results in activation or repression of the target genes. The main reactions in this cascade are activation of the nuclear receptor by its cognate ligand, and dimerization and binding of the activated receptor to specific hormone response elements in DNA, resulting in negative or positive effects on transcription of the target genes (for a review see Aranda and Pascual 2001). A model for VDR action is presented in Figure 3.

3.1. Ligand binding

Binding of 1α,25$(OH)_2D_3$ to VDR induces a conformational change within a ligand binding domain of VDR. Helix 12 in the extreme C-terminus of the VDR functions like a lid closing the hydrophobic ligand binding pocket (Moras and Gronemeyer 1998). This conformational change induces dissociation of co-repressors, such as the nuclear receptor co-repressor (NCoR) or Alien, and facilitates the interaction of the AF-2 domain with co-activator proteins such as members of the p160 family, e.g. steroid receptor coactivator 1 (SRC-1), transcription intermediary factor 2 (TIF2) and receptor-associated coactivator 3 (RAC3) (Hörlein et al. 1995, Chen and Li 1998, Dressel et al. 1999, Polly et al. 2000). The VDR co-activator interaction further facilitates recruitment of other factors to form a larger complex which modulates the chromatin structure and initiates transcription (Spencer et al. 1997). Although the principal natural ligand of VDR is 1α,25$(OH)_2D_3$, other vitamin D metabolites such as 25$(OH)D_3$ and 24,25$(OH)_2D_3$ may bind to VDR, albeit with lower affinity (Costa et al. 1985, Collins and Norman 2001). The relative binding affinity of 25$(OH)D_3$ to VDR is about 700-fold lower than that of 1α,25$(OH)_2D_3$ (Collins and Norman 2001).

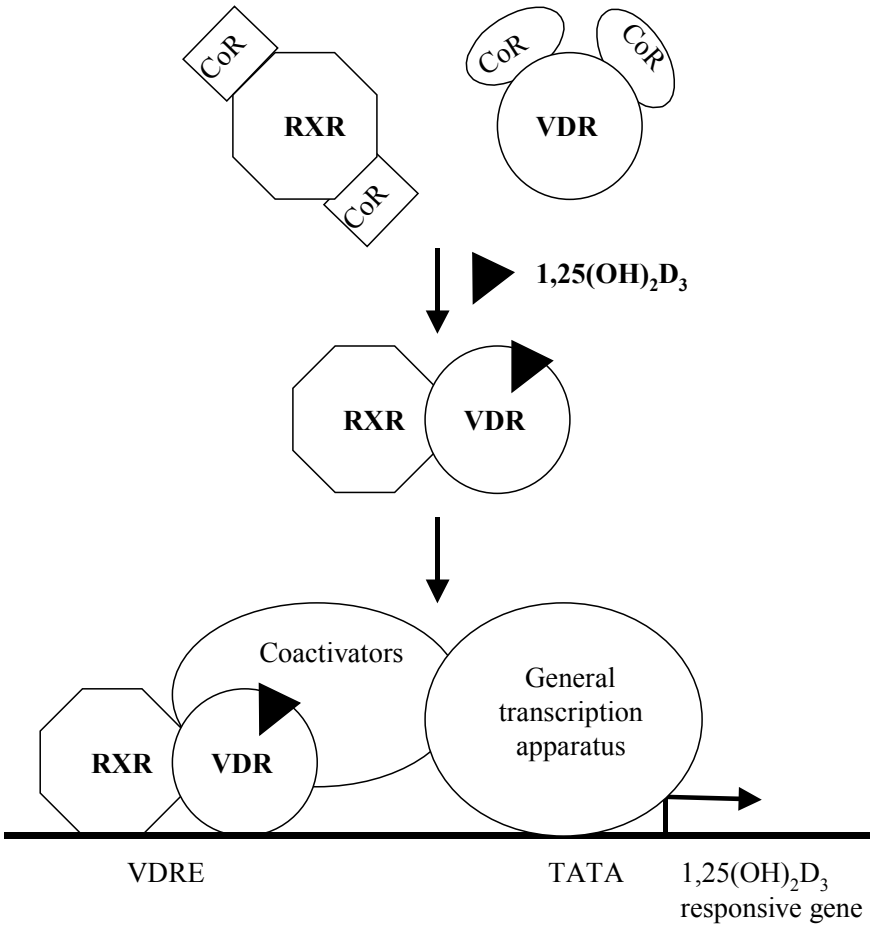


Figure 3. A model for vitamin D receptor (VDR) action. The details of the model are discussed in the text. Briefly, binding of $1\alpha,25(OH)_2D_3$ to VDR induces a conformational change which leads to dissociation of co-repressor proteins (CoR) and facilitation of heterodimerization with RXR. Transcriptional activation of $1\alpha,25(OH)_2D_3$ responsive genes is achieved by interaction of VDR-RXR heterodimer with a specific DNA sequence, the vitamin D response element, and recruitment of coactivators which bridge the contact between the receptors and general transcription apparatus.

The subcellular distribution of VDR may change upon ligand binding. Unoccupied VDR exists in equilibrium between the cytosol and the nucleus, the minor (Berger et al. 1988, Clemens et al. 1988, Milde et al. 1989) or substantial (Barsony et al. 1997)

portion of VDR being in the cytosol. Upon ligand binding, VDR is redistributed to the nucleus (Barsony et al. 1990, Barsony et al. 1997) and in the ligand-bound state VDR is predominantly a nuclear protein. VDR, like most steroid hormone receptors, is phosphorylated hormone-dependently (Pike and Sleator 1985, Brown and DeLuca 1990). The function of VDR phosphorylation is poorly understood, although it might represent a means for signals from the cell membrane to modulate nuclear hormone receptor function in response to growth factor stimulation (Issa et al. 1998).

Although nuclear receptors generally need the ligand for transcriptional regulation, some receptors which heterodimerize with RXR, for example the retinoic acid receptor (RAR) and thyroid hormone receptor (TR), act as silencers in the absence of the ligand repressing basal transcription of target genes (Chen and Evans 1995, Hörlein et al. 1995). The silencing activity is reported to be mediated by the co-repressor protein NCoR and the silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (Horwitz et al. 1996). In early reports, it has been suggested that VDR might not interact with co-repressors and act as a transcriptional silencer (Hörlein et al. 1995, Chen et al. 1996), whereas later reports show the opposite (Dwivedi et al. 1998, Tagami et al. 1998, Polly et al. 2000).

3.2. DNA-binding and dimerization

The affinity of monomeric VDR to specific DNA target sequences is not sufficient for the formation of a stable protein-DNA complex and thus formation of homo- and/or heterodimeric complexes with a second partner receptor is required for efficient DNA binding (Carlberg and Polly 1998). Like other members of nuclear receptor subfamily 1, VDR preferentially forms heterodimeric complexes with RXR, which is a nuclear receptor for 9-*cis* retinoic acid (Levin et al. 1992, Carlberg et al. 1993). Binding of the VDR-RXR heterodimer to DNA is enhanced by 1α,25$(OH)_2D_3$, whereas 9-*cis* retinoic acid inhibits this association, this possibly being related to the role of this retinoid in facilitating RXR homodimer formation (MacDonald et al. 1993, Kimmel-Jehan et al. 1997, Lemon et al. 1997, Jensen et al. 1998, Thompson et al. 1998). However, some reports indicate that 9-*cis* retinoic acid might be necessary for heterodimer formation (Carlberg et al. 1993, Dong and Noy 1998). If VDR before complexing with RXR occupies 1α,25$(OH)_2D_3$, the resulting heterodimer may be relatively resistant to dissociation and diversion to other pathways by 9-*cis* retinoic acid (Thompson et al.

1998). There might thus be hormonal crosstalk between metabolites of vitamins A and D in controlling VDR-mediated transcriptional regulation. Although the main partner of VDR is RXR, VDR also dimerizes with RAR, TR and VDR itself (Carlberg and Polly 1998). Monomeric binding of VDR to the promoter region of the granulocyte-macrophage colony-stimulating factor, leading to repression of gene transcription, has been reported (Towers and Freedman 1998).

Once heterodimeric VDR-RXR or the homodimeric VDR complex is formed, it modulates transcription by binding to specific DNA elements in the promoter regions of vitamin D responsive genes termed VDREs. Although there is considerable variation between natural VDREs, a consensus positive VDRE can be defined as a direct repeat (DR) of two half-elements of the sequence AGGTCA, separated by three intervening nucleotides (DR3) (Umesono et al. 1991). Other VDRE structures, for example direct repeats with four or six spacing nucleotides (DR4- and DR6-type respectively), inverted palindromes with nine intervening nucleotides (IP9-type) and more complex VDREs consisting of three core binding motifs, have been reported (Carlberg and Polly 1998, Toell et al. 2000). In addition to positive VDREs, genes which are negatively regulated by vitamin D are reported to contain VDREs differing from positive VDREs both in sequence composition and in the requirement for particular cellular factors other than VDR for repression of transcription (Demay et al. 1992, Mackey et al. 1996, Nishishita et al. 1998). It has been reported that the polarity of VDR-RXR heterodimer may be preferentially 5'-RXR-VDR-3', and reversed in specific VDREs (Carlberg and Polly 1998, Brown et al. 1999).

3.3. Regulation of gene expression

The effects of nuclear receptors on gene expression are mediated through recruitment of co-regulators, co-repressors and co-activators (Aranda and Pascual 2001). Unliganded VDR is bound to co-repressor proteins such as NCoR or Alien (Polly et al. 2000), which are found within multicomponent complexes containing histone deacetylase activity (Aranda and Pascual 2001). Deacetylation of histones leads to chromatin compaction and transcriptional repression. Binding of $1\alpha,25(OH)_2D_3$ to VDR induces a conformational change leading to dissociation of co-repressors and facilitating heterodimerization with RXR and interaction of the AF-2 domains with co-activator proteins, such as activator-recruited cofactor/ vitamin D receptor-

interacting protein (ARC/DRIP) complexes and members of the p160 family SRC-1, TIF2 and RAC3 (Chen and Li 1998, Rachez et al. 1999). Some of the co-activator proteins are chromatin-remodeling factors or posses histone acetylase activity, whereas others may interact directly with the basic transcriptional machinery (Aranda and Pascual 2001). Transcription factors and co-activators bridge contact between the VDRE-bound receptors, histone acetylases and RNA polymerase II (Issa et al. 1998). Recruitment of co-activator complexes to the target promoter leads to chromatin decondensation, allowing for binding and assembly of the transcription machinery and finally initiation of transcription (Aranda and Pascual 2001).

Transcriptional repression by nuclear hormone receptors occurs through a number of mechanisms (Issa et al. 1998). Inhibition of transcription factor function may be one mechanism by which VDR exerts transcriptional repression (Alroy et al. 1995, Kim et al. 1996). VDR may also inhibit the transcriptional function of TR, RAR and growth hormone receptor (GR) by competing for dimerization with RXR or possibly squelching co-activators (Garcia-Villalba et al. 1996, Yen et al. 1996, Polly et al. 1997).

4. NON-GENOMIC ACTIONS OF VITAMIN D

Steroid hormones can also have responses which occur within a few minutes after addition of a hormone and are therefore too rapid to involve changes in gene expression. These rapid actions are mediated by mechanisms other than nuclear receptors. The non-genomic actions of $1\alpha,25(OH)_2D_3$ include rapid increases in intracellular calcium levels, stimulation of intestinal calcium transport and phosphate fluxes, changes in phosphoinositide metabolism, elevation of cyclic 3',5'-guanosine monophosphate (cGMP) levels and activation of protein kinase C (reviewed in Brown et al. 1999). It has been suggested that the plasma membrane protein annexin II might be the membrane receptor which mediates the rapid actions of $1\alpha,25(OH)_2D_3$ (Baran et al. 2000a, Baran et al. 2000b). The rapid non-genomic effects may modulate the genomic actions of $1\alpha,25(OH)_2D_3$ (Bouillon et al. 1995).

5. VITAMIN D IN PROSTATE CANCER

Numerous in vitro studies have shown that 1α,25$(OH)_2D_3$ regulates growth and differentiation in a variety of normal and cancerous cells. Several mechanisms are thought to be involved in the anti-cancer effects of vitamin D, for example modulation of the cell cycle, apoptosis, growth factor and hormone secretion, telomerase activity, differentiation, angiogenesis, invasion and metastasis (van Leeuwen and Pols 1997, Hansen et al. 2001). Use of 1α,25$(OH)_2D_3$ in vivo is however limited due to its hypercalcemic side-effects, and therefore several hundred vitamin D analogs have been developed with the aim of separating growth-regulating effects from the calcemic adverse effects.

5.1. Prostate cancer

Prostate cancer is the most frequently diagnosed cancer in males in Finland and in many Western countries; its etiology remains nonetheless unclear. Epidemiological studies have suggested that the cause of prostate cancer is multifactorial, involving both genetic and environmental risk factors including aging, dark skin, a family history of prostate cancer, living in northern latitudes, and diet (Zhao and Feldman 2001). The majority (90%) of prostate cancers are sporadic and approximately 10% are hereditary; a number of genes have been held to be associated with prostate cancer risk (Zhao and Feldman 2001). Although sex hormones possibly contribute to prostatic carcinogenesis, circulating sex hormone levels in adult males have not been established as definitive etiological factors for prostate cancer (Taplin and Ho 2001).

5.2. Hormonal factors in prostate cancer

The prostate gland is stimulated to grow during development, is maintained in size and function by the presence of serum testosterone produced in the testis (Frick and Aulitzky 1991). In the prostate, the major proportion of testosterone is converted to the more potent intracellular androgen, dihydrotestosterone, by 5α-reductase enzyme activity. Androgens can bind and activate the androgen receptor, which like VDR, belongs to the superfamily of nuclear receptors. Androgens and AR are most likely involved in the genesis and progression of prostate cancer (Bosland 2000, Taplin and Ho 2001). Most prostate carcinomas react to generally used primary androgen ablation therapy by temporary involution and apoptosis of prostatic epithelial cells,

but usually subsequently relapse to an androgen-insensitive state, which is a major challenge in prostate cancer management (Bosland 2000).

Other hormonal factors like estrogen, vitamin D, prolactin, growth hormone and luteinizing hormone may act alone or in concert with androgens both in the normal prostate and in prostate cancer development and progression (Reiter et al. 1999, Taplin and Ho 2001).

5.3. Vitamin D in the etiology of prostate cancer

Epidemiological interest in the role of vitamin D in the etiology of prostate cancer was in 1990, when Schwartz and Hulka (1990) published their hypothesis that vitamin D deficiency might be a risk factor for the condition. According to this conception all known risk factors for clinical prostate cancer (aging, black race and living in northern latitudes) are associated with decreased synthesis of vitamin D. In contrast to clinical prostate cancer, subclinical prostate carcinoma is geographically virtually universal, this possibly reflecting factors which influence tumor progression. Results from geographical studies support the suggestion of an inverse relationship between the level of solar UV radiation and prostate cancer mortality (Hanchette and Schwartz 1992, Grant 2002). However, although Corder and coworkers (1993) were able to show an association between low serum $1\alpha,25(OH)_2D_3$ and prostate cancer risk, subsequent analytical epidemiological studies on the role of vitamin D in the etiology of prostate cancer have failed to prove the vitamin D hypothesis (Braun et al. 1995, Gann et al. 1996, Nomura et al. 1998). Dietary calcium obtained from dairy products has been shown in epidemiological studies to increase the risk of prostate cancer, possibly by suppressing the enzymatic activity of 1α-hydroxylase and thereby lowering the serum $1\alpha,25(OH)_2D_3$ concentration (Chan and Giovannucci 2001). The results of some, although not all, epidemiological studies show that polymorphisms in the VDR gene may contribute to the risk of prostate cancer (Taylor et al. 1996, Ingles et al. 1997, Kibel et al. 1998, Correa-Cerro et al. 1999, Furuya et al. 1999, Watanabe et al. 1999, Blazer et al. 2000, Habuchi et al. 2000, Chokkalingam et al. 2001, Hamasaki et al. 2001).

5.4. In vitro and in vivo studies

Despite controversial results in analytical epidemiological studies of the role of vitamin D in prostate cancer, numerous experimental studies have provided evidence supporting the vitamin D hypothesis. The finding of VDR within epithelial and stromal cells in the human prostate as well as in human prostate cancer cells confirmed that $1\alpha,25(OH)_2D_3$ might play a direct role in prostate biology (Miller et al. 1992, Skowronski et al. 1993, Peehl et al. 1994). Though the effect of $1\alpha,25(OH)_2D_3$ on human prostatic cells is generally growth-inhibitory in vitro, mitogenic effects have been reported with low concentrations of $1\alpha,25(OH)_2D_3$ using charcoal-stripped serum in cell culture medium (Gross et al. 1997). Studies on rat prostates in vivo have shown that in addition to its inhibitory effects on epithelial cells $1\alpha,25(OH)_2D_3$ may promote the growth of prostate size in pubertal and adult rats when $1\alpha,25(OH)_2D_3$ is administered in high doses prenatally, and the growth of stromal cells in castrated rats (Konety et al. 1996, Konety et al. 1999, Krill et al. 1999). Stimulation of prostatic stromal cells by $1\alpha,25(OH)_2D_3$ has also been reported in primary cultures of human cells (Krill et al. 1999). The growth inhibition by $1\alpha,25(OH)_2D_3$ in human prostate cancer cells is mediated via VDR and may involve both androgen-dependent and androgen-independent mechanisms; the response is modulated by factors which are cell line-specific (Hedlund et al. 1996a, Hedlund et al. 1996b, Yang et al. 2002, Zhao et al. 1997, Zhao et al. 2000, Zhuang et al. 1997).

In addition to its antiproliferative effects, $1\alpha,25(OH)_2D_3$ may stimulate cellular differentiation. It has been reported that $1\alpha,25(OH)_2D_3$ induces expression of prostate-specific antigen (PSA), a marker of the differentiated prostatic phenotype in LNCaP prostate cancer cells (Skowronski et al. 1993, Skowronski et al. 1995, Hsieh et al. 1996, Campbell et al. 1997, Zhao et al. 1997), and E-cadherin, a cell adhesion protein which may act as a putative tumor suppressor, in LNCaP and PC-3 cells (Campbell et al. 1997). In combination, $1\alpha,25(OH)_2D_3$ and dihydrotestosterone (DHT) may synergistically enhance PSA secretion (Zhao et al. 1997). An in vivo study on castrated rats has also shown that $1\alpha,25(OH)_2D_3$ in concert with testosterone may play an important role in the differentiation of the normal prostate (Konety et al. 1996). Furthermore, $1\alpha,25(OH)_2D_3$ has been shown to inhibit the invasiveness and reduce the adhesion and migration of prostatic cancer cells in vitro and inhibit metastasis in vivo in a rat model (Schwartz et al. 1997, Lokeshwar et al. 1999, Sung and Feldman 2000).

