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Regulation of Growth Factor Signaling by Vitamin D in Human Prostate Cancer Cells

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ACADEMIC DISSERTATION
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To My Family

CONTENTS

ABSTRACT

The present study was undertaken to reveal growth factor signaling pathways mediating antiproliferative action of calcitriol in human prostate cancer LNCaP cells. Calcitriol (1α,25-dihydroxyvitamin D_3) is a hormonally active metabolite of vitamin D. It controls proliferation, differentiation and apoptosis of prostate cancer cells via molecular mechanisms which are not completely understood. The antiproliferative action of calcitriol is mediated by nuclear vitamin D receptor (VDR), ligand-dependent transcription factor, which selectively regulates expression of target genes. The set of genes regulated by VDR is cell type specific and includes genes encoding components of numerous signaling pathways.

In this study the initial screening for potential calcitriol target genes in human prostate cancer LNCaP cells was performed using cDNA microarray technique followed by analysis of candidate gene expression with real-time RT-PCR and Western blotting methods. The biological significance of regulation of candidate gene expression by calcitriol was investigated in cell growth assays. cDNA microarray screening revealed a set of calcitriol-regulated genes, of which two genes, namely prostate-derived factor (PDF) gene, and platelet-derived growth factor receptor β (PDGFRβ) gene, were chosen as being implicated in the antiproliferative action of calcitriol. PDF is a growth factor belonging to the transforming growth factor-β (TGF-β) superfamily. It was shown that calcitriol induced the expression of PDF gene and evidence was provided that the induction of PDF gene expression mediated the antiproliferative action of calcitriol in LNCaP cells. PDGFRβ is a transmembrane tyrosine-kinase receptor, which mediates the action of platelet-derived growth factor (PDGF), a member of the PDGF/VEGF superfamily. It was shown that calcitriol down-regulated the expression of PDGFRβ gene in LNCaP cells induced with epidermal growth factor (EGF) and in human prostate primary stromal cells. Calcitriol counteracted the cell growth-promoting effect of PDGF-BB isoform in stromal cells. Overall these results suggested two novel points of crosstalk between signaling pathways of vitamin D and growth factors of TGF-β and PDGF/VEGF superfamilies, further confirming the important role of vitamin D in growth factor signaling.

Crosstalk between calcitriol and androgens in regulating calcitriol catabolism and growth factor signaling in human prostate cancer LNCaP cells was studied further. In the early stages the development of prostate cancer is driven by androgens, steroid hormones, which are essential for the development and maintenance of adult prostate growth and differentiation. Calcitriol and androgen signaling pathways interact in regulating prostate cancer cell growth via mechanisms which are not completely understood. In this study crosstalk between calcitriol and 5α-dihydrotestosterone (DHT), the primary active androgen in human prostate, was investigated. It was demonstrated that DHT inhibited the activity of 24-hydroxylase, the key enzyme in calcitriol inactivation, by repressing calcitriol-mediated expression of 24-hydroxylase gene. Down-regulation of 24-hydroxylase gene by DHT slowed down the inactivation of calcitriol and maintained higher concentrations of calcitriol in culture medium, suggesting that DHT potentiated calcitriol action in androgen sensitive prostate cancer cells. On the other hand it was shown that the induction of PDF gene expression by calcitriol was affected neither by DHT nor by the antiandrogen Casodex. Taken together these data support the hypothesis that the antiproliferative action of calcitriol in prostate cancer cells is mediated via both androgen dependent and androgen independent mechanisms.

ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3, calcitriol
1α-hydroxylase	$25OHD_3$-1α-hydroxylase
24-hydroxylase	$25OHD_3$-24-hydroxylase
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25OHD_3$	25-hydroxyvitamin D_3, calcidiol
ActR	activin receptor
AF	activation function
Alk	activin receptor-like kinase
AR	androgen receptor
ATPase	adenosine triphosphatase
BMP	bone morphogenic protein
BmpR	bone morphogenic protein receptor
BPH	benign prostate hyperplasia
BSA	bovine serum albumin
C-terminus	carboxyl terminus
CaMK	Ca^{2+}-calmodulin-dependent protein kinase
CBP	cAMP-response element-binding protein
CDK	cyclin-dependent kinase
DBD	DNA-binding domain
DBP	vitamin D binding protein
DCC-FBS	dextran-treated charcoal-stripped fetal bovine serum
DHT	5α-dihydrotestosterone
DRIP	VDR-interacting protein
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
FBS	fetal bovine serum
GTPase	guanosine triphospatase
HAT	histone acetyl transferase
HDAC	hystone deacetylase
HPLC	high performance liquid chromatography
HrEGF	human recombinant epidermal growth factor
HrPDGF	human recombinant platelet-derived growth factor
IDBP	intracellular vitamin D binding protein
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
K_d	dissociation constant
LBD	ligand-binding domain
MAPK	mitogen-activated protein kinase
MAPKKK	MAPK kinase kinase
NCoR	nuclear receptor corepressor
N-terminus	amino terminus
NR	nuclear receptor
NSAID	non-steroid anti-inflammatory drug
nVDRE	negative vitamin D responsive element
OD	optical density

PBS	phosphate-buffered saline
PDF	prostate-derived factor
PI3K	phosphatidylinositol-3-kinase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PKA	protein kinase A
PKC	protein kinase C
PSA	prostate-specific antigen
PTH	parathyroid hormone
RAR	retinoic acid receptor
RPLP0	human acidic ribosomal phosphoprotein P0
RXR	retinoid X receptor
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH domain	src-homology domain
SMAD	mothers against decapentaplegic
SRC	steroid receptor coactivator
TBS	tris-HCl buffered saline
TGF-β	transforming growth factor-β
TβR	transforming growth factor-β receptor
TK domain	tyrosine kinase domain
UVB	ultraviolet light, type B (280 to 320 nm)
VDR	vitamin D receptor
VDRE	vitamin D-response element
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following articles, referred to in the text by their Roman numerals:

I. Nazarova N, Qiao S, Golovko O, Lou YR, Tuohimaa P (2004) Calcitriol-induced prostate-derived factor: autocrine control of prostate cancer cell growth. Int J Cancer 112(6):951-8.

II. Nazarova N, Golovko O, Bläuer M, Tuohimaa P (2005) Calcitriol inhibits growth response to platelet-derived growth factor-BB in human prostate cells. J Steroid Biochem Mol Biol 94(1-3):189-96

III. Lou YR, Nazarova N, Talonpoika R, Tuohimaa P (2005) 5alpha-dihydrotestosterone inhibits 1alpha,25-dihydroxyvitamin D_3-induced expression of CYP24 in human prostate cancer cells. Prostate 63(3):222-30.

IV*. Nazarova N.Iu, Chikhirzhina G.I, Tuohimaa P. (2006) Calcitriol induces transcription of the placental transforming growth factor β gene in prostate cancer cells via an androgen independent mechanism. Molecular Biology (Mosc) 40 (1):72-76

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* Placental transforming growth factor β in article IV refers to the same growth factor as prostate-derived factor in article I

INTRODUCTION

Prostate cancer is currently the most common form of cancer in the Western world. According to the Finnish Cancer Registry 4233 cases of prostate cancer were registered in Finland in 2003, comprising 33.9% of all cancer incidences. Moreover, the number of cases is most likely underestimated, since clinically silent prostate cancer is common. The incidence of prostate cancer shows a strong tendency to rise every year, and although in the majority of cases prostate cancer develops slowly, mortality from this disease is so considerable that it makes prostate cancer the second leading cause of cancer deaths in men. Chemotherapy with natural or artificial antineoplastic substances would make an attractive alternative to currently available strategies for prostate cancer treatment such as surgical techniques (prostatectomy), androgen ablation (by surgical removal of the testicles, by taking female sex hormones, or antiandrogens) and radiation therapy.

Despite the highly focused research in this field the etiology of prostate cancer remains largely unknown. Prostate cancer is a sporadic, heterogeneous and multi-stage disease, both at the clinical and histological level. The majority of prostate tumors (>95%) develop from the epithelial cells of the prostate gland and are classified as adenocarcinomas. The high incidence and long latency period of prostate cancer make it an excellent target for primary chemopreventive strategies and the identification of risk factors is of primary importance for chemoprevention. The pathogenesis of prostate cancer involves interplay between environmental and genetic factors and it is estimated that about 42% of the risk of prostate cancer may be inherited. Besides advanced age and black skin, that are established risk factors for prostate cancer, there are many putative risk factors, including nutritional factors, inflammation, obesity, physical activity, and others, whose role in prostate cancer etiology remain unclear. Currently the evidence is growing for a positive role of vitamin D substances in prostate cancer prevention and treatment.