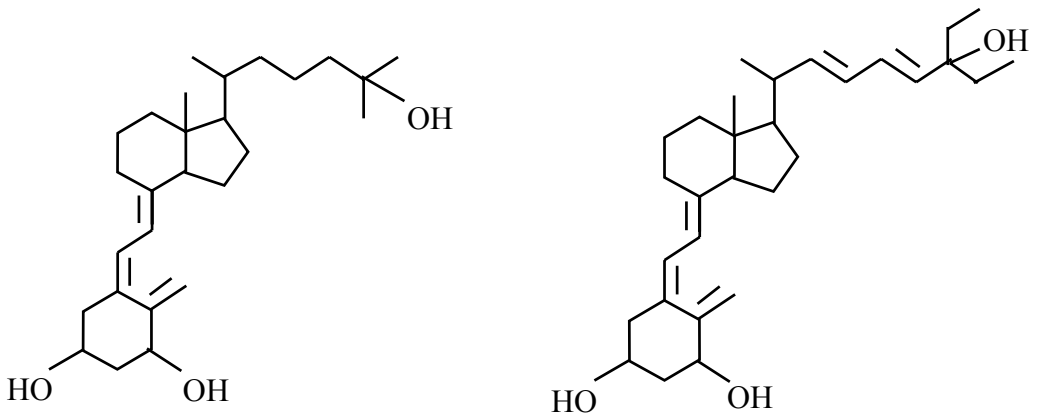
The response to vitamin D can be modulated in prostatic cells by vitamin D hydroxylating enzymes. Induction of the 1α,25$(OH)_2D_3$-inactivating 24-hydroxylase enzyme has been reported in human prostate cancer cell lines after 1α,25$(OH)_2D_3$ treatment; growth inhibition of the cell lines by 1α,25$(OH)_2D_3$ was inversely related to 24-hydroxylase activity (Skowronski et al. 1993, Miller et al. 1995). Ketoconazole is used for secondary hormonal manipulation in the treatment of prostate cancer, because, at high doses, it blocks both testicular and adrenal synthesis of testosterone (Peehl et al. 2001). It inhibits the cytochrome P450 enzymes needed for androgen biosynthesis but also for vitamin D metabolism (Loose et al. 1983). Treatment combining ketoconazole with 1α,25$(OH)_2D_3$ is held to have additional beneficial effects due to inhibition of 1α,25$(OH)_2D_3$ inactivation (Peehl et al. 2001). Liarozole is another inhibitor of cytochrome P450 enzymes which has been shown to act synergistically with 1α,25$(OH)_2D_3$ to inhibit the growth of human prostate cancer cell line DU 145 (Ly et al. 1999). In a study of 50 primary organ-confined prostate adenocarcinomas a minimal overlapping region of gain, indicative of the presence of oncogene, was found at 20q13.3-qter, which is a chromosomal region containing the 24-hydroxylase gene (Wolter et al. 2002). In addition to inactivation of 1α,25$(OH)_2D_3$, prostate cells have been shown to 1α-hydroxylate the prohormone, 25-hydroxyvitamin D_3 to active 1α,25$(OH)_2D_3$ form (Schwartz et al. 1998, Barreto et al. 2000, Hsu et al. 2001). The ability of 25(OH)D to inhibit the growth of prostatic epithelial cells in vitro has been reported (Barreto et al. 2000, Chen et al. 2000, Hsu et al. 2001). However in cancer cells, the antiproliferative action of 25(OH)D was shown to be reduced compared to that of 1α,25$(OH)_2D_3$ due to deficient 1α-hydroxylase activity in these cells (Hsu et al. 2001). Taken together, these in vitro and in vivo findings show that the prostate may be a target organ of 1α,25$(OH)_2D_3$ and may in addition both activate and inactivate vitamin D compounds.

5.5. Clinical trials and vitamin D analogs

The efficacy of vitamin D treatment has been tested in a few clinical trials. An open label, non-randomized pilot trial on seven early recurrent prostate cancer patients by Gross and colleagues (1998) provided evidence that 1α,25$(OH)_2D_3$ may effectively slow the rate of PSA rise in select cases, although dose-dependent calciuric side-effects limit its clinical usefulness. Later, a phase I trial of 1α,25$(OH)_2D_3$ in patients with advanced solid tumors, including six patients with prostate cancer, indicated that

substantial doses of 1α,25$(OH)_2D_3$ could be administered subcutaneously with tolerable toxicity (Smith et al. 1999). Van Veldhuizen and coworkers (2000) have conducted a phase II trial and reported that a major part of advanced hormone refractory prostate cancer patients (seven out of 16 studied) may have decreased baseline vitamin D levels and that supplementation with vitamin D_2 (ergocalciferol) may be a useful adjunct for alleviating pain associated with prostate cancer bone metastasis, and improving muscle strength and quality of life in these patients. Preliminary results from a phase II study on androgen-independent prostate cancer patients suggest that combinatory treatment with 1α,25$(OH)_2D_3$ and docetaxel may reduce prostate-specific antigen levels by at least 50% with 5 patients studied (Beer et al. 2001).

The above results are of course preliminary in view of the limited number of prostate cancer patients involved in these studies, but may nonetheless prove useful in the treatment of prostate cancer. The problem is that effective doses are difficult to administer without inducing hypercalcemia, and several hundred vitamin D analogs have been synthesized with the aim of separating the growth-regulating effects from the adverse calcemic effects (Mørk Hansen et al. 2001). One of the most promising synthetic analogs to date is EB 1089 (Leo Pharmaceutical Products), which has an altered side-chain structure with 26,27 dimethyl groups, insertion of an extra carbon atom (24a) at C-24, and two double bonds at C-22,23 and C-24,24a (Colston et al. 1992). The structure of EB 1089 is presented in Figure 4. In vitro studies have shown that this analog is 50-200 times more potent than 1α,25$(OH)_2D_3$ in regulating cell growth and differentiation, while its calcemic effects are approximately 50% weaker than those of 1α,25$(OH)_2D_3$ in vivo in rats (Mørk Hansen et al. 2000). The biological half-life of EB 1089 is similar to that of 1α,25$(OH)_2D_3$ (Mørk Hansen et al. 2001), but its side-chain metabolism may differ from 1α,25$(OH)_2D_3$ and may not involve 24-hydroxylation (Kissmeyer et al. 1997, Shankar et al. 1997). Another strategy is to develop VDR modulators structurally distinct from the secosteroid 1α,25$(OH)_2D_3$. A nonsecosteroidal VDR modulator, LG190119, was recently shown to inhibit LNCaP xenograft tumor growth without increased serum calcium levels or any other apparent side-effects (Polek et al. 2001). However, more studies are needed to confirm the safety and efficacy of these compounds in humans.



1α,25-dihydroxyvitamin D_3 **EB 1089**

Figure 4. Structures of 1α,25-dihydroxyvitamin D_3 and vitamin D analog EB 1089.

5.6. Mechanisms of growth regulation by vitamin D

Although there are abundant data on the antiproliferative action of vitamin D, the underlying mechanisms are less fully known. It has been suggested that growth inhibition by 1α,25$(OH)_2D_3$ might involve, e.g., regulation of the cell cycle, induction of apoptosis and modulation of growth factor, oncogene and tumor suppressor gene expression.

Studies on the LNCaP prostate cancer cell line have shown that the growth inhibitory action of 1α,25$(OH)_2D_3$ may be due to both accumulation of cells in the G0/G1 phase of the cell cycle and induction of programmed cell death, apoptosis (Blutt et al. 1997, Hsieh and Wu 1997, Blutt et al. 2000, Yang et al. 2002). The blocking of cells by 1α,25$(OH)_2D_3$ in the G0/G1 phase may be associated with alterations of important cell cycle regulators. In LNCaP cells, the activity of cyclin-dependent kinase 2 has been found to be reduced, the cyclin-dependent kinase inhibitors p21 and p27 upregulated and phosphorylation of retinoblastoma protein reduced by 1α,25$(OH)_2D_3$ or analog (Campbell et al. 1997, Zhuang and Burnstein 1998, Yang et al. 2002). In ALVA-31, PC-3 and DU-145 cells, the expression of cyclin-dependent kinase inhibitor p21 has been shown to be either up-regulated or not induced by 1α,25$(OH)_2D_3$ or analog (Campbell et al. 1997, Zhuang and Burnstein 1998, Moffatt et al. 2001). The apoptotic effect of 1α,25$(OH)_2D_3$ in LNCaP cells is accompanied by a down-regulation of Bcl-2 and Bcl-X(L) proteins, both of which protect cells from undergoing apoptosis (Blutt et al. 2000). In a culture of cells derived from benign

prostatic hyperplasia patients and in human prostate cancer cell line DU 145, a vitamin D analog induced apoptosis in both unstimulated and keratinocyte growth factor-stimulated cells, which was associated with decreased basal bcl-2 expression (Crescioli et al. 2002). EB1089 is reported to induce apoptosis in vivo in rats (Nickerson and Huynh 1999).

In addition, $1\alpha,25(OH)_2D_3$ may modulate the expression of several growth factors possibly participating in the progression of prostate cancer. The results of a study by Huynh and coworkers (1998) showed that $1\alpha,25(OH)_2D_3$ and EB1089 inhibit the insulin-like growth factor (IGF) system of mitogens in PC-3 prostate cancer cells by up-regulating IGF binding protein-3 and down-regulating IGF-II expression. However, $1\alpha,25(OH)_2D_3$ had no effect on IGF-binding protein-3 secretion in LNCaP cells (Goossens et al. 1999). In primary cultures of non-transformed prostate epithelial cells, the growth inhibition induced by $1\alpha,25(OH)_2D_3$ has been accompanied by an up-regulation of IGF binding protein 3 (Sprenger et al. 2001). In the rat, EB1089 has been shown to induce a regression of the ventral prostate which was associated with up-regulation of IGF- binding proteins 2, 3, 4 and 5 as well as IGF-I expression levels (Nickerson and Huynh 1999). One study on a rat epithelial prostate cell line has shown that cellular differentiation promoted by $1\alpha,25(OH)_2D_3$ may be mediated through transforming growth factor β (TGFβ) 2 and 3 (Danielpour 1996). However, TGFβ may promote a smooth muscle cell phenotype in human prostatic stromal cells while $1\alpha,25(OH)_2D_3$ may not be involved in this differentiation process (Peehl and Sellers 1997). In vitro studies on human prostate cells have shown that $1\alpha,25(OH)_2D_3$ and analog may counteract the mitogenic activity of keratinocyte growth factor (Crescioli et al. 2000, Crescioli et al. 2002). The expression of parathyroid hormone-related protein (PTHrP), an epidermal growth factor-regulated secretory product of human prostatic epithelial cells, is inhibited by $1\alpha,25(OH)_2D_3$ in several cell types but possibly not in epithelial cells of the prostate (Cramer et al. 1996). On the other hand, a recent study by Sepulveda and Falzon (2002) has shown that the expression of PTHrP is down-regulated by $1\alpha,25(OH)_2D_3$ in PC-3 cells.

It is further conceivable that, $1\alpha,25(OH)_2D_3$ may regulate specific oncogenes and tumor suppressor genes in the prostate. Campbell and colleagues (2000) have studied several prostate cancer cell lines and shown that the anti-proliferative effects of

$1\alpha,25(OH)_2D_3$ may be mediated, in part, by the induction of breast cancer susceptibility gene 1 (BRCA1) expression. BRCA1 has been implicated in the maintenance of global genome stability, possibly controlling both DNA damage repair and the transcription of DNA damage-inducible genes (Zheng et al. 2001).

6. VITAMIN D IN OVARIAN CANCER

6.1. Ovarian cancer

Ovarian cancer is the fourth most frequently diagnosed cancer in females in Finland and in many Western countries. The survival of patients with ovarian cancer is low as the condition is diagnosed late in its course, which may be asymptomatic until advanced state. The etiology of ovarian cancer is poorly understood. The results of epidemiological studies suggest that both genetic and environmental risk factors are involved in its pathogenesis. Multiparity, the use of oral contraceptives, tubal ligation and hysterectomy are associated with a reduced risk of ovarian cancer, whereas aging, nulliparity and genital talc application are thought to increase the risk (Daly and Obrams 1998, Riman et al. 1998, La Vecchia and Franceschi 1999, Holschneider and Berek 2000). The majority of ovarian cancer cases are sporadic and about 5 % are familial (Kristensen and Trope 1997, Holschneider and Berek 2000). The oncogenes or tumor suppressor genes associated with sporadic ovarian cancer include human epidermal growth factor receptor-2 (HER-2/neu), c-myc and K-ras, whereas familial ovarian cancer is associated with BRCA1 and BRCA2 tumor suppressor genes (Aunoble et al. 2000, Holschneider and Berek 2000). The majority (80 – 90%) of ovarian tumors arise from the ovarian surface epithelium and a minority from germ cells or sex cord/stromal cells (Kristensen and Trope 1997).

6.2. Hormonal factors in ovarian cancer

In women of fertile age, the ovaries are the major sites of sex steroid hormone production. In addition to progesterone and estrogenic hormones, the ovaries secrete significant amounts of the androgenic hormones androstendion and testosterone. The menstrual cycle, the maturation of follicles in the ovaries, is regulated by the concerted action of gonadotropin-releasing hormone, follicle-stimulating hormone, luteinizing hormone, estrogens, progesterone and inhibin. Ovarian androgens may directly augment granulosa cell responsiveness to follicle-stimulating hormone, serve

as substrates for estrogen synthesis in the preovulatory follicle and play a role in the promotion of atresia in ovarian follicles (Armstrong and Papkoff 1976, Chryssikopoulos 2000). In the menopause, the production of ovarian estrogens and progesterone decreases and the postmenopausal ovaries produce mainly androgens (androstendion) (Adashi 1994). The majority of ovarian cancer cases are diagnosed in postmenopausal women aged 50-60 years.

The mechanisms of ovarian carcinogenesis are not fully understood. In view of the higher prevalence of ovarian cancer in nulliparous than in multiparous women and the lower frequency of disease in users of oral contraceptives compared with non-users, it has been suggested that the risk of epithelial ovarian cancer could be related to repetitious ovulatory activity (Kristensen and Trope 1997, Risch 1998). The traumatized ovarian surface epithelium is recurrently repaired and exposed to estrogen-rich follicular fluid which may increase genetic abnormalities and lead to malignant transformation (Kristensen and Trope 1997, Riman et al. 1998). Excessive gonadotropin and androgen stimulation of the ovary have been postulated as contributing factors (Risch 1998, Holschneider and Berek 2000), whereas progesterone may have a protective role in the etiology of ovarian cancer (Risch 1998). The production of androgens in the post-menopausal ovaries as well as the predominance of AR and the production of androgens in ovarian cancer tissue suggests a role for androgens in the etiology of the malignancy (Mahlck et al. 1986, Kuhnel et al. 1987, Slotman et al. 1989, Adashi 1994, Ilekis et al. 1997, Cardillo et al. 1998).

6.3. Vitamin D in the etiology of ovarian cancer

It has been observed that the ovarian cancer incidence and mortality are higher in northern than southern latitudes. Geographical studies have provided evidence that sunlight may be protective against ovarian cancer mortality (Lefkowitz and Garland 1994, Grant 2002). As UV radiation is needed for vitamin D synthesis in the skin, it has been suggested that the beneficial effect of sunlight might be mediated through the action of vitamin D. However, analytical epidemiological studies measuring vitamin D levels in serum, as yet not available, are needed to evaluate the possible relationship.

6.4. Vitamin D in non-malignant ovaries

The ovary is reported to be a target organ for vitamin D as vitamin D receptor has been shown using ligand-binding assay and autoradiography in the ovaries of hamsters, hens and mice (Dokoh et al. 1983, Stumpf and Denny 1989). In the rat ovary, the presence of VDR has been shown by immunohistochemistry (Johnson et al. 1996). In addition, the ovaries might be capable of catalyzing 25-hydroxylation of vitamin D_3 as has been shown in rats (Su et al. 1990). Furtermore, vitamin D metabolites (25(OH)D, 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$) have been shown in the follicular fluid of human ovaries, albeit in lower concentrations than in serum (Potashnik et al. 1992). The polycystic ovarian syndrome is one of the most common female endocrine disorders and is characterized by hyperandrogenic chronic anovulation with infertility, irregular menses and dysfunctional uterine bleeding (Franks 1995). Treatment of polycystic ovarian syndrome patients, of whom the majority evince low serum 25(OH)D concentrations, with vitamin D and calcium has resulted in normalized menstrual cycles, which suggests that abnormalities in calcium homeostasis might be partially responsible for the arrested follicular development in women suffering from this syndrome (Thys-Jacobs et al. 1999).

Studies on female rats have shown that vitamin D deficiency markedly reduces fertility, and the situation is not corrected by normalizing hypocalcemia, but requires 1α,25$(OH)_2D_3$ (Halloran and DeLuca 1980, Kwiecinksi et al. 1989). However, the high doses of 1α,25$(OH)_2D_3$ which bring about hypercalcemia might disturb the estrous cycle, as reported in the rat (Horii et al. 1992). Studies on null mutant mice have suggested that 1α,25$(OH)_2D_3$ may play a role in ovarian function, as infertility and impaired folliculogenesis were seen in both VDR and 1α-hydroxylase null mutant mice (Yoshizawa et al. 1997, Panda et al. 2001). The effect of 1α,25$(OH)_2D_3$ on ovarian function remains, however, unclear, as dietary supplementation with calcium, which corrected hypocalcemia, partially normalized fertility and the aromatase enzyme needed for estrogen biosynthesis in the VDR null mutant mice (Kinuta et al. 2000, Johnson and DeLuca 2001). Moreover, female mice in a study using different VDR null mutant mice model were fertile (Li et al. 1997).

6.5. Vitamin D in human ovarian cancer

The receptor for vitamin D has also been reported in four human ovarian cancer tissues out of nine studied and in the ovarian cancer cell line OVCAR-3, using ligand binding assay (Frampton et al. 1982, Saunders et al. 1992). Induction of 24-hydroxylase activity by $1\alpha,25(OH)_2D_3$ has been shown in ovarian neoplasms, indicating that the tissue is responsive to $1\alpha,25(OH)_2D_3$ (Christopherson et al. 1986). Inhibition of ovarian cancer cell growth by $1\alpha,25(OH)_2D_3$ has been reported (Saunders et al. 1992, Saunders et al. 1995). The growth-inhibitory action of $1\alpha,25(OH)_2D_3$ in ovarian cells may be mediated through down-regulation of proto-oncogene c-myc (Saunders et al. 1993). Amplification of chromosomal region 20q12-q13, containing the 24-hydroxylase gene, has been reported in ovarian cancer (Iwabuchi et al. 1995, Tanner et al. 2000); possible amplification of 24-hydroxylase gene has not however been confirmed. Amplification of the 24-hydroxylase gene has been reported in breast cancer and it has been suggested that 24-hydroxylase might act as an oncogene limiting and abrogating the growth control mediated by $1\alpha,25(OH)_2D_3$ (Albertson et al. 2000).

A clinical trial has been conducted on the efficacy of $1\alpha,25(OH)_2D_3$ combined with isotretinoin in the treatment of epithelial ovarian cancer patients after chemotherapy (Rustin et al. 1996). The investigators treated 22 women with $1\alpha,25(OH)_2D_3$ and isotretinoin for up to 74 weeks and tumor response was monitored using serum cancer antigen CA 125 level as a marker of tumor progression. Treatment produced no responses or change in the tumor growth rate based on CA 125 except in one patient with borderline ovarian serous carcinoma, which was behaving in a malignant invasive manner. However, the VDR status was not studied in these patients. More studies are needed to evaluate the effects of $1\alpha,25(OH)_2D_3$ and its analogs in ovarian cancer.

AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. to raise and characterize a specific antibody to the VDR which can be used to analyze VDR protein expression by immunohistochemistry and Western blot in human tissues (I).

2. to examine the cellular distribution of VDR in the prostate, and compare relative VDR abundances between different human prostate samples (I).

3. to study the expression and regulation of VDR and AR proteins by cognate ligands in human ovarian cancer tissue and ovarian cancer cell line OVCAR-3 (II).

4. to study the effects of vitamin D compounds (1α,25$(OH)_2D_3$, EB 1089 and 25$(OH)D_3$) and androgen (DHT) on the growth of OVCAR-3 cells (II, III).

4. to evaluate the possible association between serum vitamin D status (25-hydroxyvitamin D concentration) and the risk of prostate cancer (IV).

MATERIALS AND METHODS

1. ANTIBODIES (I, II)

1.1. VDR antibody

Antibody R112 was raised in a rabbit by immunization of a synthetic peptide corresponding to the N-terminal residues 10-24 of the human VDR. A male New Zealand white rabbit was injected subcutaneously with 100 μg of peptide conjugated to thyroglobulin. The first injection was given in 0.6 ml of Freund´s complete adjuvant and the subsequent three in 0.6 ml of Freund´s incomplete adjuvant at 1-month intervals. The serum was precipitated by addition of saturated ammonium sulphate to a final volume of 40%, and the resulting IgG fraction was further characterized for VDR specificity. The immunoreactivity of the IgG fraction was tested against the corresponding ovalbumin-conjugated peptide (0.5 μg/ml) immobilized on microtiter plates (Nunc-Immuno Plate, MaxiSorp, Nunc, Roskilde, Denmark).

1.2. AR antibody

A polyclonal antibody, AR70, was produced in rabbits against a synthetic peptide corresponding to the N-terminal residues 1 to 17 of human AR. This sequence is identical in human and rat AR (Tähkä et al. 1997).

2. CELL CULTURE (I, II, III)

2.1. MG-63 human osteosarcoma cells

MG-63 cells (ATCC, Manassas, USA), which are known to express VDR (Franceschi et al. 1985), were grown in RPMI medium (Gibco, NY, USA) supplemented with 7% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a humidified 95% air/ 5% CO_2 incubator. To enhance VDR expression, as previously reported (Costa and Feldman 1986, Mahonen et al. 1990, Mahonen and Mäenpää 1994), 10 nM 1α,25-dihydroxyvitamin D_3 (Hoffman LaRoche, Basel, Switzerland) was added to the culture medium. Treatments were carried out in RPMI 1640 medium containing 2% charcoal-treated FBS.