Since its first publication in 1990 by Schwartz and Hulka in a paper entitled “Is vitamin D deficiency a risk factor for prostate cancer (Hypothesis)?” the idea of vitamin D involvement in the maintenance of normal prostate growth and differentiation gained a support from numerous epidemiological and experimental studies. Schwartz and Hulka suggested that vitamin D maintains the differentiated state of prostate cells and that vitamin D deficiency permits subclinical prostate cancer to progress to clinical disease. This hypothesis supported earlier findings on the antineoplastic action of vitamin D compounds in other cancer types such as colon and mammary gland cancer. Numerous *in vitro* studies reported the role of vitamin D metabolites in controlling prostate cancer cell proliferation, differentiation and apoptosis as well as such cancer-related processes as cell migration and angiogenesis. Unravelling of molecular mechanisms of action of vitamin D in prostate cells is important for the appropriate use of vitamin D metabolites and their analogs in prostate cancer prevention and anticancer therapy.

REVIEW OF THE LITERATURE

1. DISCOVERY AND CHEMISTRY OF VITAMIN D

Vitamin D was first discovered by Sir Edward Mellanby (Mellanby 1919) and McCollum (McCollum et al. 1922) as an essential nutritional substance present in cod liver oil and active in healing rickets. Later it was found that the substance is not actually a vitamin because vitamin D activity could be induced by irradiation of experimental animals or food with ultraviolet (Steenbock and Black 1924). At the same time it was noticed that rickets in children could be prevented and cured by exposing them to ultraviolet light (Chick et al. 1923). Later the two most abundant vitamin D forms were isolated and their structures were identified. Vitamin D_2, ergocalciferol, was isolated from irradiation of plant sterols (Askew et al. 1931, Windaus and Linsert 1928). Soon after 7-dehydrocholesterol, the precursor of vitamin D_3, was isolated from skin (Windaus et al. 1935) and converted to vitamin D_3, cholecalciferol. A Nobel Prize in chemistry was awarded to Windaus in 1938 for his contribution to the isolation, identification and chemical synthesis of vitamin D. By 1967 it became clear that vitamin D is converted to polar metabolites which are more biologically active and act more rapidly than vitamin D itself (Lund and DeLuca 1966, Morii et al. 1967). These metabolites, calcidiol (25-hydroxyvitamin D_3, $25OHD_3$) (Blunt and DeLuca 1969, Blunt et al. 1968) and calcitriol (1α,25-dihydroxyvitamin D_3, $1,25OH_2D_3$) (Holick et al. 1971, Lawson et al. 1969, Semmler et al. 1972) were isolated in pure forms, chemically identified and synthesized. Thus nowadays vitamin D_2 and vitamin D_3 are considered as steroid prohormones. Proof that calcitriol is the primary active metabolite of vitamin D was obtained from studies in anephric animals which responded to physiological doses of calcitriol by increasing intestinal absorption of calcium and bone calcium mobilization but did not respond to calcidiol (Boyle et al. 1972, Wong et al. 1972). Calcitriol is now generally accepted as the primary hormonally active form of vitamin D_3.

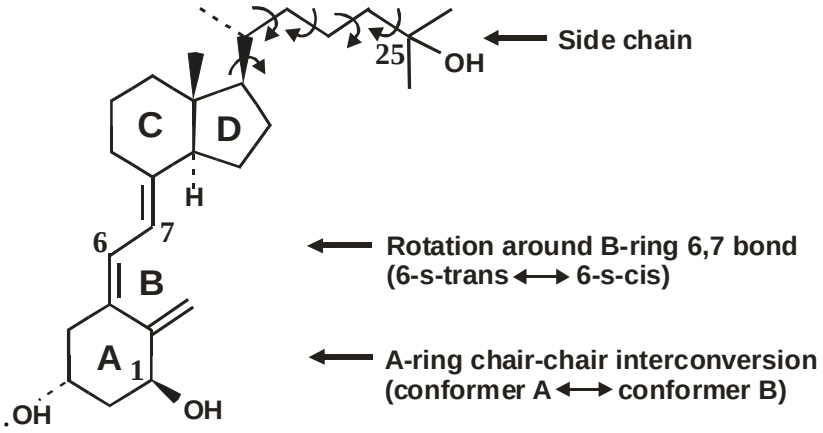

Figure 1. Scheme of calcitriol structure with the illustration of its conformational flexibility. The dynamic rotation of the cholesterol-like side chain of calcitriol with 360° rotations about the 5 C-C bonds are indicated by the curved arrow (modified from Norman et al. 1996).