2.2. OVCAR-3 human ovarian adenocarcinoma cells

OVCAR-3 cells (ATCC) were grown in RPMI 1640 medium (Sigma Aldrich, St. Louis, USA) supplemented with 10% FBS, 10 μg/ml insulin and antibiotics at 37°C in a humidified 95% air/ 5% CO_2 incubator. For cell growth assays, OVCAR-3 cells were seeded at 1500 (II) or 2000 (III) cells/ well in 96-well culture plates. After allowing the cells to attach for 24 h, they were treated with ethanol vehicle or 10 nM or 100 nM 1α,25$(OH)_2D_3$ (a kind gift from Leo Pharmaceuticals, Ballerup, Denmark) and/or DHT (5α-androstan-17β-ol-3-one; Fluka, Buchs, Switzerland). Media containing ethanol vehicle and/or hormones were changed every third day. Cell growth was analyzed for 0, 1, 3, 5, 7 or 9 days after hormone treatment under non-confluent conditions.

For Western blot analysis, near-confluent cells were treated for 24 h with 10 nM or 100 nM 1α,25$(OH)_2D_3$ and/or DHT. Control cells received ethanol vehicle at a concentration equal to that in hormone-treated cells (0.1% vol/vol). Cell pellets were collected for Western blot analysis.

2.3. HaCaT human keratinocyte cells and COS monkey kidney cells

HaCaT cells were grown in DMEM supplemented with 10% FBS and antibiotics and COS cells in DMEM/F12 supplemented with 5% FBS and antibiotics (Sigma Aldrich). The cells were kept at 37°C in a humidified 95% air/ 5% CO_2 incubator.

2.4. Cell fractionation and protein extraction

Nuclear and cytosolic protein extracts were prepared from cell pellets according to a modified version of the method of Hurst and colleagues (1990), omitting the heat-denaturation step. The cells were washed twice with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 7.8 mM $Na_2HPO_4*2H_2O$), harvested with a cell scraper, and resuspended in 300 μl of ice-cold lysis buffer (20 mM Hepes [pH 8.0], 20 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol and protease inhibitor coctail [Roche Diagnostics, Mannheim, Germany]) and left on ice for 5 min. A nuclear pellet containing proteins tightly associated with nuclear structures was obtained by centrifugation for 1 min at maximal speed in an Eppendorf centrifuge. Supernatant was taken as a cytosolic fraction. The crude nuclear pellet was

resuspended in 60 µl of buffer (20 mM Hepes [pH 7.9], 25% [by vol.] glycerol, 0.42 M NaCl, 1.5 µM $MgCl_2$, 2 µM EDTA [pH 8.0], 1 mM dithiothreitol and protease inhibitors) and left on ice for 15 min, resuspended, and left for a further 15 min. Nuclear debris was removed by centrifugation as above and supernatant was taken as a nuclear fraction. Protein concentrations were determined using the method of Lowry (1951).

2.5. Cell growth assays

Relative cell numbers were quantified by crystal violet staining, as previously described (Kueng et al. 1989). Briefly, cells were fixed by addition of 10 µl of an 11% glutaraldehyde solution to 100 µl of medium. After being shaken for 15 min (500 cycles/min), the plates were washed with de-ionized water and air-dried, then stained by addition of 100 µl of a 0.1% solution of crystal violet dissolved in de-ionized water. After incubation for 20 min, excess dye was removed by extensive washing with de-ionized water. The plates were air-dried prior to bound-dye solubilization in 100 µl of 10% acetic acid. The optical density of dye extracts was measured directly in the plates using a Victor 1420 Multilabel Counter (Wallac, Turku, Finland). The wavelength selected was 590 nm. The growth curves were presented differently in studies II and III. In study II, A_{590nm} values were taken as such as relative cell numbers, whereas in study III the A_{590nm} value on day 0 (overnight culture of 2000 cells) was set as 0 by subtracting it from all other values (day 1-11). The time taken by OVCAR-3 cells to double in number (cellular doubling time) was calculated in the logarithmic growth phase (II).

Statistical analysis. Cell growth experiments with different hormone concentrations were repeated one to four times, 7 (II) or 8 (III) replicates each. Relative cell numbers (II) or relative cell growth (III) was compared using a two-tailed Student's t-test.

3. TISSUE SAMPLES (I, II)

3.1. Human tissue samples

Human skin donors were healthy volunteers (I). Prostatic tissues were obtained from 8 men undergoing radical prostatectomy for urinary bladder cancer (I). Patient ages

varied from 69 to 75 years, mean 72 years. Ovarian cancer tissues were obtained from 14 women undergoing radical ovariectomy for ovarian cancer (II); 9 serous cystadenocarcinomas, one mucinous cystadenocarcinoma, 3 endometrioid carcinomas and one granulosa cell tumor. Patient ages varied from 42 to 84 years, mean 63 years. Some of the tissues were collected between 1995 and 1997 in the Department of Pathology, and some were obtained directly from the Department of Obstetrics and Gynecology, Tampere University Hospital. The local ethical committee approved these studies.

3.2. Rat tissue samples

Kidney, intestine and prostate were taken from adult male rats (I). Ovaries were taken from adult female rats (II).

4. WESTERN BLOT ANALYSIS (I, II)

Protein samples were mixed 1:2 with sample buffer (125 mM Tris, 20% glycerol, 4% SDS, 5% (I) or 10% (II) β-mercaptoethanol, 0.05% bromophenolblue) and boiled for 5 min. The proteins (40 μg/lane) were resolved according to their electrophoretic mobility in 12% (for VDR)(I, II) or 7.5% (for AR)(II) polyacrylamide running gels according to the method of Laemmli (1970) and transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) on ice. The membranes were incubated for 1 h at 37°C with TBST (50 mM Tris, 0.9% NaCl, 0.05% Tween 20, pH 8.0) containing 5% non-fat milk to block unspecific binding of proteins, and thereafter with primary antibody (5 μg/ml) in TBS (50 mM Tris, 0.9% NaCl) (I) or in TBST (II) containing 1% non-fat milk overnight at 4°C. After washing with TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (Cappel, West Chester, USA) diluted 1:10000 for 1 hr at room temperature. Immunoreactive protein bands were detected using the enhanced chemiluminescence (ECL) method (Amersham, Aylesbury, UK). Relative band intensities and areas were measured using the SigmaScan program (II).

The molecular weight standards (BioRad Laboratories, Richmond, USA) were myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase b (M_r 97,400), bovine

serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), trypsin inhibitor (M_r 21,500) and lysozyme (M_r 14,400).

4.1. Controls for Western blot

To verify the specificity of the VDR band, primary antibody was presaturated with an excess of the corresponding peptide for 5 h at 4°C (I). Human VDR produced *in vitro* in the TnT-coupled rabbit reticulocyte lysate system (Promega, Madison, USA) according to manufacturer's instructions was used as a positive control (I, II).

5. IMMUNOHISTOCHEMISTRY (I, II)

5.1. Cells

MG-63 cells were plated on glass slides and grown to about 50% confluency. The cells were washed with PBS, fixed with 4% paraformaldehyde in PBS on ice, followed by permeabilization with 0.5% Triton X-100 in PBS for 40 min at room temperature. The slides were incubated with 2.5% non-fat milk in PBS for 30 min, and thereafter with primary antibody (10 μg/ml) in PBS containing 0.5% non-fat milk at 4°C overnight. Biotinylated secondary antibody (goat anti-rabbit IgG, Vector Laboratories, Burlingame, USA) was incubated for 30 min and finally the avidin biotin-peroxidase complex (Vectastain Elite, ABC Kit, Vector, Burlingame, USA) was incubated for 30 min. Peroxidase activity was visualized by an incubation in 0.02% 3,3´diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA), 0.01% imidazole, and 0.02% H_2O_2 in 0.5 M Tris, pH 7.6, for 5-7 min. The specimens were washed in PBS after every incubation step, except after blocking.

5.2. Tissues

5.2.1. Tissue fixation

Tissue samples were immersion-fixed with 4% paraformaldehyde in PBS or Baker´s fluid (4% paraformaldehyde and 1% $CaCl_2$ in distilled water, pH 7.6) at 4°C for 1.5-2 h (I) or at room temperature for 18-24 h (II). Thereafter the samples were dehydrated and embedded in paraffin.

5.2.2. Immunostaining

Paraffin sections (5 μm) were deparaffinized with xylene and rehydrated in a graded ethanol series. The antigens (VDR and AR) were unmasked by heating the specimens in 0.01 M sodium citrate buffer (pH 6.0) at 100°C for 10 min, followed by cooling for 20 min at room temperature and rinsing in TBS buffer (pH 7.6). Endogenous peroxidase activity was eliminated by treatment with 0.5% H_2O_2 in methanol for 15 min. After washing in distilled water and PBS, non-specific binding was eliminated with 10% normal goat serum in PBS, followed by incubation with primary antibody (5 μg/ml) overnight at 4°C. Biotinylated secondary antibody (goat anti-rabbit IgG; Vector) was incubated for 30 min, and finally, avidin biotin-peroxidase complex (Vectastain Elite, ABC Kit; Vector) was incubated for 30 min. Alternatively (III), histochemical staining was performed using the Histostain-Plus Kit (Zymed, San Francisco, USA). Peroxidase activity was visualized by an incubation in 0.02% 3,3´diaminobenzidine tetrahydrochloride (Sigma), 0.01% imidazole, and 0.02% H_2O_2 in 0.5 M Tris, pH 7.6, for 10 min.

5.3. Immunohistochemical controls

Control experiments included presaturation of the primary antibody (R112) with an excess of the corresponding peptide (I, II), substitution of R112 with PBS (II) or normal rabbit IgG at the same concentration as R112 (I, II) and serial dilutions of R112 up to extinction (I).

6. RT-PCR ANALYSIS (III)

Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect 1α-hydroxylase and 24-hydroxylase mRNAs. The culture medium of cells grown to 70% confluence was replaced with medium containing 100 nM 1α,25$(OH)_2D_3$, 25$(OH)D_3$ or EB 1089. Ethanol was used as vehicle and was also added to control cells. After 4, 6 and 24 h treatment RNA was extracted using TRIZOL reagent (Life Technologies, Carlsbad, USA). The integrity of RNA samples was confirmed on gel electrophoresis.

The specific oligonucleotide primers for RT-PCR were synthesized by Amersham Bioscience. To detect 1α-hydroxylase RT-PCR was performed according to

manufacturer's instructions from 1 μg total RNA (RobusT RT-PCR Kit, Finnzymes, Espoo, Finland). The RT-PCR protocol was as follows: reverse transcription at 48°C for 30 min and a denaturation step at 94°C for 2 min followed by 30 cycles with denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. The final extension step after the cycles was at 72°C for 7 min. Total RNA (0.5 μg) from monkey kidney COS cells transfected with human 1α-hydroxylase cDNA (a kind gift from Shigeaki Kato, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) using Lipofectamine reagent (Life Technologies) was used as positive control. A functional control included in the RT-PCR kit was carried out in the same run as the other samples. Agarose gel electrophoresis (1.5% gel) was used to detect RT-PCR products. These were extracted from the gel after electrophoresis and the sequences verified by hybridization with a [^{32}P]-labelled probe made from 1α-hydroxylase cDNA.

The reactions for 24-hydroxylase were performed in the LightCycler instrument (Roche Diagnostics) from 300 ng total RNA and PBGD was used as an external control. A master mix of the following components was prepared in a 20 μl volume: 0.3 μM 24-hydroxylase primers or 0.5 μM PBGD primers and 3.25 Mn^{2+} for 24-hydroxylase or 3.5 mM Mn^{2+} for PBGD. Nucleotides, *Tth* DNA polymerase with reverse transcriptase activity, SYBR Green I and reaction buffer were included in the LightCycler-RNA Master SYBR Green I kit (Roche Diagnostics). For preparation of the standard curve, total RNA from HaCaT cells known to express 24-hydroxylase RNA (Harant et al. 2000) was amplified in the same run as the samples. The RT-PCR protocol was as follows: reverse transcription at 61°C for 20 min and denaturation at 95°C for 30 s followed by 45 cycles with denaturation at 95°C for 1 s, annealing at 57°C for 24-hydroxylase or at 62°C for PBGD for 7 s and extension at 72°C for 12 s. Detection of fluorescent product was performed at the end of the extension step of each cycle. To verify specific products, melting curve analysis and gel electrophoresis were applied. The data were quantified by the Fit Points method with LightCycler Data Analysis software. The amplification efficiency and the relative expression ratio of 24-hydroxylase were calculated according to the method of Pfaffl (2001).

7. A CASE-CONTROL STUDY (IV)

7.1. Study population

The association between vitamin D status and the risk of prostate cancer was examined in a case-control study nested within a cohort of 18,966 men aged 40-55 who participated in the first health examination for the Helsinki Heart Study in 1981-1982 and had not previously had clinically detected prostate cancer. The Helsinki Heart Study is a randomized double-blind primary prevention trial on the efficacy of lowering serum lipid levels with gemfibrozil to reduce coronary heart disease risk (Frick et al. 1987). The participants were selected by screening from employees in two governmental agencies and five industrial companies. The workplaces were located in different parts in Finland.

7.2. Selection of cases and controls

For the present study the participants were followed up for prostate cancer diagnosed from 1981-1982 to the end of 1995 by record linkage to the countrywide population-based Finnish Cancer Registry. During the follow-up, 158 cases of prostate cancer were identified in the cohort. Baseline serum sample was available from 149 of them. The controls were selected by cumulative incidence sampling from those with no prostate cancer during the follow-up and alive at the time of diagnosis of the corresponding cases. They were matched (1:4) for age at entry (±2 years), time of sample retrieval (±2 months) and residence. Some of the stored frozen samples had been accidentally thawed. Of the 149 samples from prostate cancer cases, 58 were among those thawed. The samples from the controls had been stored in the same freeze room as those of the corresponding cases, and were thus subject to the same temperature changes, which eliminated the potential effect of thawing. The mean (SD) levels of 25(OH)D among cases were 39.1 (17.4) nmol/l among those thawed and 42.8 (22.4) nmol/l among those not thawed, while among controls the levels were 42.5 (17.3) nmol/l and 45.1 (18.8) nmol/l.

7.3. Measurement of serum 25-hydroxyvitamin D

Serum concentrations of 25(OH)D were analyzed by radioimmunoassay (Incstar Corporation, Stillwater, USA). The method measures both $25(OH)D_2$ and $25(OH)D_3$. The samples were analyzed blinded, without knowledge of the case-control status and

using the same lot of assay kits. Cases and their corresponding controls were analyzed in the same run. The coefficients of intra- and interassay variations for 25(OH)D assay were 8.5% and 16%, respectively. The cut-off points of the quartiles of 25(OH)D concentrations were based on the total original cohort.

7.4. Statistical methods

All analyses of the association of 25(OH)D level and risk of prostate cancer were performed using conditional logistic regression analysis on the SAS program package (SAS Institute, Gary, NC, USA); the matching status could thus be maintained. In this kind of analysis, differences between case and the control values of the covariate are considered instead of the actual concentrations. This is an advantage when strong potential confounders are matched for. The 25(OH)D levels were categorized by quartiles to explore the pattern of association with prostate cancer risk. In addition to the factors matched for, adjustment was made for the following potential confounders: smoking (non, past or current); treatment with gemfibrozil (no, yes); and a number of factors (body mass index, systolic blood pressure, and level of HDL cholesterol) related to the insulin resistance syndrome (as continuous variables).

SUMMARY OF RESULTS

1. VDR ANTIBODY R112 (I)

1.1. Characterization of the immunoreactivity of R112 in Western blot

A polyclonal antibody R112 was raised in a rabbit by immunization of a synthetic peptide corresponding to the N-terminal residues 10-24 of the human VDR. The selected peptide sequence is located in the A/B domain of the receptor and is identical in human, rat, mouse and bovine VDR. The immunoreactivity of R112 in Western blot was tested with hVDR expressed in vitro in a reticulocyte lysate system and with untreated and $1\alpha,25(OH)_2D_3$-treated MG-63 osteosarcoma cells. Antibody R112 recognized a protein of 48 kDa in all samples. The protein was up-regulated by $1\alpha,25(OH)_2D_3$ in MG-63 cells. No signal was detected in the negative control (the transcription/translation reaction in vitro performed without added DNA), and the excess of the corresponding peptide saturated the primary antibody R112 completely.

1.2. Optimization of the immunohistochemical method for paraffin-embedded tissues

Tissues known to express VDR protein were selected to test the capacity of R112 to perform immunohistochemistry on paraffin-embedded tissue samples. No VDR-positive cells were found after the conventional staining procedure without pretreatment of the paraffin sections. Several fixation fluids and unmasking methods were therefore tested in different combinations to optimize the efficiency of the staining. The optimal combination was found to be a formalin-based fixative (Baker´s fluid or 4% paraformaldehyde in PBS) and heating in citrate buffer (0.01 M, pH 6.0) for 10 min.

1.3. Characterization of the immunoreactivity of R112 in immunohistochemistry

The classical vitamin D target organs, intestine, kidney and skin, were used to verify immunohistochemical staining with R112. The distribution of VDR in these tissues was in agreement with that previously observed (Stumpf et al. 1984, Berger et al. 1988, Milde et al. 1991, Kumar et al. 1994).

2. VDR EXPRESSION IN HUMAN AND RAT PROSTATE (I)

VDR was localized in the nuclei of secretory epithelial cells and a few stromal cells of the human and rat prostate. In the rat prostate, weak immunostaining was also observed in the cytoplasm of secretory epithelial cells. The nuclear staining in the secretory epithelial cells was concentrated near the nuclear membrane and in discrete foci in the nucleoplasm. In the rat prostate, virtually all secretory epithelial cells showed nuclear staining, whereas in human samples the staining was more focal, only a few cells in one acinus being VDR-positive. In the human prostate, the number of VDR-positive cells/acinus was higher in acini with cuboidal than in those with columnar epithelium. Screening of different human prostatic tissues (n = 8) revealed marked variation in VDR expression between individual samples, ranging from strong positive staining to no immunoreactivity.

3. EXPRESSION AND REGULATION OF VDR AND AR PROTEINS IN OVARY (II)

3.1. VDR and AR in human ovarian cancer tissue

VDR and AR protein distributions were studied immunohistochemically on 14 ovarian cancer cases (aged 42-84 years) whose stage, differentiation and diagnosis were assigned following the recommendations of the World Health Organization.

VDR. Six of the 14 ovarian cancer cases studied expressed VDR, 3 showing moderate staining and 3 weak staining. Immunoreactivity was located mainly in the nuclei of cancer cells with some cytoplasmic staining. In some cases, only stromal cells adjacent to cancer cells showed immunoreactivity, while cancer cells showed none. In VDR-positive cases, tumor cells displayed a rather heterogeneous staining pattern. The mean age of patients with VDR-positive (+ and +/-) tumors was 74 years compared to 55 years in patients with undetectable VDR levels. Five out of six VDR-positive cases were in stage I or II, one was in stage IV.

AR. Nine of the 14 cases studied expressed AR, 6 showing moderate and 3 weak staining. Immunoreactivity was located mainly in the nuclei of cancer cells. Some cells showed weak immunoreactivity in the cytoplasm. The intensity of nuclear AR

staining varied markedly from sample to sample and within a given sample from cell to cell. Three cases expressed both AR and VDR. The mean age of patients with AR-positive (+ and +/-) tumors was 64 years compared to 62 years in patients with undetectable AR levels. Five out of nine AR positive cases were in stage I or II, three in stage III or IV.

3.2. VDR and AR in rat ovary

Both VDR and AR were observed in varying staining intensities in the nuclei of most cell types of the rat ovary. AR staining was evenly distributed throughout the nucleus, whereas VDR staining was concentrated in discrete foci in the nucleoplasm. The number of VDR foci varied according to cell type.

VDR. More abundant VDR protein expression was seen in the theca cells of primary and secondary follicles, theca lutein cells of the corpus luteum and granulosa cells of atretic follicles. In the surface epithelium, in the follicular cells of primordial follicles and in the granulosa cells of primary and secondary follicles and of the corpus luteum, VDR protein expression was moderate. Weak VDR immunoreactivity was seen in the ovarian stroma and endothelial cells of blood vessels. Epithelial cells of the oviduct showed strong VDR staining, whereas stromal and muscle cells showed moderate staining.

AR. Most intense AR staining was seen in the granulosa cells of primary and secondary follicles. Moderate AR immunoreactivity was detected in the granulosa cells of atretic follicles, in the follicular cells of primordial follicles, in the theca cells of primary and secondary follicles and of the corpus luteum, in the surface epithelium and ovarian stroma. Weakly AR-positive cells were observed in the granulosa lutein cells of the corpus luteum and in the endothelial cells of blood vessels. Weak AR staining was shown in muscle and stromal cells of the oviduct, whereas no staining was seen in epithelial cells.

3.3. Regulation of VDR and AR protein expression in OVCAR-3 cells

Homologous and heterologous regulation of VDR and AR protein levels by $1\alpha,25(OH)_2D_3$ and/or DHT were studied using cell fractionation and Western blot analysis.

VDR. A 48 kDa VDR protein was detected only in the nuclear fraction of OVCAR-3 cells. A dose-dependent increase in VDR protein was seen after treatment with 10 and 100 nM 1α,25$(OH)_2D_3$ (4.7-fold and 7-fold, respectively), suggesting homologous up-regulation. DHT also induced VDR protein in a dose-dependent manner, but the effect was weaker than that of 1α,25$(OH)_2D_3$ (heterologous regulation). Combination of 10 nM 1α,25$(OH)_2D_3$ and 10 nM DHT slightly enhanced the effect of 10 nM 1α,25$(OH)_2D_3$ alone. However, treatment with 10 nM 1α,25$(OH)_2D_3$ and 100 nM DHT reduced VDR expression induced by either hormone alone. Combination of 100 nM 1α,25$(OH)_2D_3$ and 10 nM DHT slightly lowered the effect of 100 nM 1α,25$(OH)_2D_3$ alone.

AR. A 110 kDa AR protein was detected in both the nuclear and cytoplasmic fractions of OVCAR-3 cells. A dose-dependent increase in AR expression was seen after DHT treatment in both fractions, which suggests homologous up-regulation of AR in ovarian cells. Nuclear AR protein was also induced by 100 nM 1α,25$(OH)_2D_3$ (heterologous regulation). Combination of 100 nM DHT and 100 nM 1α,25$(OH)_2D_3$ increased AR protein above the level induced by 100 nM DHT alone.

4. REGULATION OF OVCAR-3 CELL GROWTH BY DHT AND VITAMIN D COMPOUNDS (II, III)

The growth-regulatory effects of dihydrotestosterone and various vitamin D compounds were studied on a human ovarian adenocarcinoma cell line, OVCAR-3, using cell growth assays. It should be noted that increase/reduction in relative cell numbers (%) are presented in study II, whereas growth inhibition/stimulation (%) are shown in study III based on differently presented values on the growth curve.

4.1. Growth stimulation by DHT

The growth-stimulatory effect of DHT on OVCAR-3 cells was seen with both concentrations (10 and 100 nM) after 5 days of exposure, being more evident after 7 days. At the end of the study (day 9), 10 nM DHT increased relative cell numbers 41%, and 100 nM DHT 48%, above that of controls. DHT shortened the cellular doubling time from 83 h (control cells) to about 70 h at both 10 and 100 nM concentrations.

4.2. Combinatory effects of 1α,25(OH)$_2$D$_3$ and DHT

Combination of 1α,25(OH)$_2$D$_3$ with DHT showed that 1α,25(OH)$_2$D$_3$ clearly reduces the growth-stimulatory effect of DHT on OVCAR-3 cells. The effect was observed 3 or 7 days after treatment with 100 or 10 nM 1α,25(OH)$_2$D$_3$, respectively, combined with both DHT concentrations. At the end of the study, 10 nM 1α,25(OH)$_2$D$_3$ combined with 10 or 100 nM DHT reduced the effect of DHT alone by 12% or 22%, respectively. At the same time point, 100 nM 1α,25(OH)$_2$D$_3$ combined with 10 or 100 nM DHT inhibited the growth-stimulatory effect of DHT by 56 or 59%, respectively. 1α,25(OH)$_2$D$_3$ inhibited the effect of DHT on the cellular doubling time.

4.3. Growth modulation by 1α,25(OH)$_2$D$_3$ and vitamin D analog EB 1089

A dose-dependent growth modulation of OVCAR-3 cells was seen with different concentrations of 1α,25(OH)$_2$D$_3$. Growth of OVCAR-3 cells was inhibited with 100 nM 1α,25(OH)$_2$D$_3$. The effect was already to be observed 5 days after 1α,25(OH)$_2$D$_3$ exposure. After 9 days 100 nM 1α,25(OH)$_2$D$_3$ reduced relative cell numbers to 27% of those in control cells (II) and at the end of the study (day 11) the cell number was 26% of that in control (III). A weak growth-promoting effect was seen with 10 nM 1α,25(OH)$_2$D$_3$ on days 5 (III) and 7 (II) but at the end of the study (day 11) 10 nM 1α,25(OH)$_2$D$_3$ inhibited growth by 8% when compared to control (III). With a 1 nM concentration, 1α,25(OH)$_2$D$_3$ had no effect on cell growth and the physiological concentration of 1α,25(OH)$_2$D$_3$ (0.1 nM) stimulated the growth by 23% after 11 days of treatment (III).

Studies on vitamin D analog EB 1089 showed it to be more potent than the parent hormone, 1α,25(OH)$_2$D$_3$, in inhibiting the growth of OVCAR-3 cells (III). Similar growth inhibition was seen with both 1 nM and 100 nM concentrations, which equaled the growth inhibition induced by 100 nM 1α,25(OH)$_2$D$_3$. However, the lowest EB 1089 concentration tested (0.01 nM) stimulated growth by 22% by the end of the study (day 11).

4.4. Growth stimulation by 25(OH)D$_3$

In the range of 10 nM to 500 nM, 25(OH)D$_3$ stimulated the growth of OVCAR-3 cells. With lower concentrations (10 to 200 nM) growth stimulation was between 32%

and 41%, whereas the highest concentration used (500 nM) stimulated growth by 11% when compared to control.

5. EXPRESSION AND REGULATION OF 1α-HYDROXYLASE AND 24-HYDROXYLASE mRNA IN OVCAR-3 CELLS (III)

The response of cells to vitamin D is dependent in part on metabolism. To test whether enzymes 1α-hydroxylase and 24-hydroxylase might be involved in the metabolism of vitamin D compounds in OVCAR-3 cells, the expression and regulation of these enzymes by vitamin D compounds were studied at mRNA level.

5.1. 1α-hydroxylase

The expression of the 25(OH)D_3-activating enzyme 1α-hydroxylase in OVCAR-3 cells was studied using RT-PCR. An RT-PCR product of 303 bp was detected in both untreated and vitamin D-treated OVCAR-3 cells, suggesting that mRNA for 1α-hydroxylase was expressed in these cells. The expression level was not altered by the vitamin D compounds (25(OH)D_3, 1α,25$(OH)_2D_3$ and EB 1089) and the concentration (100 nM) tested after 4 h.

5.2. 24-hydroxylase

The expression of 24-hydroxylase enzyme, which inactivates 1α,25$(OH)_2D_3$, was studied in OVCAR-3 cells using quantitative RT-PCR. An RT-PCR product of 212 bp was detected in both untreated and vitamin D-treated cells, suggesting that 24-hydroxylase mRNA was expressed in these cells. Expression of 24-hydroxylase mRNA was induced in these cells 650-fold by 1α,25$(OH)_2D_3$ and 600-fold by EB 1089 after 6 h treatment and after 24 h induction was 1100-fold and 1000-fold, respectively, compared to control. After 6 h treatment with 25(OH)D_3 the 24-hydroxylase mRNA expression was slightly up-regulated, but declined to basal level after 24 h treatment.

6. ASSOCIATION BETWEEN SERUM 25(OH)D LEVEL AND RISK OF PROSTATE CANCER (IV)

Prostate cancer risk, analyzed by quartiles of the 25(OH)D levels, was inversely related to serum 25(OH)D. The risk was, however, on the increase only among values lower than the median of 40 nmol/l. Adjustment for factors possibly affecting the prostate cancer risk did not essentially change the pattern of risk. The difference in the mean levels of serum 25(OH)D between cases and controls was restricted to subjects aged <52 years at entry. The mean level of serum 25(OH)D was 6.2 nmol/l lower among cases younger at entry (<52 years) compared to their controls (37.1 vs. 43.3 nmol/l), whereas among those older than 51 at entry there was no difference (44.9 vs. 44.6 nmol/l). Among the younger subjects (<52 years) the odds ratio (OR) for prostate cancer associated with low 25(OH)D level (≤ 40 nmol/l) was 3.07 (p = 0.001) compared to those with high levels of 25(OH)D (> 40 nmol/l), when no covariates were adjusted for, and 3.5 (p = 0.0006) when adjusting for factors related to insulin resistance syndrome. The corresponding values among those older than 51 at entry were both 1.2.

The lowest mean serum level of 25(OH)D (32.7 nmol/l, OR 6.3) was seen in those younger at entry and with a non-localized cancer. However, since there was a high proportion of cases with unknown stage, no firm conclusions can be drawn.

The mean age at diagnosis of the patients with 25(OH)D levels above the median was 1.8 years higher than that of patients with 25(OH)D below the median (63.1 vs. 61.3 years, p = 0.02). More than 75% of cases were diagnosed during the last 6 follow-up years, and therefore there is no great variation in the follow-up time between entry to study and diagnosis of cancer.

DISCUSSION

1. VDR PROTEIN IS EXPRESSED IN VARYING ABUNDANCE IN HUMAN PROSTATE TISSUES

Immunohistochemical analysis of the rat and human prostate revealed that VDR was located in the nuclei of secretory epithelial and a few stromal cells. The finding is in agreement with that in a previous report demonstrating VDR by ligand-binding and Northern blot analysis mainly in the epithelial but also in the stromal compartment of the human prostate (Peehl et al. 1994). In the secretory epithelial cells of the rat prostate, strong VDR staining was shown here in the nucleus and weak in the cytoplasm, whereas a previous report has indicated strong cytoplasmic staining in these cells (Johnson et al. 1996). This discrepancy might be due to different specifities of the polyclonal VDR antibodies used. In the human prostate, VDR was located in the nuclear compartment here as well as in a recent study (Krill et al. 2001).

In addition to possible species-differences in VDR distribution, the lower frequency of VDR-positive epithelial cells in the human as against the rat prostate detected in the present study may be related to the advanced age of the human sample donors (mean 72 years). Previously, advancing age has been shown to result in a reduction in intestinal and bone VDR in the rat and in some, although not all, human studies (Horst et al. 1990, Ebeling et al. 1992, Kinyamu et al. 1997, Martinez et al. 2001). The marked variations in VDR protein abundance between prostatic samples from men aged 69-75 years observed in this study might be due to homologous and heterologous regulation of VDR by differing endocrine status (Krishnan and Feldman 1997). Recently, an immunohistochemical study on variations in VDR expression in males of different ages has shown that prostatic VDR expression may be lower in young (10-19 years) as well as in aged (60-70 years) males and higher in the fifth decade (Krill et al. 2001). Krill's group also reported that VDR protein might be more abundant in the peripheral zone, the region where the majority of prostate cancers occur, compared to the central zone.

The finding of VDR within epithelial and a few stromal cells of the human prostate suggests that $1\alpha,25(OH)_2D_3$ may play a direct role in prostate biology. Previous

experimental data showing that vitamin D may possess anti-cancer activity in prostate cells (Zhao and Feldman 2001) and the location of VDR mainly in the cells from which the majority of prostate cancers arise suggest that 1α,25$(OH)_2D_3$ might be a protective factor in the pathogenesis of prostate cancer. Study of possible changes in VDR protein expression during carcinogenesis might provide information on the role of VDR in prostate cancer.

2. VDR AND AR PROTEINS ARE EXPRESSED IN HUMAN OVARIAN CANCER TISSUE; THE EXPRESSION IS REGULATED BY 1α,25$(OH)_2D_3$ AND DHT IN OVCAR-3 CELLS

Immunohistochemical analysis of the rat ovary here showed that VDR and AR proteins are widely expressed in this tissue. VDR protein was most abundant in the theca cells; in the granulosa cells and in the surface epithelium VDR density was moderate and in the ovarian stroma low. AR staining was most intense in granulosa cells, whereas in theca cells, in the ovarian surface epithelium and stroma staining was moderate. In human ovarian cancer, the expression of VDR and AR proteins was less abundant than in rat ovaries, with 43% (6/14) of cancer tissues expressing VDR and 64% (9/14) expressing AR protein. In OVCAR-3 ovarian cancer cells, VDR protein was up-regulated by 1α,25$(OH)_2D_3$ and DHT, as was AR by both of these ligands.

The distribution of VDR and AR proteins in the rat ovary observed in our study was in general agreement with previous immunohistochemical findings, although relative staining intensities have been somewhat different (Takeda et al. 1990, Tetsuka et al. 1995, Johnson et al. 1996). In contrast to previous immunohistochemical studies, we showed AR also in thecal cells and in the surface epithelium of the ovaries (Takeda et al. 1990, Tetsuka et al. 1995). The most intense AR staining was seen in granulosa cells here as in a previous study (Tetsuka et al. 1995). The widespread expression of VDR and AR in rat ovarian cells observed in our study and reported elsewhere suggests important roles for vitamin D and androgen in ovarian function. Studies on mice and rats have suggested that vitamin D may be important for female fertility, especially for normal folliculogenesis (Halloran and DeLuca 1980, Kwiecinksi et al. 1989, Li et al. 1997, Yoshizawa et al. 1997, Kinuta et al. 2000, Johnson and DeLuca 2001, Panda et al. 2001). The effects of vitamin D might be mediated partially via

maintenance of calcium homeostasis (Kinuta et al. 2000, Johnson and DeLuca 2001). A study on polycystic ovarian syndrome patients has suggested that vitamin D and calcium might be necessary for normal menstrual cycles in humans (Thys-Jacobs et al. 1999). Direct regulation of vitamin D responsive genes involved in female fertility awaits further investigation. Androgens have been shown to modulate oocyte maturation in humans and frogs (Franke and Berendonk 1997, Nelson et al. 1999, Lutz et al. 2001). In frogs, androgens may signal through AR to promote oocyte maturation (Lutz et al. 2001).

Previously, VDR expression has been shown in four ovarian cancer tissues out of nine in studies using ligand binding assay (Frampton et al. 1982, Saunders et al. 1992); the figure is quite similar to our result (six out of 14) based on immunohistochemistry. A recent immunohistochemical study has shown that the nuclei of epithelial cells in ovarian tumors might be strongly VDR-positive; however, the number of cases studied was not indicated (Krill et al. 2001). In our study, loss of VDR may not be related to the advanced age of the ovarian cancer patients, as patients with undetectable VDR levels in their tumors were younger than those with VDR-positive tumors (mean age 55 vs. 74 years). It is more likely that VDR protein expression is associated with the lower stage of the tumor, as five out of six VDR-positive tumors here were in stage I or II. Although the number of ovarian cancer cases studied was rather small and the result possibly due to selection of the material, it is conceivable that loss of VDR protein may contribute to ovarian tumor progression. In our study, AR protein was detected in nine ovarian cancer cases out of 14 studied, which is in agreement with previous results suggesting that the majority of ovarian cancer cases express AR protein (Kuhnel et al. 1987, Slotman et al. 1989, Ilekis et al. 1997, Cardillo et al. 1998). The mean age of the patients whose tumors showed or did not show AR immunoreactivity was quite similar, but AR was more frequently expressed in stage I/II compared to stage III/IV tumors. Similarly to loss of VDR, loss of AR might contribute to tumor progression. Altered expression of nuclear receptor genes has previously been observed in tumor development and progression, but the underlying mechanisms are poorly known. One suggested mechanism is aberrant cytosine methylation of the promoter regions, which may silence the nuclear receptor genes (Berger and Daxenbichler 2002). VDR protein expression has been reported to be considerably lower in chemically induced murine colon cancer compared to normal

mucosa, this being associated with a high frequency of VDR gene methylation in cancer tissue (Smirnoff et al. 1999). Methylation of AR gene promoter has been shown in AR-negative metastatic prostate cancer cells and biallelic methylation in 80% of AR-negative uterine endometrial cancers (Jarrard et al. 1998, Kinoshita et al. 2000, Sasaki et al. 2000). In females, one allele of the X-chromosomal AR gene is always methylated.

Ligand-dependent homologous regulation of steroid receptor levels is one way to modulate steroid hormone-mediated responses. Homologous up-regulation of VDR protein by $1\alpha,25(OH)_2D_3$ has been reported in many models (Krishnan and Feldman 1997), which is in line with our result on OVCAR-3 cells. The relatively low abundance of VDR in ovarian cancer tissues may be up-regulated by $1\alpha,25(OH)_2D_3$ similarly to OVCAR-3 cells, and in this way cancer tissue might respond to $1\alpha,25(OH)_2D_3$ treatment. A previous study, together with the present result, suggests that androgens may up-regulate AR protein in the human ovaries (Chadha et al. 1994). However, the effect of androgen on AR mRNA may be up-regulation, as reported on primate granulosa cells (Weil et al. 1998) or down-regulation as observed in rat granulosa cells (Tetsuka and Hillier 1996). Steroid receptor abundance is also regulated by factors other than their own ligands, for example other steroid hormones (heterologous regulation). Our finding that androgen (DHT) may up-regulate VDR protein is in agreement with that in a previous study on T 47D breast cancer cells (Escaleira et al. 1993). The up-regulation of AR protein by $1\alpha,25(OH)_2D_3$ observed here has been previously reported in LNCaP prostate cancer cells (Hsieh et al. 1996, Zhao et al. 1997, Zhao et al. 1999). The stimulatory effect of $1\alpha,25(OH)_2D_3$ on AR gene expression might be indirect, as a protein synthesis inhibitor has been shown to abolish the induction of AR mRNA by $1\alpha,25(OH)_2D_3$ (Zhao et al. 1999); however, future studies must clarify the underlying mecahnisms. The regulation of VDR by DHT and AR by $1\alpha,25(OH)_2D_3$ suggest possible cross-talk between $1\alpha,25(OH)_2D_3$ and androgens and their cognate receptors.

3. THE GROWTH OF OVCAR-3 CELLS IS MODULATED BY DHT AND VITAMIN D COMPOUNDS 1α,25(OH)$_2$D$_3$, EB 1089 AND 25(OH)D$_3$

3.1. The growth of OVCAR-3 cells is stimulated by DHT

In the present study, DHT at 10 and 100 nM concentrations stimulated the growth of OVCAR-3 cells. Previous studies of human ovarian cancer cell lines using androgens have shown either no effect on cell growth rates or growth inhibition with high concentrations (1-100 μM) (Thompson and Adelson 1993, Karlan et al. 1995). Anti-androgen treatment has been shown to inhibit ovarian cancer cell proliferation in culture, which implies a role for androgen in ovary cell growth stimulation (Slotman and Rao 1989). In one recent study, growth stimulation of ovarian cancer cells was observed with androgen treatment, one cell line being however nonresponsive to androgen stimulation (Syed et al. 2001). The divergent results may be explained by the different androgen concentrations used and cell line-specific factors, such as different patterns of AR protein. Together, the results suggest that androgens may regulate the growth of ovarian cells. The growth-promoting effect of DHT would be in keeping with a suggested role of androgen as a contributing factor in ovarian carcinogenesis (Risch 1998, Holschneider and Berek 2000).

3.2. 1α,25(OH)$_2$D$_3$ can antagonize the growth-promoting effects of DHT

The ability observed in this study of 1α,25(OH)$_2$D$_3$ to efficiently antagonize the growth-promoting effects of DHT in OVAR-3 cells is in agreement with a previous study on prostate cancer cell line LNCaP (Esquenet et al. 1996). In primary cultures of human prostate epithelial cells the growth inhibitory action of 1α,25(OH)$_2$D$_3$ has been seen to be maintained in the presence of DHT (Krill et al. 1999). Growth inhibition of LNCaP cells by 1α,25(OH)$_2$D$_3$ may be mediated by an androgen-dependent mechanism and is reported to be preceded by the induction of AR gene expression (Zhao et al. 1997, Zhao et al. 1999) as was also observed in the present study. Further studies on different prostate cancer cell lines have shown that antiandrogen may or may not block the antiproliferative effect of 1α,25(OH)$_2$D$_3$, indicating androgen-dependent and androgen-independent actions of 1α,25(OH)$_2$D$_3$ in different prostate cancer cell lines (Zhao et al. 2000). However, the combined growth effects of 1α,25(OH)$_2$D$_3$ and androgen are poorly understood and have not been

previously studied in ovarian cells. Further studies are needed to elucidate the mechanisms underlying the effects.