The calcitriol molecule is unique in flexibility compared to other steroid hormones due to seco B-ring, which enables rotation about C6-C7 bond, and A-ring, which undergoes conformational chair-chair transitions (Norman et al. 1996). It was suggested that such structural flexibility enables calcitriol to adopt several functionally different conformations which preferentially bound to different transport and receptor proteins (Norman et al. 1996).

2. METABOLISM AND TRANSPORT OF VITAMIN D

The liver was found to be important for the production of calcidiol, the major circulating form of vitamin D_3 (Ponchon et al. 1969). Several hepatic cytochrome P-450s have been shown to 25-hydroxylate vitamin D compounds, but of these only CYP2R1 appears to be critical in vitamin D metabolism (Cheng et al. 2003, Cheng et al. 2004). The 25-hydroxylation is roughly regulated and thus the levels of circulatory calcidiol increase in proportion to vitamin D intake (Holick 1981). For this reason, plasma calcidiol level is commonly used as an indicator of vitamin D status. The major site of specific hydroxylation of calcidiol at the 1α-position is kidney (Fraser and Kodicek 1970). Renal 1α-hydroxylase activity, as opposed to hepatic 25-hydroxylase, is highly regulated to keep the potent activity of calcitriol in calcium homeostasis under strict control. Dietary calcium and phosphate are potent regulators of the enzyme activity. Calcium regulates the activity of 1α-hydroxylase both directly and indirectly by altering parathyroid hormone (PTH) levels (Omdahl et al. 1972). Dietary phosphate restriction increases 1α-hydroxylase gene expression (Shinki et al. 1997) and activity (Tanaka and Deluca 1973) indirectly, probably via regulation of so-called phosphaturic factors like Fibroblast growth factor 23 (Gutierrez et al. 2005, Perwad et al. 2005, Shimada et al. 2004), frizzled-related protein-4 (Berndt et al. 2003), and matrix extracellular phosphoglycoprotein (Rowe et al. 2000, Rowe et al. 2004). Another factor that appears to control renal production of calcitriol is protein encoded by *klotho* gene (Razzaque and Lanske 2006, Yoshida et al. 2002), which is a potent negative regulator of 1α-hydroxylase (Tsujikawa et al. 2003). Importantly, calcitriol restricts its own circulating level via feedback regulation that reduces the risk of vitamin D intoxication. This feedback regulation seems to be mediated via inhibition of PTH-induced 1α-hydroxylase gene expression or via activation of *klotho* gene expression (Brenza et al. 1998). In specific conditions such as pregnancy (Gray et al. 1979), chronic renal failure (Dusso et al. 1988, Zerwekh et al. 1983), tuberculosis (Cadranel et al. 1990), and rheumatoid arthritis (Shaw et al. 1994) extrarenally produced calcitriol can also contribute to circulating calcitriol level.

Nowadays it is clear that local synthesis of calcitriol (hydroxylation of calcidiol at 1α-position) occurs in an autocrine or paracrine fashion in many non-renal tissues, including prostate, breast, colon, lung pancreatic β cells, monocytes, keratinocytes, and parathyroid cells (Zehnder et al. 2001). The extrarenally produced calcitriol primarily serves as an autocrine/paracrine factor with cell-specific functions and the regulation of 1α-hydroxylase at extrarenal sites is different from that of renal enzyme. The rates of local synthesis and degradation of calcitriol are under the strict control of multiple cytokines and growth factors that optimize the levels of calcitriol for cell-specific functions via mechanisms incompletely understood. Separate regulation of circulating and local calcitriol production constitutes a double control over calcitriol levels in target tissues.

Inactivation of calcitriol occurs primarily in a series of oxidation reactions at carbon 24 and 23, leading to side chain cleavage, catalyzed by vitamin D 24-hydroxylase. 24-hydroxylase gene expression is regulated in a reciprocal manner to 1α-hydroxylase by phosphate (Tanaka and Deluca 1973, Wu et al. 1996), PTH (Henry and Norman 1984) and calcitriol (Armbrecht and Boltz 1991, Chen and DeLuca 1995, Ohyama et al. 1994).