3.3. 1α,25(OH)$_2$D$_3$ and its analog EB 1089 can inhibit the growth of OVCAR-3 cells

The growth inhibition of OVCAR-3 cells by 100 nM 1α,25(OH)$_2$D$_3$ observed in this study is in agreement with previous results on OVCAR-3 cells (Saunders et al. 1992, Saunders et al. 1993) and many other cancer cell lines (Christakos et al. 1996, Gross et al. 1997), and supports the hypothesis that vitamin D might be a factor protecting against ovarian cancer (Lefkowitz and Garland 1994, Grant 2002). Vitamin D analogue EB 1089 was a potent inhibitor of OVCAR-3 cell growth already at a 1 nM concentration in this study. Studies on other cancer cell types have suggested that EB 1089 may be 50-200 times more potent than 1α,25(OH)$_2$D$_3$ in the regulation of cell growth (Hansen et al. 2000), this being again in accord with our result showing similar growth inhibition with 1 nM EB 1089 and 100 nM 1α,25(OH)$_2$D$_3$ in OVCAR-3 cells. Mitogenic effects of 1α,25(OH)$_2$D$_3$ have been reported with low 1α,25(OH)$_2$D$_3$ concentrations in prostate (Gross et al. 1997) and other cancer cell lines (Munker et al. 1986, Love-Schimenti et al. 1996) as well as in the ovarian cancer cell line in the present study. A very low concentration of EB 1089 (0.01 nM) also stimulated growth slightly in this study. In addition to 1α,25(OH)$_2$D$_3$, the growth response to other steroids such as androgen, estrogen, progesterone and glucocorticoid in some cell culture models is reported to be biphasic: growth stimulation with low concentrations and inhibition with higher concentrations (Chalbos et al. 1982, Hiraoka et al. 1987, Langeler et al. 1993, Landsman et al. 2001, Syed et al. 2001). However, the local metabolism possibly modulating intracellular hormone concentrations is poorly understood.

The relatively high doses of 1α,25(OH)$_2$D$_3$ needed to obtain an inhibitory growth response may be due to the 1α,25(OH)$_2$D$_3$-inactivating enzyme 24-hydroxylase, which was highly inducible by 1α,25(OH)$_2$D$_3$ and EB 1089 (1100-fold and 1000-fold, respectively) in this study on OVCAR-3 cells. The ability of 1α,25(OH)$_2$D$_3$ to induce 24-hydroxylase through a vitamin D receptor-dependent process is well known and is used as a marker of 1α,25(OH)$_2$D$_3$ action (Chen and DeLuca 1995, Kerry et al. 1996, Ohyama et al. 1996). Previously, induction of specific activity of 24-hydroxylase by

$1\alpha,25(OH)_2D_3$ has been shown in ovarian cancer cells (Christopherson et al. 1986). Although EB 1089 up-regulated 24-hydroxylase mRNA expression here, the metabolism of EB 1089 may not primarily involve 24-hydroxylase due to the altered side-chain of the compound, and this may explain the higher potency of EB 1089 compared to $1\alpha,25(OH)_2D_3$ (Shankar et al. 1997). Amplification of the 24-hydroxylase gene has recently been reported in breast cancer, which may provide cancer cells a means to escape vitamin D-mediated growth control (Albertson et al. 2000). The chromosomal region containing the 24-hydroxylase gene has been shown to be amplified in various ovarian cancers (Iwabuchi et al. 1995, Tanner et al. 2000), but this has not been studied in OVCAR-3 cells. The recently developed specific inhibitors of 24-hydroxylase might allow use of lower $1\alpha,25(OH)_2D_3$ concentrations to obtain growth inhibitory effects (Schuster et al. 2001a, Schuster et al. 2001b).

3.4. Prohormone $25(OH)D_3$ is growth stimulatory

The finding here that $25(OH)D_3$ stimulates the growth of OVCAR-3 cells is at odds with previous results showing inhibition of proliferation by $25(OH)D_3$ in cultured prostate cells (Barreto et al. 2000, Chen et al. 2000, Hsu et al. 2001) and in colon tissue in vivo (Holt et al. 2002). The effect of $25(OH)D_3$ may be cell type- and cell line-specific and depend e.g. on the status of the VDR and vitamin D metabolizing enzymes, 1α-hydroxylase and 24-hydroxylase, within cells. The activity of the $25(OH)D_3$-activating enzyme 1α-hydroxylase may be different in these cells. In OVCAR-3 cells, 1α-hydroxylase activity may be so low that only minimal amounts of $1\alpha,25(OH)_2D_3$ are produced and a low $1\alpha,25(OH)_2D_3$ concentration was shown to be growth-stimulatory in this study. The expression level of 1α-hydroxylase was not changed by treatment with the vitamin D compounds tested, which is in line with previous studies showing constitutive 1α-hydroxylase expression in extra-renal tissues (Cross et al. 1997, Jones et al. 1999, Schuessler et al. 2001). In the early phase of human colorectal cancer genesis, the expression of 1α-hydroxylase and VDR mRNA are up-regulated whereas in poorly differentiated late-stage carcinomas only low levels of the respective mRNAs can be detected (Cross et al. 2001). In prostate cancer cells, the activity of 1α-hydroxylase was reported 10- to 20-fold lower than in normal prostate cells, leading to reduced antiproliferative action of $25(OH)D_3$ (Hsu et al. 2001). The results suggest that in the early stages of malignancy cancer cells might be able to increase their potential for an autocrine counter-regulatory response to

neoplastic cell growth by up-regulating 1α-hydroxylase and VDR expression, whereas cancer cells in later stages might limit the growth-regulatory action of $25(OH)D_3$ by down-regulating the expression of 1α-hydroxylase.

A minor part of the mitogenic effects of $25(OH)D_3$ might be mediated through 24-hydroxylated products, $24,25(OH)_2D_3$ and $1\alpha,24,25(OH)_3D_3$, or directly by binding to VDR. The growth-modulating actions of 24-hydroxylated products of vitamin D are poorly known, but $1\alpha,24,25(OH)_3D_3$ and the further oxidized product, $1\alpha,25(OH)_2$-24-oxo-vitamin D_3, might exert growth-modulating effects (Campbell et al. 1999, Rashid et al. 2001). The relative binding affinity of $25(OH)D_3$ to VDR is about 700-fold lower than that of $1\alpha,25(OH)_2D_3$ (Collins and Norman 2001), but $25(OH)D_3$ might bind to VDR, activate it and have effects of its own. The direct role of $25(OH)D_3$ in the regulation of vitamin D target genes is poorly known, but it has been reported that $25(OH)D_3$ may up-regulate VDR expression, although to a much lesser extent than $1\alpha,25(OH)_2D_3$ (Costa et al. 1985). Regulation of VDR expression has however been studied in pig kidney cells and human skin fibroblasts which are later reported to contain enzyme 1α-hydroxylase, suggesting that the effect on VDR up-regulation may be mediated by $1\alpha,25(OH)_2D_3$ rather than $25(OH)D_3$ (Wikvall 2001).

The results here together with previous findings suggest that vitamin D may modulate the growth of ovarian cancer cells (Saunders et al. 1992, Saunders et al. 1993). Studies on cell cycle regulators and different growth factors may clarify the mechanisms of growth inhibition and stimulation of ovarian cancer cells by vitamin D compounds. The possible mitogenic effects of vitamin D should be considered when critically evaluating the possible treatment of ovarian cancer patients with vitamin D.

4. LOW SERUM 25(OH)D CONCENTRATION IS ASSOCIATED WITH INCREASED RISK OF PROSTATE CANCER

An inverse association between serum 25(OH)D concentration, the main indicator of vitamin D status (Fraser 1995), and prostate cancer risk was shown in this study. The highest prostate cancer risk (more than 3-fold) was among men younger than 52 years at entry who had low serum 25(OH)D concentrations (≤ 40 nmol/l). Among men

older at entry the risk associated with low serum 25(OH)D concentration was minimal, this possibly attributable to decreasing androgen values during the andropause (Lund et al. 1999). After 40 years of age, serum testosterone levels start to decrease and after 50 years the decrease might be significant, since androgen replacement therapy appears to be reasonable thereafter. Younger men in our study might have had an unopposed androgen effect on the prostate when vitamin D levels were low and this may have led to initiation of carcinogenesis. The ability of $1\alpha,25(OH)_2D_3$ to efficiently antagonize the growth-promoting effects of androgens has previously been reported on prostate cancer cell line LNCaP (Esquenet et al. 1996) and in our study on OVCAR-3 cells. In primary cultures of human prostate epithelial cells the growth inhibitory action of $1\alpha,25(OH)_2D_3$ was maintained in the presence of DHT (Krill et al. 1999). The growth-inhibitory effect of vitamin D may involve both androgen-dependent and independent mechanisms (Zhao et al. 2000).

The present results suggest that vitamin D can affect the invasiveness of prostate cancer. The men with low serum 25(OH)D concentration and younger than 52 years at entry had an increased risk of non-localized prostate cancer (adjusted relative risk 6.3), which is in agreement with experimental results suggesting that vitamin D may prevent invasion and metastasis of prostate cancer cells (Schwartz et al. 1997, Lokeshwar et al. 1999, Sung and Feldman 2000). However, a high proportion of prostate cancer cases in our study were of unknown state and further studies are therefore called for to confirm this finding. Shifting the cut-off point confirmed our conclusion that the excess risk of prostate cancer associated with low serum 25(OH)D concentration was almost entirely confined to those younger than 51-53 years at entry. Our material was, however, too small to be analysed in greater detail by age, and therefore larger cohorts are needed in order to clarify this aspect.

An inverse (Corder et al. 1993) or no association (Braun et al. 1995, Gann et al. 1996, Nomura et al. 1998) has previously been observed between the serum concentrations of the active form of vitamin D, $1\alpha,25(OH)_2D_3$ and prostate cancer risk. We were interested in 25(OH)D because it had been reported that prostate cells may synthesize $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$ (Schwartz et al. 1998) and because tightly regulated serum $1\alpha,25(OH)_2D_3$, which has a short half-life (Breslau and Zerwekh 1997), may not correlate with intraprostatic $1\alpha,25(OH)_2D_3$ concentrations. The reliability of a

single 25(OH)D determination can be questioned in that there is high seasonal variation in serum 25(OH)D concentrations. Both age and time of blood retrieval were used as matching variables, so that their confounding effect was excluded, but a modifying effect may remain.

Since the serum samples here were collected for the study of coronary heart disease (Frick et al. 1987), relevant confounders for prostate cancer were not measured. However, in consequence of this background the men were exceptionally young for a prostate cancer study, and we were therefore able to ascertain a high risk of prostate cancer among men younger than 52 years at entry. Results of earlier studies on serum 25(OH)D and the risk of prostate cancer have shown no correlation (Corder et al. 1993, Braun et al. 1995, Gann et al. 1996, Nomura et al. 1998). In the present series, more than half of the men had serum 25(OH)D concentrations below 50 nmol/l, which has been suggested to indicate vitamin D deficiency (Malabanan et al. 1998). In previous studies, the levels of 25(OH)D have been higher than here; only one quarter or less of the samples having had levels below 50 nmol/l (20 ng/ml). This difference in the 25(OH)D levels may partially account for the discrepancy between the previous negative results and our findings. However, the main reason for it is probably the older age and the cut-off point at older age in the earlier studies (Corder et al. 1993, Braun et al. 1995, Gann et al. 1996, Nomura et al. 1998).

Taken together, our results suggest that vitamin D may be one of the components involved in the multifactorial process of prostate carcinogenesis. The increased relative risk of prostate cancer in men with low vitamin D levels, and younger at entry, is consistent with the hypothesis that vitamin D might be involved directly in the initiation (antiandrogenic effect) and in the regulation of the growth rate (antiproliferative effect) of prostate cancer. As previous epidemiological data on the issue are inconsistent, studies on larger populations with a focus on middle-aged men might clarify the possible increased risk of prostate cancer associated with low serum vitamin D level. Studies of the joint effects of vitamin D and other possible risk factors for prostate cancer, for example dietary factors and sex hormones, might provide valuable information on the role of vitamin D in the etiology of prostate cancer.

SUMMARY AND CONCLUSIONS

1. This paper describes the production and characterization of a novel VDR antibody employed to study the cellular distribution of VDR protein in the prostate. This approach on the human prostate provides evidence that VDR may be located within epithelial and a few stromal cells of the human prostate and suggests that $1\alpha,25(OH)_2D_3$ may play a direct role in prostate biology. Moreover, the results show that there may be strong variations in VDR protein expression between prostatic samples. Previous experimental data on the anti-cancer activities of vitamin D and the location of VDR mainly in epithelial cells whence the majority of prostate cancers arise suggest that $1\alpha,25(OH)_2D_3$ might be involved in the pathogenesis of prostate cancer.

2. To gain an insight into the possible role of vitamin D and androgens in ovarian cancer, the expression and regulation of their receptors were studied in human ovarian cancer tissue and ovarian cancer cells. The expression of VDR or AR proteins in part of human ovarian cancer tissues suggests that these ovarian tumors may respond to vitamin D or androgen. VDR and AR protein expression seems to be associated with lower stage of the tumor, suggesting that loss of VDR and AR might contribute to ovarian tumor progression. The regulation of VDR and AR proteins by cognate ligands in ovarian cancer cell line OVCAR-3 sheds light on the possible mechanisms modulating the amplitude of responses to $1\alpha,25(OH)_2D_3$ and androgen in ovarian cancer cells. The revealed up-regulation of VDR by androgen and of AR by $1\alpha,25(OH)_2D_3$ in OVCAR-3 cells suggests possible cross-talk between $1\alpha,25(OH)_2D_3$ and androgens and their cognate receptors.

3. The two studies provide evidence that the growth of OVCAR-3 cells may be modulated by various vitamin D compounds ($1\alpha,25(OH)_2D_3$, EB 1089 and $25(OH)D_3$) and by androgen (DHT). The effect of DHT and $25(OH)D_3$ as well as low concentrations of $1\alpha,25(OH)_2D_3$ and EB 1089, was mitogenic in these studies. Higher concentrations of $1\alpha,25(OH)_2D_3$ and EB 1089 significantly inhibited growth. The newly found mRNA expression of both vitamin D-activating and -inactivating enzymes, 1α-hydroxylase and 24-hydroxylase, may modulate the growth response to vitamin D compounds in OVCAR-3 cells. The simultaneous expression of inducible

VDR and 24-hydroxylase and constitutive 1α-hydroxylase in OVCAR-3 cells suggests that they represent a means for in situ regulation of the response to vitamin D. The possible mitogenic effects of vitamin D compounds should be considered when critically evaluating the possibility of treating ovarian cancer patients with vitamin D.

4. The availability of stored serum samples collected from men with prostate cancer on average 11 years later and from men who remained cancer-free for the same period, provided an opportunity to evaluate the relationship between vitamin D status and risk of prostate cancer. The inverse association between serum 25(OH)D concentration and prostate cancer risk shown in this study suggests that vitamin D may be one of the elements involved in the multifactorial process of prostate carcinogenesis. The highest prostate cancer risk was among men younger than 52 years at entry who had low serum 25(OH)D concentration (≤ 40 nmol/l), whereas among older men the risk due to low serum 25(OH)D concentration was minimal. Together with previous experimental data our results suggest that one regulatory mechanism of vitamin D in prostate cancer might be inhibition of invasion and metastasis. The increased relative risk of prostate cancer in men with low vitamin D levels, and younger at entry, is consistent with the hypothesis that vitamin D might be directly involved in the initiation (antiandrogenic effect) and in the regulation of the growth rate (antiproliferative effect) of prostate cancer.

Taken together, the data obtained provide evidence that vitamin D, VDR and vitamin D-metabolizing enzymes might constitute one of the factors involved in the complicated process of prostate and ovarian carcinogenesis.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Anatomy, Medical School, University of Tampere, as part of a project in the Graduate School of Steroid Research.

I wish above all to express my gratitude to my supervisor, Professor Pentti Tuohimaa, M.D., Ph.D. for offering me the possibility to work in his group and for the idea of studying the effects of vitamin D in cancer. I am also grateful to Professor Timo Ylikomi, M.D., Ph.D. for sharing his expert knowledge of molecular biology and for his constructive criticism of the manuscript of the thesis.

I express my thanks to the official reviewers of the thesis, Professor Carsten Carlberg, Ph.D. and Docent Christel Lamberg-Allardt, Ph.D. (Agr.For.), M.Sc. for their careful review and valuable comments on this manuscript.

My sincere thanks are due to my co-authors, and to other co-workers at the Department of Anatomy, whose pleasant co-operation and encouragement made this work possible. I am deeply indebted to Heimo Syvälä, Ph.D. for his practical guidance throughout. The practical help of Merja Bläuer, Ph.D., Ya-Hua Zhuang, M.D., Ph.D. and Professor Markku Pelto-Huikko, M.D., Ph.D., with writing, computers and photography, has been of great importance. Leena Tenkanen, Ph.D. and Professor Matti Hakama, Sc.D. were of irreplaceable help with statistical analyses. I owe my special thanks to my ”room mates” Susanna Miettinen, M.Sc., Satu Passinen, M.D. and Susanna Pasanen, M.D., Ph.D., and to Marjo Aitola, M.D., for many fruitful discussions on all aspects of life.

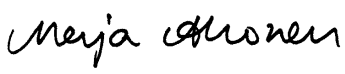
It has been a pleasure to work together with Tytti Ahola, M.D., Niina Alkio, B.M., Marjaana Haverinen, M.Sc., Pirjo Koikkalainen, M.Sc., Ilkka Laaksi, M.D., Yan-Ru Lou, M.Sc., Tommi Manninen, B.Sc., Pasi Pennanen, M.Sc., Sami Purmonen, M.Sc., Rami Puustinen, M.D., Riina Sarkanen, M.Sc., Nanna Sarvilinna, M.D., Annika Vienonen, M.Sc. and Johanna Väliaho, M.Sc.

I am grateful to Mrs. Hilkka Mäkinen, Mrs. Tarja Arvela, Ms. Taina Eskola, Mrs. Janette Hinkka, Ms. Riina Mahlanen, Ms. Carita Osmovaara, Ms. Arja Ahola, Ms.

Ulla Jukarainen, Mika Wallen, Ph.D. and Pekka Vilja, Ph.D. for their expert technical assistance. The excellent secretarial help of Ms. Marja Eloranta is appreciated.

My warmest thanks go to my family and friends for their support and encouragement during these years. Most of all, I wish to express my deepest gratitude to my dear husband, Matti, for his patience and love. My sweet child, Iida, helped me to totally forget all scientific thought and opened my eyes to the priorities of life. I am grateful to my parents, Asta and Onni Kivineva for their unstinting support during my studies and to my brother, Topi Kivineva for his knowledge in computers. I also want to thank my 89-year old grandmother, Aino Moilanen, for teaching me the way of positive thinking and my sister-in-law, Petra Ahonen, for sharing an interest in children and research.

I wish to thank Robert MacGilleon, M.A. for revising the language of this thesis. This study was financially supported by grants from the Academy of Finland, the Finnish Cancer Foundation, the Yrjö Jahnsson Foundation, the Sigrid Juselius Foundation, the Medical Research Fund of Tampere University Hospital, the University of Tampere and the Scientific Foundation of Tampere City. Permission to reproduce the original articles was kindly granted by Elsevier Science (I), John Wiley & Sons, Inc. (II) and Kluwer Academic Publishers (IV).



Tampere, August 2002

REFERENCES

Adashi EY (1994): The climacteric ovary as a functional gonadotropin-driven androgen-producing gland. Fertil Steril 62:20-27.

Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW and Pinkel D (2000): Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 25:144-146.

Alroy I, Towers TL and Freedman LP (1995): Transcriptional repression of the interleukin-2 gene by vitamin D_3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. Mol Cell Biol 15:5789-5799.

Aranda A and Pascual A (2001): Nuclear hormone receptors and gene expression. Physiol Rev 81:1269-1304.

Arbour NC, Prahl JM and DeLuca HF (1993): Stabilization of the vitamin D receptor in rat osteosarcoma cells through the action of 1,25-dihydroxyvitamin D_3. Mol Endocrinol 7:1307-1312.

Armbrecht HJ and Hodam TL (1994): Parathyroid hormone and 1,25-dihydroxyvitamin D synergistically induce the 1,25-dihydroxyvitamin D-24-hydroxylase in rat UMR-106 osteoblast-like cells. Biochem Biophys Res Commun 205:674-679.

Armbrecht HJ, Okuda K, Wongsurawat N, Nemani RK, Chen ML and Boltz MA (1992): Characterization and regulation of the vitamin D hydroxylases. J Steroid Biochem Mol Biol 43:1073-1081.

Armbrecht HJ, Wongsurawat VJ, Hodam TL and Wongsurawat N (1996): Insulin markedly potentiates the capacity of parathyroid hormone to increase expression of 25-hydroxyvitamin D_3-24-hydroxylase in rat osteoblastic cells in the presence of 1,25-dihydroxyvitamin D_3. FEBS Lett 393:77-80.

Armbrecht HJ, Hodam TL, Boltz MA, Partridge NC, Brown AJ and Kumar VB (1998): Induction of the vitamin D 24-hydroxylase (CYP24) by 1,25-dihydroxyvitamin D_3 is regulated by parathyroid hormone in UMR106 osteoblastic cells. Endocrinology 139:3375-3381.

Armstrong DT and Papkoff H (1976): Stimulation of aromatization of exogenous and endogenous androgens in ovaries of hypophysectomized rats in vivo by follicle-stimulating hormone. Endocrinology 99:1144-1151.

Aunoble B, Sanches R, Didier E and Bignon YJ (2000): Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer (review). Int J Oncol 16:567-576.

Axen E, Postlind H, Sjoberg H and Wikvall K (1994): Liver mitochondrial cytochrome P450 CYP27 and recombinant-expressed human CYP27 catalyze 1α-hydroxylation of 25-hydroxyvitamin D_3. Proc Natl Acad Sci U S A 91:10014-10018.

Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J and O'Malley BW (1988): Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci U S A 85:3294-3298.

Baran DT, Quail JM, Ray R and Honeyman T (2000a): Binding of 1α,25-dihydroxyvitamin D_3 to annexin II: effect of vitamin D metabolites and calcium. J Cell Biochem 80:259-265.

Baran DT, Quail JM, Ray R, Leszyk J and Honeyman T (2000b): Annexin II is the membrane receptor that mediates the rapid actions of 1α,25-dihydroxyvitamin D_3. J Cell Biochem 78:34-46.

Barreto AM, Schwartz GG, Woodruff R and Cramer SD (2000): 25-hydroxyvitamin D_3, the prohormone of 1,25-dihydroxyvitamin D_3, inhibits the proliferation of primary prostatic epithelial cells. Cancer Epidemiol Biomarkers Prev 9:265-270.

Barsony J, Pike JW, DeLuca HF and Marx SJ (1990): Immunocytology with microwave-fixed fibroblasts shows 1α,25-dihydroxyvitamin D_3-dependent rapid and estrogen-dependent slow reorganization of vitamin D receptors. J Cell Biol 111:2385-2395.

Barsony J, Renyi I and McKoy W (1997): Subcellular distribution of normal and mutant vitamin D receptors in living cells. Studies with a novel fluorescent ligand. J Biol Chem 272:5774-5782.

Beer TM, Hough KM, Garzotto M, Lowe BA and Henner WD (2001): Weekly high-dose calcitriol and docetaxel in advanced prostate cancer. Semin Oncol 28:49-55.

Berger J and Daxenbichler G (2002): DNA methylation of nuclear receptor genes - possible role in malignancy. J Steroid Biochem Mol Biol 80:1-11.

Berger U, Wilson P, McClelland RA, Colston K, Haussler MR, Pike JW and Coombes RC (1988): Immunocytochemical detection of 1,25-dihydroxyvitamin D receptors in normal human tissues. J Clin Endocrinol Metab 67:607-613.

Bikle DD and Gee E (1989): Free, and not total, 1,25-dihydroxyvitamin D regulates 25-hydroxyvitamin D metabolism by keratinocytes. Endocrinology 124:649-654.

Blazer DG, 3rd, Umbach DM, Bostick RM and Taylor JA (2000): Vitamin D receptor polymorphisms and prostate cancer. Mol Carcinog 27:18-23.

Blutt SE, Allegretto EA, Pike JW and Weigel NL (1997): 1,25-dihydroxyvitamin D_3 and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1. Endocrinology 138:1491-1497.

Blutt SE, McDonnell TJ, Polek TC and Weigel NL (2000): Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. Endocrinology 141:10-17.

Bosland MC (2000): The role of steroid hormones in prostate carcinogenesis. J Natl Cancer Inst Monogr 27:39-66.

Bouillon R, Okamura WH and Norman AW (1995): Structure-function relationships in the vitamin D endocrine system. Endocr Rev 16:200-257.

Braun MM, Helzlsouer KJ, Hollis BW and Comstock GW (1995): Prostate cancer and prediagnostic levels of serum vitamin D metabolites (Maryland, United States). Cancer Causes Control 6:235-239.

Breslau NA (1988): Normal and abnormal regulation of 1,25-$(OH)_2D$ synthesis. Am J Med Sci 296:417-425.

Breslau NA and Zerwekh JE (1997): Pharmacology of vitamin D preparations. In: Vitamin D, pp. 607-618. Eds. D Feldman, FH Glorieux and JW Pike, Academic Press, New York.

Brown AJ, Dusso A and Slatopolsky E (1999): Vitamin D. Am J Physiol 277:F157-175.

Brown TA and DeLuca HF (1990): Phosphorylation of the 1,25-dihydroxyvitamin D_3 receptor. A primary event in 1,25-dihydroxyvitamin D_3 action. J Biol Chem 265:10025-10029.

Campbell MJ, Elstner E, Holden S, Uskokovic M and Koeffler HP (1997): Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D_3 analogue involves the induction of $p21^{(waf1)}$, $p27^{(kip1)}$ and E-cadherin. J Mol Endocrinol 19:15-27.

Campbell MJ, Gombart AF, Kwok SH, Park S and Koeffler HP (2000): The anti-proliferative effects of $1\alpha,25(OH)_2D_3$ on breast and prostate cancer cells are associated with induction of BRCA1 gene expression. Oncogene 19:5091-5097.

Cardillo MR, Petrangeli E, Aliotta N, Salvatori L, Ravenna L, Chang C and Castagna G (1998): Androgen receptors in ovarian tumors: correlation with oestrogen and progesterone receptors in an immunohistochemical and semiquantitative image analysis study. J Exp Clin Cancer Res 17:231-237.

Carlberg C and Polly P (1998): Gene regulation by vitamin D_3. Crit Rev Eukaryot Gene Expr 8:19-42.

Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF and Hunziker W (1993): Two nuclear signalling pathways for vitamin D. Nature 361:657-660.

Chadha S, Pache TD, Huikeshoven JM, Brinkmann AO and van der Kwast TH (1994): Androgen receptor expression in human ovarian and uterine tissue of long-term androgen-treated transsexual women. Hum Pathol 25:1198-1204.

Chalbos D, Vignon F, Keydar I and Rochefort H (1982): Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J Clin Endocrinol Metab 55:276-283.

Chan JM and Giovannucci EL (2001): Dairy products, calcium, and vitamin D and risk of prostate cancer. Epidemiol Rev 23:87-92.

Chawla A, Repa JJ, Evans RM and Mangelsdorf DJ (2001): Nuclear receptors and lipid physiology: opening the X-files. Science 294:1866-1870.

Chen JD and Evans RM (1995): A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454-457.

Chen JD and Li H (1998): Coactivation and corepression in transcriptional regulation by steroid/nuclear hormone receptors. Crit Rev Eukaryot Gene Expr 8:169-190.

Chen JD, Umesono K and Evans RM (1996): SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. Proc Natl Acad Sci U S A 93:7567-7571.

Chen KS and DeLuca HF (1995): Cloning of the human 1α,25-dihydroxyvitamin D_3 24-hydroxylase gene promoter and identification of two vitamin D-responsive elements. Biochim Biophys Acta 1263:1-9.

Chen TC, Schwartz GG, Burnstein KL, Lokeshwar BL and Holick MF (2000): The in vitro evaluation of 25-hydroxyvitamin D_3 and 19-nor-1α,25-dihydroxyvitamin D_2 as therapeutic agents for prostate cancer. Clin Cancer Res 6:901-908.

Chokkalingam AP, McGlynn KA, Gao YT, Pollak M, Deng J, Sesterhenn IA, Mostofi FK, Fraumeni JF, Jr. and Hsing AW (2001): Vitamin D receptor gene polymorphisms, insulin-like growth factors, and prostate cancer risk: a population-based case-control study in China. Cancer Res 61:4333-4336.

Christakos S, Raval-Pandya M, Wernyj RP and Yang W (1996): Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D_3. Biochem J 316:361-371.

Christopherson WA, Porter JC, MacDonald PC and Casey ML (1986): Responsiveness of human carcinoma cells of gynecologic origin to 1,25-dihydroxycholecalciferol. Am J Obstet Gynecol 155:1293-1296.

Chryssikopoulos A (2000): The potential role of intraovarian factors on ovarian androgen production. Ann N Y Acad Sci 900:184-192.

Clemens TL, Garrett KP, Zhou XY, Pike JW, Haussler MR and Dempster DW (1988): Immunocytochemical localization of the 1,25-dihydroxyvitamin D_3 receptor in target cells. Endocrinology 122:1224-1230.

Collins ED and Norman AW (2001): Vitamin D. In: Handbook of vitamins, pp. 51-113. Eds. RB Rucker, JW Suttie, DB McCormick and LJ Machlin, Marcel Dekker, Inc., New York.

Colston KW, Mackay AG, James SY, Binderup L, Chander S, Coombes RC (1992): EB 1089: a new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. Biochem Pharmacol 44:2273-2280.

Corder EH, Guess HA, Hulka BS, Friedman GD, Sadler M, Vollmer RT, Lobaugh B, Drezner MK, Vogelman JH and Orentreich N (1993): Vitamin D and prostate cancer: a prediagnostic study with stored sera. Cancer Epidemiol Biomarkers Prev 2:467-472.

Correa-Cerro L, Berthon P, Haussler J, Bochum S, Drelon E, Mangin P, Fournier G, Paiss T, Cussenot O and Vogel W (1999): Vitamin D receptor polymorphisms as markers in prostate cancer. Hum Genet 105:281-287.

Costa EM and Feldman D (1986): Homologous up-regulation of the $1,25(OH)_2$ vitamin D_3 receptor in rats. Biochem Biophys Res Commun 137:742-747.

Costa EM, Hirst MA and Feldman D (1985): Regulation of 1,25-dihydroxyvitamin D_3 receptors by vitamin D analogs in cultured mammalian cells. Endocrinology 117:2203-2210.

Cramer SD, Peehl DM, Edgar MG, Wong ST, Deftos LJ and Feldman D (1996): Parathyroid hormone-related protein (PTHrP) is an epidermal growth factor-regulated secretory product of human prostatic epithelial cells. Prostate 29:20-29.

Crescioli C, Maggie M, Vannelli GB, Luconi M, Salerno R, Barni T, Gulisano M, Forti G and Serio M (2000): Effect of a vitamin D_3 analogue on keratinocyte growth factor-induced cell proliferation in benign prostate hyperplasia. J Clin Endocrinol Metab 85:2576-2583.

Crescioli C, Maggi M, Luconi M, Vannelli GB, Salerno R, Sinisi AA, Bonaccorsi L, Ferruzzi P, Barni T, Forti G and Serio M (2002): Vitamin D_3 analogue inhibits keratinocyte growth factor signaling and induces apoptosis in human prostate cancer cells. Prostate 50:15-26.

Cross HS, Peterlik M, Reddy GS and Schuster I (1997): Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: expression of 25-hydroxyvitamin D_3-1α-hydroxylase activity and regulation of side-chain metabolism. J Steroid Biochem Mol Biol 62:21-28.

Cross HS, Bareis P, Hofer H, Bischof MG, Bajna E, Kriwanek S, Bonner E and Peterlik M (2001): 25-Hydroxyvitamin D_3-1α-hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis. Steroids 66:287-292.

Daly M and Obrams GI (1998): Epidemiology and risk assessment for ovarian cancer. Semin Oncol 25:255-264.

Danielpour D (1996): Induction of transforming growth factor-beta autocrine activity by all-trans-retinoic acid and 1α,25-dihydroxyvitamin D_3 in NRP-152 rat prostatic epithelial cells. J Cell Physiol 166:231-239.

Demay MB, Kiernan MS, DeLuca HF and Kronenberg HM (1992): Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D_3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci U S A 89:8097-8101.

Dokoh S, Donaldson CA, Marion SL, Pike JW and Haussler MR (1983): The ovary: a target organ for 1,25-dihydroxyvitamin D_3. Endocrinology 112:200-206.

Dong D and Noy N (1998): Heterodimer formation by retinoid X receptor: regulation by ligands and by the receptor's self-association properties. Biochemistry 37:10691-10700.

Dressel U, Thormeyer D, Altincicek B, Paululat A, Eggert M, Schneider S, Tenbaum SP, Renkawitz R and Baniahmad A (1999): Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. Mol Cell Biol 19:3383-3394.

Dusso A, Brown A and Slatopolsky E (1994): Extrarenal production of calcitriol. Semin Nephrol 14:144-155.

Dwivedi PP, Muscat GE, Bailey PJ, Omdahl JL and May BK (1998): Repression of basal transcription by vitamin D receptor: evidence for interaction of unliganded vitamin D receptor with two receptor interaction domains in RIP13Δ1. J Mol Endocrinol 20:327-335.

Ebeling PR, Sandgren ME, DiMagno EP, Lane AW, DeLuca HF and Riggs BL (1992): Evidence of an age-related decrease in intestinal responsiveness to vitamin D: relationship between serum 1,25-dihydroxyvitamin D_3 and intestinal vitamin D receptor concentrations in normal women. J Clin Endocrinol Metab 75:176-182.

Elaroussi MA, Prahl JM and DeLuca HF (1994): The avian vitamin D receptors: primary structures and their origins. Proc Natl Acad Sci U S A 91:11596-11600.

Escaleira MT, Sonohara S and Brentani MM (1993): Sex steroids induced up-regulation of 1,25-$(OH)_2$ vitamin D_3 receptors in T 47D breast cancer cells. J Steroid Biochem Mol Biol 45:257-263.

Esquenet M, Swinnen JV, Heyns W and Verhoeven G (1996): Control of LNCaP proliferation and differentiation: actions and interactions of androgens, 1α,25-dihydroxycholecalciferol, all-trans retinoic acid, 9-cis retinoic acid, and phenylacetate. Prostate 28:182-194.

Frampton RJ, Suva LJ, Eisman JA, Findlay DM, Moore GE, Moseley JM and Martin TJ (1982): Presence of 1,25-dihydroxyvitamin D_3 receptors in established human cancer cell lines in culture. Cancer Res 42:1116-1119.

Franceschi RT, James WM and Zerlauth G (1985): 1α,25-dihydroxyvitamin D_3 specific regulation of growth, morphology, and fibronectin in a human osteosarcoma cell line. J Cell Physiol 123:401-409.

Franke WW and Berendonk B (1997): Hormonal doping and androgenization of athletes: a secret program of the German Democratic Republic government. Clin Chem 43:1262-1279.

Franks S (1995): Polycystic ovary syndrome. N Engl J Med 333:853-861.

Fraser DR (1995): Vitamin D. Lancet 345:104-107.

Freedman LP (1992): Anatomy of the steroid receptor zinc finger region. Endocr Rev 13:129-145.

Frick J and Aulitzky W (1991): Physiology of the prostate. Infection 19:S115-118.

Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, Huttunen JK, Kaitaniemi P, Koskinen P, Manninen V, Mäenpää H, Mälkönen M, Mänttäri M, Norola S, Pasternack A, Pikkarainen J, Romo M, Sjöblom T and Nikkilä EA (1987): Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 317:1237-1245.

Furuya Y, Akakura K, Masai M and Ito H (1999): Vitamin D receptor gene polymorphism in Japanese patients with prostate cancer. Endocr J 46:467-470.

Gampe RT Jr, Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, Kliewer SA, Willson TM and Xu HE (2000): Asymmetry in the PPARgamma/RXRα crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. Mol Cell 5:545-555.

Gann PH, Ma J, Hennekens CH, Hollis BW, Haddad JG and Stampfer MJ (1996): Circulating vitamin D metabolites in relation to subsequent development of prostate cancer. Cancer Epidemiol Biomarkers Prev 5:121-126.

Garcia-Villalba P, Jimenez-Lara AM and Aranda A (1996): Vitamin D interferes with transactivation of the growth hormone gene by thyroid hormone and retinoic acid. Mol Cell Biol 16:318-327.

Gascon-Barre M, Demers C, Ghrab O, Theodoropoulos C, Lapointe R, Jones G, Valiquette L and Menard D (2001): Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney. Clin Endocrinol (Oxf) 54:107-115.

Goossens K, Esquenet M, Swinnen JV, Manin M, Rombauts W and Verhoeven G (1999): Androgens decrease and retinoids increase the expression of insulin-like growth factor-binding protein-3 in LNcaP prostatic adenocarcinoma cells. Mol Cell Endocrinol 155:9-18.

Grant WB (2002): An estimate of premature cancer mortality in the U.S. due to inadequate doses of solar ultraviolet-B radiation. Cancer 94:1867-1875.

Gross C, Peehl DM and Feldman D (1997): Vitamin D and prostate cancer. In: Vitamin D, pp. 1125-1139. Eds. D Feldman, FH Glorieux and JW Pike, Academic Press, San Diego.

Gross C, Stamey T, Hancock S and Feldman D (1998): Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D_3 (calcitriol). J Urol 159:2035-2039; discussion 2039-2040.

Habuchi T, Suzuki T, Sasaki R, Wang L, Sato K, Satoh S, Akao T, Tsuchiya N, Shimoda N, Wada Y, Koizumi A, Chihara J, Ogawa O and Kato T (2000): Association of vitamin D receptor gene polymorphism with prostate cancer and benign prostatic hyperplasia in a Japanese population. Cancer Res 60:305-308.

Halloran BP and DeLuca HF (1980): Effect of vitamin D deficiency on fertility and reproductive capacity in the female rat. J Nutr 110:1573-1580.

Hamasaki T, Inatomi H, Katoh T, Ikuyama T and Matsumoto T (2001): Clinical and pathological significance of vitamin D receptor gene polymorphism for prostate cancer which is associated with a higher mortality in Japanese. Endocr J 48:543-549.

Hanchette CL and Schwartz GG (1992): Geographic patterns of prostate cancer mortality. Evidence for a protective effect of ultraviolet radiation. Cancer 70:2861-2869.

Harant H, Spinner D, Reddy GS and Lindley IJ (2000): Natural metabolites of 1α,25-dihydroxyvitamin D_3 retain biologic activity mediated through the vitamin D receptor. J Cell Biochem 78:112-120.

Haussler MR, Jurutka PW, Hsieh JC, Thompson PD, Selznick SH, Haussler CA and Whitfield GK (1995): New understanding of the molecular mechanism of receptor-mediated genomic actions of the vitamin D hormone. Bone 17:33S-38S.

Hedlund TE, Moffatt KA and Miller GJ (1996a): Stable expression of the nuclear vitamin D receptor in the human prostatic carcinoma cell line JCA-1: evidence that the antiproliferative effects of 1α,25-dihydroxyvitamin D_3 are mediated exclusively through the genomic signaling pathway. Endocrinology 137:1554-1561.

Hedlund TE, Moffatt KA and Miller GJ (1996b): Vitamin D receptor expression is required for growth modulation by 1α,25-dihydroxyvitamin D_3 in the human prostatic carcinoma cell line ALVA-31. J Steroid Biochem Mol Biol 58:277-288.

Heikinheimo RJ, Haavisto MV, Harju EJ, Inkovaara JA, Kaarela RH, Kolho LA and Rajala SA (1991): Serum vitamin D level after an annual intramuscular injection of ergocalciferol. Calcif Tissue Int 49:S87.

Hewison M, Zehnder D, Bland R and Stewart PM (2000): 1α-hydroxylase and the action of vitamin D. J Mol Endocrinol 25:141-148.

Hiraoka D, Nakamura N, Nishizawa Y, Uchida N, Noguchi S, Matsumoto K and Sato B (1987): Inhibitory and stimulatory effects of glucocorticoid on androgen-induced growth of murine Shionogi carcinoma 115 in vivo and in cell culture. Cancer Res 47:6560-6564.

Holick MF (2001): Sunlight "D"ilemma: risk of skin cancer or bone disease and muscle weakness. Lancet 357:4-6.

Holschneider CH and Berek JS (2000): Ovarian cancer: epidemiology, biology, and prognostic factors. Semin Surg Oncol 19:3-10.

Holt PR, Arber N, Halmos B, Forde K, Kissileff H, McGlynn KA, Moss SF, Fan K, Yang K and Lipkin M (2002): Colonic epithelial cell proliferation decreases with increasing levels of serum 25-hydroxyvitamin D. Cancer Epidemiol Biomarkers Prev 11:113-119.

Horii I, Takizawa S and Fujii T (1992): Effect of 1,25-dihydroxyvitamin D_3 on the female reproductive system in rats. J Toxicol Sci 17:91-105.

Hörlein AJ, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Söderström M, Glass CK and Rosenfeld MG (1995): Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397-404.

Horst RL and Reinhardt TA (1997): Vitamin D metabolism. In: Vitamin D, pp. 13-31. Eds. D Feldman, FH Glorieux and JW Pike, Academic Press, New York.

Horst RL, Goff JP and Reinhardt TA (1990): Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. Endocrinology 126:1053-1057.

Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS and Tung L (1996): Nuclear receptor coactivators and corepressors. Mol Endocrinol 10:1167-1177.

Hsieh JC, Jurutka PW, Selznick SH, Reeder MC, Haussler CA, Whitfield GK and Haussler MR (1995): The T-box near the zinc fingers of the human vitamin D receptor is required for heterodimeric DNA binding and transactivation. Biochem Biophys Res Commun 215:1-7.

Hsieh JC, Shimizu Y, Minoshima S, Shimizu N, Haussler CA, Jurutka PW and Haussler MR (1998): Novel nuclear localization signal between the two DNA-binding zinc fingers in the human vitamin D receptor. J Cell Biochem 70:94-109.

Hsieh T and Wu JM (1997): Induction of apoptosis and altered nuclear/cytoplasmic distribution of the androgen receptor and prostate-specific antigen by 1α,25-dihydroxyvitamin D_3 in androgen-responsive LNCaP cells. Biochem Biophys Res Commun 235:539-544.

Hsieh TY, Ng CY, Mallouh C, Tazaki H and Wu JM (1996): Regulation of growth, PSA/PAP and androgen receptor expression by 1α,25-dihydroxyvitamin D_3 in the androgen-dependent LNCaP cells. Biochem Biophys Res Commun 223:141-146.

Hsu JY, Feldman D, McNeal JE and Peehl DM (2001): Reduced 1α-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D_3-induced growth inhibition. Cancer Res 61:2852-2856.

Hulla W, Kallay E, Krugluger W, Peterlik M and Cross HS (1995): Growth control of human colon-adenocarcinoma-derived Caco-2 cells by vitamin-D compounds and extracellular calcium in vitro: relation to c-myc-oncogene and vitamin-D-receptor expression. Int J Cancer 62:711-716.

Hurst HC, Masson N, Jones NC and Lee KA (1990): The cellular transcription factor CREB corresponds to activating transcription factor 47 (ATF-47) and forms complexes with a group of polypeptides related to ATF-43. Mol Cell Biol 10:6192-6203.

Huynh H, Pollak M and Zhang JC (1998): Regulation of insulin-like growth factor (IGF) II and IGF binding protein 3 autocrine loop in human PC-3 prostate cancer cells by vitamin D metabolite $1,25(OH)_2D_3$ and its analog EB1089. Int J Oncol 13:137-143.

Ilekis JV, Connor JP, Prins GS, Ferrer K, Niederberger C and Scoccia B (1997): Expression of epidermal growth factor and androgen receptors in ovarian cancer. Gynecol Oncol 66:250-254.

Ingles SA, Ross RK, Yu MC, Irvine RA, La Pera G, Haile RW and Coetzee GA (1997): Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. J Natl Cancer Inst 89:166-170.

Issa LL, Leong GM and Eisman JA (1998): Molecular mechanism of vitamin D receptor action. Inflamm Res 47:451-475.

Iwabuchi H, Sakamoto M, Sakunaga H, Ma YY, Carcangiu ML, Pinkel D, Yang-Feng TL and Gray JW (1995): Genetic analysis of benign, low-grade, and high-grade ovarian tumors. Cancer Res 55:6172-6180.

Jarrard DF, Kinoshita H, Shi Y, Sandefur C, Hoff D, Meisner LF, Chang C, Herman JG, Isaacs WB and Nassif N (1998): Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. Cancer Res 58:5310-5314.

Jensen TJ, Henriksen LO, Solvsten H and Kragballe K (1998): Inhibition of the 1,25-dihydroxyvitamin D_3-induced increase in vitamin D receptor (VDR) levels and binding of VDR-retinoid X receptor (RXR) to a direct repeat (DR)-3 type response element by an RXR-specific ligand in human keratinocyte cultures. Biochem Pharmacol 55:767-773.

Jin CH, Kerner SA, Hong MH and Pike JW (1996): Transcriptional activation and dimerization functions in the human vitamin D receptor. Mol Endocrinol 10:945-957.

Johnson JA, Grande JP, Roche PC and Kumar R (1996): Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D_3 receptor in rat reproductive tissues. Histochem Cell Biol 105:7-15.

Johnson LE and DeLuca HF (2001): Vitamin D receptor null mutant mice fed high levels of calcium are fertile. J Nutr 131:1787-1791.

Jones G, Ramshaw H, Zhang A, Cook R, Byford V, White J and Petkovich M (1999): Expression and activity of vitamin D-metabolizing cytochrome P450s (CYP1α and CYP24) in human nonsmall cell lung carcinomas. Endocrinology 140:3303-3310.

Jurutka PW, Hsieh JC, Remus LS, Whitfield GK, Thompson PD, Haussler CA, Blanco JC, Ozato K and Haussler MR (1997): Mutations in the 1,25-dihydroxyvitamin D_3 receptor identifying C-terminal amino acids required for transcriptional activation that are functionally dissociated from hormone binding, heterodimeric DNA binding, and interaction with basal transcription factor IIB, in vitro. J Biol Chem 272:14592-14599.

Kain SR and Henry HL (1989): 25-hydroxyvitamin D_3 metabolism in mitochondria from primary renal cultures. Mol Cell Endocrinol 64:63-69.

Karlan BY, Jones J, Greenwald M and Lagasse LD (1995): Steroid hormone effects on the proliferation of human ovarian surface epithelium in vitro. Am J Obstet Gynecol 173:97-104.

Kerry DM, Dwivedi PP, Hahn CN, Morris HA, Omdahl JL and May BK (1996): Transcriptional synergism between vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase (CYP24) promoter. J Biol Chem 271:29715-29721.

Kibel AS, Isaacs SD, Isaacs WB and Bova GS (1998): Vitamin D receptor polymorphisms and lethal prostate cancer. J Urol 160:1405-1409.

Kim RH, Li JJ, Ogata Y, Yamauchi M, Freedman LP and Sodek J (1996): Identification of a vitamin D_3-response element that overlaps a unique inverted TATA box in the rat bone sialoprotein gene. Biochem J 318:219-226.

Kimmel-Jehan C, Jehan F and DeLuca HF (1997): Salt concentration determines 1,25-dihydroxyvitamin D_3 dependency of vitamin D receptor-retinoid X receptor-vitamin D-responsive element complex formation. Arch Biochem Biophys 341:75-80.

Kinoshita H, Shi Y, Sandefur C, Meisner LF, Chang C, Choon A, Reznikoff CR, Bova GS, Friedl A and Jarrard DF (2000): Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer. Cancer Res 60:3623-3630.

Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S and Seino Y (2000): Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. Endocrinology 141:1317-1324.

Kinyamu HK, Gallagher JC, Prahl JM, DeLuca HF, Petranick KM and Lanspa SJ (1997): Association between intestinal vitamin D receptor, calcium absorption, and serum 1,25 dihydroxyvitamin D in normal young and elderly women. J Bone Miner Res 12:922-928.

Kissmeyer AM, Binderup E, Binderup L, Mork Hansen C, Andersen NR, Makin HL, Schroeder NJ, Shankar VN and Jones G (1997): Metabolism of the vitamin D analog EB 1089: identification of in vivo and in vitro liver metabolites and their biological activities. Biochem Pharmacol 53:1087-1097.

Kizaki M, Norman AW, Bishop JE, Lin CW, Karmakar A and Koeffler HP (1991): 1,25-dihydroxyvitamin D_3 receptor RNA: expression in hematopoietic cells. Blood 77:1238-1247.

Konety BR, Schwartz GG, Acierno JS, Jr., Becich MJ and Getzenberg RH (1996): The role of vitamin D in normal prostate growth and differentiation. Cell Growth Differ 7:1563-1570.

Konety BR, Nangia AK, Nguyen TS, Thomas A and Getzenberg RH (1999): Effect of prenatal vitamin D (calcitriol) exposure on the growth and development of the prostate. Prostate 41:181-189.

Krill D, Stoner J, Konety BR, Becich MJ and Getzenberg RH (1999): Differential effects of vitamin D on normal human prostate epithelial and stromal cells in primary culture. Urology 54:171-177.

Krill D, DeFlavia P, Dhir R, Luo J, Becich MJ, Lehman E and Getzenberg RH (2001): Expression patterns of vitamin D receptor in human prostate. J Cell Biochem 82:566-572.

Krishnan AV and Feldman D (1997): Regulation of vitamin D receptor abundance. In: Vitamin D, pp. 179-200. Eds. D Feldman, FH Glorieux and JW Pike, Academic Press, San Diego.

Kristensen GB and Trope C (1997): Epithelial ovarian carcinoma. Lancet 349:113-117.

Kueng W, Silber E and Eppenberger U (1989): Quantification of cells cultured on 96-well plates. Anal Biochem 182:16-19.

Kuhnel R, de Graaff J, Rao BR and Stolk JG (1987): Androgen receptor predominance in human ovarian carcinoma. J Steroid Biochem 26:393-397.

Kumar R, Schaefer J, Grande JP and Roche PC (1994): Immunolocalization of calcitriol receptor, 24-hydroxylase cytochrome P-450, and calbindin D28k in human kidney. Am J Physiol 266:F477-485.

Kwiecinksi GG, Petrie GI and DeLuca HF (1989): 1,25-dihydroxyvitamin D_3 restores fertility of vitamin D-deficient female rats. Am J Physiol 256:E483-487.

La Vecchia C and Franceschi S (1999): Oral contraceptives and ovarian cancer. Eur J Cancer Prev 8:297-304.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lamberg-Allardt CJ, Outila TA, Karkkainen MU, Rita HJ and Valsta LM (2001): Vitamin D deficiency and bone health in healthy adults in finland: could this be a concern in other parts of Europe? J Bone Miner Res 16:2066-2073.

Landsman T, Leitner G, Robinzon TB and Helle ED (2001): Effect of gonadal steroids on proliferative responses and subset alterations in cultured chicken lymphocytes. Poult Sci 80:1329-1338.

Langeler EG, van Uffelen CJ, Blankenstein MA, van Steenbrugge GJ and Mulder E (1993): Effect of culture conditions on androgen sensitivity of the human prostatic cancer cell line LNCaP. Prostate 23:213-223.

Lefkowitz ES and Garland CF (1994): Sunlight, vitamin D, and ovarian cancer mortality rates in US women. Int J Epidemiol 23:1133-1136.

Lehtonen-Veromaa M, Mottonen T, Irjala K, Kärkkäinen M, Lamberg-Allardt C, Hakola P and Viikari J (1999): Vitamin D intake is low and hypovitaminosis D common in healthy 9- to 15-year-old Finnish girls. Eur J Clin Nutr 53:746-751.

Lemon BD, Fondell JD and Freedman LP (1997): Retinoid X receptor:vitamin D_3 receptor heterodimers promote stable preinitiation complex formation and direct 1,25-dihydroxyvitamin D_3-dependent cell-free transcription. Mol Cell Biol 17:1923-1937.

Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A and Grippo JF (1992): 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature 355:359-361.

Li XY, Boudjelal M, Xiao JH, Peng ZH, Asuru A, Kang S, Fisher GJ and Voorhees JJ (1999): 1,25-dihydroxyvitamin D_3 increases nuclear vitamin D_3 receptors by blocking ubiquitin/proteasome-mediated degradation in human skin. Mol Endocrinol 13:1686-1694.

Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R and Demay MB (1997): Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc Natl Acad Sci U S A 94:9831-9835.

Lokeshwar BL, Schwartz GG, Selzer MG, Burnstein KL, Zhuang SH, Block NL and Binderup L (1999): Inhibition of prostate cancer metastasis in vivo: a comparison of 1,25-dihydroxyvitamin D (calcitriol) and EB1089. Cancer Epidemiol Biomarkers Prev 8:241-248.

Loose DS, Kan PB, Hirst MA, Marcus RA and Feldman D (1983): Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. J Clin Invest 71:1495-1499.

Love-Schimenti CD, Gibson DF, Ratnam AV and Bikle DD (1996): Antiestrogen potentiation of antiproliferative effects of vitamin D_3 analogues in breast cancer cells. Cancer Res 56:2789-2794.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951): Protein measurement with Folin phenol reagent. J Biol Chem 193:265-275.

Lund BC, Bever-Stille KA and Perry PJ (1999): Testosterone and andropause: the feasibility of testosterone replacement therapy in elderly men. Pharmacotherapy 19:951-956.

Luo Z, Rouvinen J and Mäenpää PH (1994): A peptide C-terminal to the second Zn finger of human vitamin D receptor is able to specify nuclear localization. Eur J Biochem 223:381-387.

Lutz LB, Cole LM, Gupta MK, Kwist KW, Auchus RJ and Hammes SR (2001): Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc Natl Acad Sci U S A 98:13728-13733.

Ly LH, Zhao XY, Holloway L and Feldman D (1999): Liarozole acts synergistically with 1α,25-dihydroxyvitamin D_3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase activity. Endocrinology 140:2071-2076.

MacDonald PN, Dowd DR, Nakajima S, Galligan MA, Reeder MC, Haussler CA, Ozato K and Haussler MR (1993): Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D_3-activated expression of the rat osteocalcin gene. Mol Cell Biol 13:5907-5917.

Mackey SL, Heymont JL, Kronenberg HM and Demay MB (1996): Vitamin D receptor binding to the negative human parathyroid hormone vitamin D response element does not require the retinoid X receptor. Mol Endocrinol 10:298-305.

MacLaughlin J and Holick MF (1985): Aging decreases the capacity of human skin to produce vitamin D_3. J Clin Invest 76:1536-1538.

Maglich JM, Sluder A, Guan X, Shi Y, McKee DD, Carrick K, Kamdar K, Willson TM and Moore JT (2001): Comparison of complete nuclear receptor sets from the human, Caenorhabditis elegans and Drosophila genomes. Genome Biol 2:research0029.1-0029.7.

Mahlck CG, Backstrom T and Kjellgren O (1986): Androstenedione production by malignant epithelial ovarian tumors. Gynecol Oncol 25:217-222.

Mahonen A and Mäenpää PH (1994): Steroid hormone modulation of vitamin D receptor levels in human MG-63 osteosarcoma cells. Biochem Biophys Res Commun 205:1179-1186.

Mahonen A, Pirskanen A, Keinanen R and Mäenpää PH (1990): Effect of 1,25$(OH)_2D_3$ on its receptor mRNA levels and osteocalcin synthesis in human osteosarcoma cells. Biochim Biophys Acta 1048:30-37.

Malabanan A, Veronikis IE and Holick MF (1998): Redefining vitamin D insufficiency. Lancet 351:805-806.

Martinez P, Moreno I, De Miguel F, Vila V, Esbrit P and Martinez ME (2001): Changes in osteocalcin response to 1,25-dihydroxyvitamin D_3 stimulation and basal vitamin D receptor expression in human osteoblastic cells according to donor age and skeletal origin. Bone 29:35-41.

Matkovits T and Christakos S (1995): Variable in vivo regulation of rat vitamin D-dependent genes (osteopontin, Ca,Mg-adenosine triphosphatase, and 25-hydroxyvitamin D_3 24-hydroxylase): implications for differing mechanisms of regulation and involvement of multiple factors. Endocrinology 136:3971-3982.

Mawer EB, Hayes ME, Heys SE, Davies M, White A, Stewart MF and Smith GN (1994): Constitutive synthesis of 1,25-dihydroxyvitamin D_3 by a human small cell lung cancer cell line. J Clin Endocrinol Metab 79:554-560.

McDonnell DP, Scott RA, Kerner SA, O'Malley BW and Pike JW (1989): Functional domains of the human vitamin D_3 receptor regulate osteocalcin gene expression. Mol Endocrinol 3:635-644.

Michigami T, Suga A, Yamazaki M, Shimizu C, Cai G, Okada S and Ozono K (1999): Identification of amino acid sequence in the hinge region of human vitamin D receptor that transfers a cytosolic protein to the nucleus. J Biol Chem 274:33531-33538.

Milde P, Merke J, Ritz E, Haussler MR and Rauterberg EW (1989): Immunohistochemical detection of 1,25-dihydroxyvitamin D_3 receptors and estrogen receptors by monoclonal antibodies: comparison of four immunoperoxidase methods. J Histochem Cytochem 37:1609-1617.

Milde P, Hauser U, Simon T, Mall G, Ernst V, Haussler MR, Frosch P and Rauterberg EW (1991): Expression of 1,25-dihydroxyvitamin D_3 receptors in normal and psoriatic skin. J Invest Dermatol 97:230-239.

Miller GJ, Stapleton GE, Ferrara JA, Lucia MS, Pfister S, Hedlund TE and Upadhya P (1992): The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1α,25-dihydroxyvitamin D_3. Cancer Res 52:515-520.

Miller GJ, Stapleton GE, Hedlund TE and Moffat KA (1995): Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1α,25-dihydroxyvitamin D_3 in seven human prostatic carcinoma cell lines. Clin Cancer Res 1:997-1003.

Miyamoto T, Kakizawa T, Ichikawa K, Nishio S, Takeda T, Suzuki S, Kaneko A, Kumagai M, Mori J, Yamashita K, Sakuma T and Hashizume K (2001): The role of hinge domain in heterodimerization and specific DNA recognition by nuclear receptors. Mol Cell Endocrinol 181:229-238.

Moffatt KA, Johannes WU, Hedlund TE and Miller GJ (2001): Growth inhibitory effects of 1α,25-dihydroxyvitamin D_3 are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. Cancer Res 61:7122-7129.

Monkawa T, Yoshida T, Hayashi M and Saruta T (2000): Identification of 25-hydroxyvitamin D_3 1α-hydroxylase gene expression in macrophages. Kidney Int 58:559-568.

Moras D and Gronemeyer H (1998): The nuclear receptor ligand-binding domain: structure and function. Curr Opin Cell Biol 10:384-391.

Mørk Hansen C, Hamberg KJ, Binderup E and Binderup L (2000): Seocalcitol (EB 1089): a vitamin D analogue of anti-cancer potential. Background, design, synthesis, pre-clinical and clinical evaluation. Curr Pharm Des 6:803-828.

Mørk Hansen C, Binderup L, Hamberg KJ and Carlberg C (2001): Vitamin D and cancer: effects of $1,25(OH)_2D_3$ and its analogs on growth control and tumorigenesis. Front Biosci 6:D820-848.

Munker R, Norman A and Koeffler HP (1986): Vitamin D compounds. Effect on clonal proliferation and differentiation of human myeloid cells. J Clin Invest 78:424-430.

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T and Kato S (1998): The promoter of the human 25-hydroxyvitamin D_3 1α-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1α,25$(OH)_2D_3$. Biochem Biophys Res Commun 249:11-16.

Nakajima S, Hsieh JC, MacDonald PN, Galligan MA, Haussler CA, Whitfield GK and Haussler MR (1994): The C-terminal region of the vitamin D receptor is essential to form a complex with a receptor auxiliary factor required for high affinity binding to the vitamin D-responsive element. Mol Endocrinol 8:159-172.

Nelson VL, Legro RS, Strauss JF, 3rd and McAllister JM (1999): Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. Mol Endocrinol 13:946-957.

Nickerson T and Huynh H (1999): Vitamin D analogue EB1089-induced prostate regression is associated with increased gene expression of insulin-like growth factor binding proteins. J Endocrinol 160:223-229.

Nishimura A, Shinki T, Jin CH, Ohyama Y, Noshiro M, Okuda K and Suda T (1994): Regulation of messenger ribonucleic acid expression of 1α,25-dihydroxyvitamin D_3-24-hydroxylase in rat osteoblasts. Endocrinology 134:1794-1799.

Nishishita T, Okazaki T, Ishikawa T, Igarashi T, Hata K, Ogata E and Fujita T (1998): A negative vitamin D response DNA element in the human parathyroid hormone-related peptide gene binds to vitamin D receptor along with Ku antigen to mediate negative gene regulation by vitamin D. J Biol Chem 273:10901-10907.

Nomura AM, Stemmermann GN, Lee J, Kolonel LN, Chen TC, Turner A and Holick MF (1998): Serum vitamin D metabolite levels and the subsequent development of prostate cancer (Hawaii, United States). Cancer Causes Control 9:425-432.

Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI and Willnow TE (1999): An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D_3. Cell 96:507-515.

Ohyama Y, Ozono K, Uchida M, Yoshimura M, Shinki T, Suda T and Yamamoto O (1996): Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. J Biol Chem 271:30381-30385.

Outila TA, Karkkainen MU and Lamberg-Allardt CJ (2001): Vitamin D status affects serum parathyroid hormone concentrations during winter in female adolescents: associations with forearm bone mineral density. Am J Clin Nutr 74:206-210.

Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN and Goltzman D (2001): Targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci U S A 98:7498-7503.

Peehl DM and Sellers RG (1997): Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells. Exp Cell Res 232:208-215.

Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA and Feldman D (1994): Antiproliferative effects of 1,25-dihydroxyvitamin D_3 on primary cultures of human prostatic cells. Cancer Res 54:805-810.

Peehl DM, Seto E and Feldman D (2001): Rationale for combination ketoconazole/ vitamin D treatment of prostate cancer. Urology 58:123-126.

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:E45-E45.

Pike JW and Sleator NM (1985): Hormone-dependent phosphorylation of the 1,25-dihydroxyvitamin D_3 receptor in mouse fibroblasts. Biochem Biophys Res Commun 131:378-385.

Pikuleva IA, Bjorkhem I and Waterman MR (1997): Expression, purification, and enzymatic properties of recombinant human cytochrome P450c27 (CYP27). Arch Biochem Biophys 343:123-130.

Polek TC, Murthy S, Blutt SE, Boehm MF, Zou A, Weigel NL and Allegretto EA (2001): Novel nonsecosteroidal vitamin D receptor modulator inhibits the growth of LNCaP xenograft tumors in athymic mice without increased serum calcium. Prostate 49:224-233.

Polly P, Carlberg C, Eisman JA and Morrison NA (1997): 1α,25-dihydroxyvitamin D_3 receptor as a mediator of transrepression of retinoid signaling. J Cell Biochem 67:287-296.

Polly P, Herdick M, Moehren U, Baniahmad A, Heinzel T and Carlberg C (2000): VDR-Alien: a novel, DNA-selective vitamin D_3 receptor-corepressor partnership. Faseb J 14:1455-1463.

Portale AA and Miller WL (2000): Human 25-hydroxyvitamin D-1α-hydroxylase: cloning, mutations, and gene expression. Pediatr Nephrol 14:620-625.

Potashnik G, Lunenfeld E, Levitas E, Itskovitz J, Albutiano S, Yankowitz N, Sonin Y, Levy J, Glezerman M and Shany S (1992): The relationship between endogenous oestradiol and vitamin D_3 metabolites in serum and follicular fluid during ovarian stimulation for in-vitro fertilization and embryo transfer. Hum Reprod 7:1357-1360.

Rachez C, Lemon BD, Suldan Z, Bromleigh V, Gamble M, Naar AM, Erdjument-Bromage H, Tempst P and Freedman LP (1999): Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. Nature 398:824-828.

Rashid SF, Mountford JC, Gombart AF and Campbell MJ (2001): 1α,25-dihydroxyvitamin D_3 displays divergent growth effects in both normal and malignant cells. Steroids 66:433-440.

Rastinejad F, Perlmann T, Evans RM and Sigler PB (1995): Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature 375:203-211.

Reiter E, Hennuy B, Bruyninx M, Cornet A, Klug M, McNamara M, Closset J and Hennen G (1999): Effects of pituitary hormones on the prostate. Prostate 38:159-165.

Renaud JP and Moras D (2000): Structural studies on nuclear receptors. Cell Mol Life Sci 57:1748-1769.

Riman T, Persson I and Nilsson S (1998): Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. Clin Endocrinol (Oxf) 49:695-707.

Risch HA (1998): Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst 90:1774-1786.

Rochel N, Wurtz JM, Mitschler A, Klaholz B and Moras D (2000): The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol Cell 5:173-179.

Rustin GJ, Quinnell TG, Johnson J, Clarke H, Nelstrop AE and Bollag W (1996): Trial of isotretinoin and calcitriol monitored by CA 125 in patients with ovarian cancer. Br J Cancer 74:1479-1481.

Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, Liebhaber SA and Cooke NE (1999): Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. J Clin Invest 103:239-251.

Sakaki T, Sawada N, Nonaka Y, Ohyama Y and Inouye K (1999): Metabolic studies using recombinant *Escherichia coli* cells producing rat mitochondrial CYP24 - CYP24 can convert 1α,25-dihydroxyvitamin D_3 to calcitroic acid. Eur J Biochem 262:43-48.

Sasaki M, Oh BR, Dharia A, Fujimoto S and Dahiya R (2000): Inactivation of the human androgen receptor gene is associated with CpG hypermethylation in uterine endometrial cancer. Mol Carcinog 29:59-66.

Saunders DE, Christensen C, Lawrence WD, Malviya VK, Malone JM, Williams JR and Deppe G (1992): Receptors for 1,25-dihydroxyvitamin D_3 in gynecologic neoplasms. Gynecol Oncol 44:131-136.

Saunders DE, Christensen C, Wappler NL, Schultz JF, Lawrence WD, Malviya VK, Malone JM and Deppe G (1993): Inhibition of c-myc in breast and ovarian carcinoma cells by 1,25-dihydroxyvitamin D_3, retinoic acid and dexamethasone. Anticancer Drugs 4:201-208.

Saunders DE, Christensen C, Williams JR, Wappler NL, Lawrence WD, Malone JM, Malviya VK and Deppe G (1995): Inhibition of breast and ovarian carcinoma cell growth by 1,25-dihydroxyvitamin D_3 combined with retinoic acid or dexamethasone. Anticancer Drugs 6:562-569.

Sawada N, Sakaki T, Ohta M and Inouye K (2000): Metabolism of vitamin D_3 by human CYP27A1. Biochem Biophys Res Commun 273:977-984.

Schuessler M, Astecker N, Herzig G, Vorisek G and Schuster I (2001): Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D_3: CYP27 in epidermis completes the set of essential vitamin D_3-hydroxylases. Steroids 66:399-408.

Schuster I, Egger H, Astecker N, Herzig G, Schussler M and Vorisek G (2001a): Selective inhibitors of CYP24: mechanistic tools to explore vitamin D metabolism in human keratinocytes. Steroids 66:451-462.

Schuster I, Egger H, Bikle D, Herzig G, Reddy GS, Stuetz A, Stuetz P and Vorisek G (2001b): Selective inhibition of vitamin D hydroxylases in human keratinocytes. Steroids 66:409-422.

Schwartz GG and Hulka BS (1990): Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). Anticancer Res 10:1307-1311.

Schwartz GG, Wang MH, Zang M, Singh RK and Siegal GP (1997): 1α,25-dihydroxyvitamin D (calcitriol) inhibits the invasiveness of human prostate cancer cells. Cancer Epidemiol Biomarkers Prev 6:727-732.

Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL and Holick MF (1998): Human prostate cells synthesize 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3. Cancer Epidemiol Biomarkers Prev 7:391-395.

Shankar VN, Dilworth FJ, Makin HL, Schroeder NJ, Trafford DJ, Kissmeyer AM, Calverley MJ, Binderup E and Jones G (1997): Metabolism of the vitamin D analog EB1089 by cultured human cells: redirection of hydroxylation site to distal carbons of the side-chain. Biochem Pharmacol 53:783-793.

Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K and Suda T (1992): Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1α,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. J Biol Chem 267:13757-13762.

Skowronski RJ, Peehl DM and Feldman D (1993): Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D_3 receptors and actions in human prostate cancer cell lines. Endocrinology 132:1952-1960.

Skowronski RJ, Peehl DM and Feldman D (1995): Actions of vitamin D_3, analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D_3. Endocrinology 136:20-26.

Slotman BJ and Rao BR (1989): Response to inhibition of androgen action of human ovarian cancer cells in vitro. Cancer Lett 45:213-220.

Slotman BJ, Kuhnel R, Rao BR, Dijkhuizen GH, de Graaff J and Stolk JG (1989): Importance of steroid receptors and aromatase activity in the prognosis of ovarian cancer: high tumor progesterone receptor levels correlate with longer survival. Gynecol Oncol 33:76-81.

Smirnoff P, Liel Y, Gnainsky J, Shany S and Schwartz B (1999): The protective effect of estrogen against chemically induced murine colon carcinogenesis is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. Oncol Res 11:255-264.

Smith DC, Johnson CS, Freeman CC, Muindi J, Wilson JW and Trump DL (1999): A Phase I trial of calcitriol (1,25-dihydroxycholecalciferol) in patients with advanced malignancy. Clin Cancer Res 5:1339-1345.

Sone T, Kerner S and Pike JW (1991): Vitamin D receptor interaction with specific DNA. Association as a 1,25-dihydroxyvitamin D_3-modulated heterodimer. J Biol Chem 266:23296-23305.

Song LN (1996): Demonstration of vitamin D receptor expression in a human megakaryoblastic leukemia cell line: regulation of vitamin D receptor mRNA expression and responsiveness by forskolin. J Steroid Biochem Mol Biol 57:265-274.

Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ and O'Malley BW (1997): Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194-198.

Sprenger CC, Peterson A, Lance R, Ware JL, Drivdahl RH and Plymate SR (2001): Regulation of proliferation of prostate epithelial cells by 1,25-dihydroxyvitamin D_3 is accompanied by an increase in insulin-like growth factor binding protein-3. J Endocrinol 170:609-618.

Stumpf WE and Denny ME (1989): Vitamin D (soltriol), light, and reproduction. Am J Obstet Gynecol 161:1375-1384.

Stumpf WE, Clark SA, Sar M and DeLuca HF (1984): Topographical and developmental studies on target sites of 1,25$(OH)_2$ vitamin D_3 in skin. Cell Tissue Res 238:489-496.

Su P, Rennert H, Shayiq RM, Yamamoto R, Zheng YM, Addya S, Strauss JF and Avadhani NG (1990): A cDNA encoding a rat mitochondrial cytochrome P450 catalyzing both the 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D_3: gonadotropic regulation of the cognate mRNA in ovaries. DNA Cell Biol 9:657-667.

Sung V and Feldman D (2000): 1,25-dihydroxyvitamin D_3 decreases human prostate cancer cell adhesion and migration. Mol Cell Endocrinol 164:133-143.

Suzuki T, Suzuki N, Srivastava AS and Kurokawa T (2000): Identification of cDNAs encoding two subtypes of vitamin D receptor in flounder, Paralichthys olivaceus. Biochem Biophys Res Commun 270:40-45.

Syed V, Ulinski G, Mok SC, Yiu GK and Ho SM (2001): Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. Cancer Res 61:6768-6776.

Tagami T, Lutz WH, Kumar R and Jameson JL (1998): The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. Biochem Biophys Res Commun 253:358-363.

Tähkä KM, Zhuang YH, Tähkä S and Tuohimaa P (1997): Photoperiod-induced changes in androgen receptor expression in testes and accessory sex glands of the bank vole, Clethrionomys glareolus. Biol Reprod 56:898-908.

Takeda H, Chodak G, Mutchnik S, Nakamoto T and Chang C (1990): Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor. J Endocrinol 126:17-25.

Tanaka Y, Castillo L and DeLuca HF (1976): Control of renal vitamin D hydroxylases in birds by sex hormones. Proc Natl Acad Sci U S A 73:2701-2705.

Tangpricha V, Flanagan JN, Whitlatch LW, Tseng CC, Chen TC, Holt PR, Lipkin MS and Holick MF (2001): 25-hydroxyvitamin D-1α-hydroxylase in normal and malignant colon tissue. Lancet 357:1673-1674.

Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg A and Isola JJ (2000): Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. Clin Cancer Res 6:1833-1839.

Taplin ME and Ho SM (2001): Clinical review 134: The endocrinology of prostate cancer. J Clin Endocrinol Metab 86:3467-3477.

Taylor JA, Hirvonen A, Watson M, Pittman G, Mohler JL and Bell DA (1996): Association of prostate cancer with vitamin D receptor gene polymorphism. Cancer Res 56:4108-4110.

Tetsuka M and Hillier SG (1996): Androgen receptor gene expression in rat granulosa cells: the role of follicle-stimulating hormone and steroid hormones. Endocrinology 137:4392-4397.

Tetsuka M, Whitelaw PF, Bremner WJ, Millar MR, Smyth CD and Hillier SG (1995): Developmental regulation of androgen receptor in rat ovary. J Endocrinol 145:535-543.

Theodoropoulos C, Demers C, Mirshahi A and Gascon-Barre M (2001): 1,25-dihydroxyvitamin D_3 downregulates the rat intestinal vitamin D_3-25-hydroxylase CYP27A. Am J Physiol Endocrinol Metab 281:E315-325.

Thompson MA and Adelson MD (1993): Aging and development of ovarian epithelial carcinoma: the relevance of changes in ovarian stromal androgen production. Adv Exp Med Biol 330:155-165.

Thompson PD, Jurutka PW, Haussler CA, Whitfield GK and Haussler MR (1998): Heterodimeric DNA binding by the vitamin D receptor and retinoid X receptors is enhanced by 1,25-dihydroxyvitamin D_3 and inhibited by 9-cis-retinoic acid. Evidence for allosteric receptor interactions. J Biol Chem 273:8483-8491.

Thys-Jacobs S, Donovan D, Papadopoulos A, Sarrel P and Bilezikian JP (1999): Vitamin D and calcium dysregulation in the polycystic ovarian syndrome. Steroids 64:430-435.

Toell A, Polly P and Carlberg C (2000): All natural DR3-type vitamin D response elements show a similar functionality in vitro. Biochem J 352:301-309.

Tovar Sepulveda VA and Falzon M (2002): Regulation of PTH-related protein gene expression by vitamin D in PC-3 prostate cancer cells. Mol Cell Endocrinol 190:115-124.

Towers TL and Freedman LP (1998): Granulocyte-macrophage colony-stimulating factor gene transcription is directly repressed by the vitamin D_3 receptor. Implications for allosteric influences on nuclear receptor structure and function by a DNA element. J Biol Chem 273:10338-10348.

Trechsel U, Bonjour JP and Fleisch H (1979): Regulation of the metabolism of 25-hydroxyvitamin D_3 in primary cultures of chick kidney cells. J Clin Invest 64:206-217.

Umesono K, Murakami KK, Thompson CC and Evans RM (1991): Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell 65:1255-1266.

Väisänen S, Peräkylä M, Kärkkäinen JI, Steinmeyer A and Carlberg C (2002): Critical role of helix 12 of the vitamin D_3 receptor for the partial agonism of carboxylic ester antagonists. J Mol Biol 315:229-238.

van den Bemd GC, Pols HA, Birkenhager JC and van Leeuwen JP (1996): Conformational change and enhanced stabilization of the vitamin D receptor by the 1,25-dihydroxyvitamin D_3 analog KH1060. Proc Natl Acad Sci U S A 93:10685-10690.

van Leeuwen JPTM and Pols HAP (1997): Vitamin D: anticancer and differentiation. In: Vitamin D, pp. 1089-1105. Eds. D Feldman, FH Glorieux and JW Pike, Academic Press, San Diego.

Van Veldhuizen PJ, Taylor SA, Williamson S and Drees BM (2000): Treatment of vitamin D deficiency in patients with metastatic prostate cancer may improve bone pain and muscle strength. J Urol 163:187-190.

Verhaeghe J and Bouillon R (1992): Calciotropic hormones during reproduction. J Steroid Biochem Mol Biol 41:469-477.

Walters MR (1992): Newly identified actions of the vitamin D endocrine system. Endocr Rev 13:719-764.

Watanabe M, Fukutome K, Murata M, Uemura H, Kubota Y, Kawamura J and Yatani R (1999): Significance of vitamin D receptor gene polymorphism for prostate cancer risk in Japanese. Anticancer Res 19:4511-4514.

Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Okafor J and Bondy CA (1998): Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J Clin Endocrinol Metab 83:2479-2485.

Whitfield GK, Hsieh JC, Nakajima S, MacDonald PN, Thompson PD, Jurutka PW, Haussler CA and Haussler MR (1995): A highly conserved region in the hormone-binding domain of the human vitamin D receptor contains residues vital for heterodimerization with retinoid X receptor and for transcriptional activation. Mol Endocrinol 9:1166-1179.

Wiese RJ, Uhland-Smith A, Ross TK, Prahl JM and DeLuca HF (1992): Up-regulation of the vitamin D receptor in response to 1,25-dihydroxyvitamin D_3 results from ligand-induced stabilization. J Biol Chem 267:20082-20086.

Wikvall K (2001): Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form (review). Int J Mol Med 7:201-209.

Wolter H, Gottfried HW and Mattfeldt T (2002): Genetic changes in stage pT2N0 prostate cancer studied by comparative genomic hybridization. BJU Int 89:310-316.

Yang ES, Maiorino CA, Roos BA, Knight SR and Burnstein KL (2002): Vitamin D-mediated growth inhibition of an androgen-ablated LNCaP cell line model of human prostate cancer. Mol Cell Endocrinol 186:69-79.

Yen PM, Liu Y, Sugawara A and Chin WW (1996): Vitamin D receptors repress basal transcription and exert dominant negative activity on triiodothyronine-mediated transcriptional activity. J Biol Chem 271:10910-10916.

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T and Kato S (1997): Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16:391-396.

Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM and Hewison M (2001): Extrarenal expression of 25-hydroxyvitamin D_3-1α-hydroxylase. J Clin Endocrinol Metab 86:888-894.

Zhao X and Feldman D (1993): Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT-29 human colon cancer cells. Endocrinology 132:1808-1814.

Zhao XY and Feldman D (2001): The role of vitamin D in prostate cancer. Steroids 66:293-300.

Zhao XY, Ly LH, Peehl DM and Feldman D (1997): 1α,25-dihydroxyvitamin D_3 actions in LNCaP human prostate cancer cells are androgen-dependent. Endocrinology 138:3290-3298.

Zhao XY, Ly LH, Peehl DM and Feldman D (1999): Induction of androgen receptor by 1α,25-dihydroxyvitamin D_3 and 9-cis retinoic acid in LNCaP human prostate cancer cells. Endocrinology 140:1205-1212.

Zhao XY, Peehl DM, Navone NM and Feldman D (2000): 1α,25-dihydroxyvitamin D_3 inhibits prostate cancer cell growth by androgen-dependent and androgen-independent mechanisms. Endocrinology 141:2548-2556.

Zheng L, Annab LA, Afshari CA, Lee WH and Boyer TG (2001): BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. Proc Natl Acad Sci U S A 98:9587-9592.

Zhuang SH and Burnstein KL (1998): Antiproliferative effect of 1α,25-dihydroxyvitamin D_3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. Endocrinology 139:1197-1207.

Zhuang SH, Schwartz GG, Cameron D and Burnstein KL (1997): Vitamin D receptor content and transcriptional activity do not fully predict antiproliferative effects of vitamin D in human prostate cancer cell lines. Mol Cell Endocrinol 126:83-90.

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ORIGINAL COMMUNICATIONS

ERRATA

There is an error in the original communication IV, page 849. In Table 2, the number (n) of controls in January-February reads 143, should read 343.