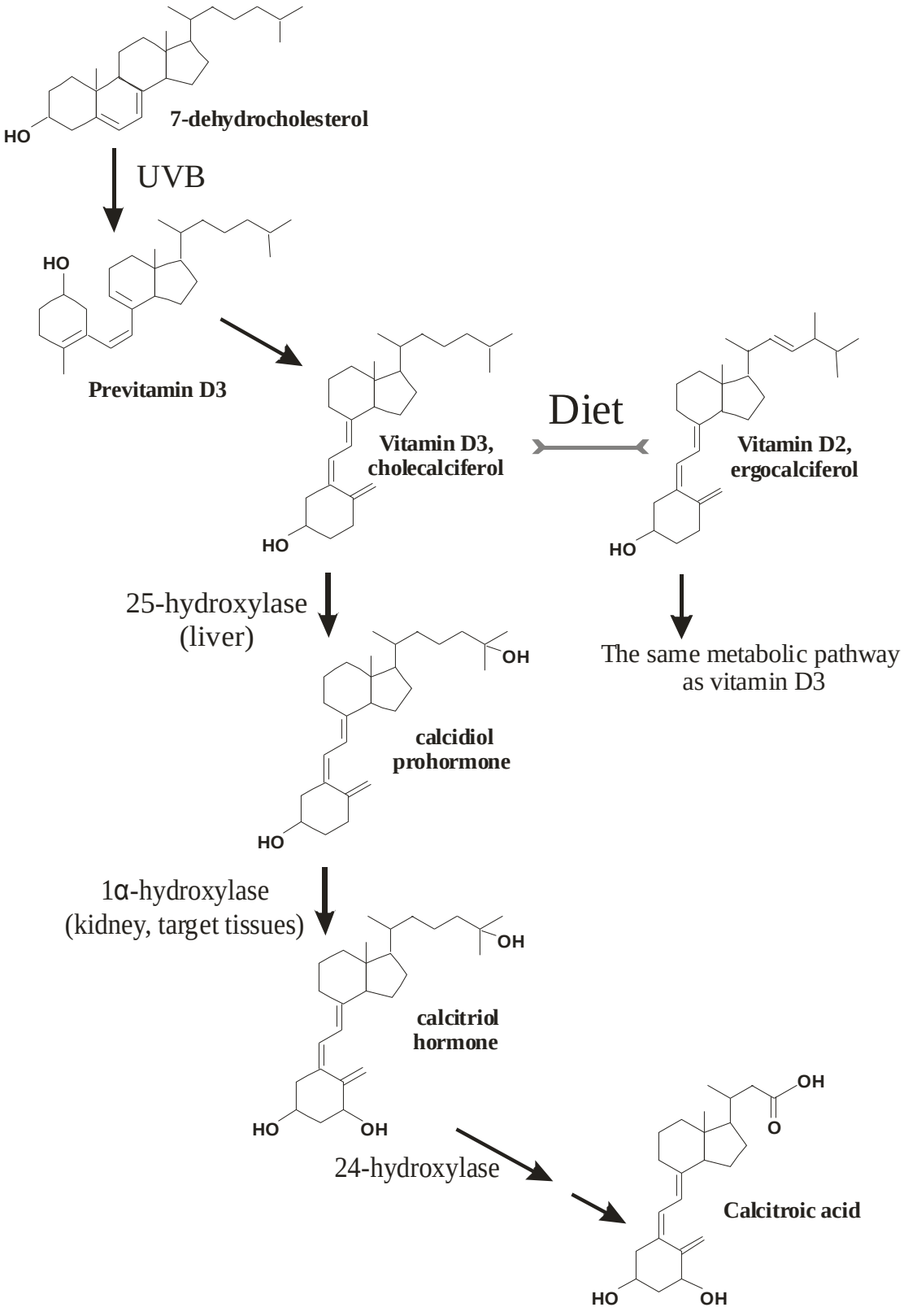

Figure 2. General scheme for vitamin D_3 metabolism. UVB catalyses conversion of skin cellular plasma membrane 7-dehydrocholesterol into provitamin D followed by its thermal isomerization into vitamin D_3 (cholecalciferol) (Holick et al. 1977, Okano et al. 1977). Vitamin D_2 and vitamin D_3 are the most abundant nutritional forms of vitamin D. Active metabolites of vitamin D, calcidiol and calcitriol are produced from cholecalciferol via consecutive hydroxylation cycles in liver and kidney. Inactivation of calcitriol occurs primarily in a series of oxidation reactions at carbon 24 and 23, leading to side chain cleavage and formation of biologically inert calcitroic acid.

In the circulation vitamin D metabolites are transported bound to plasma proteins. The most important carrier protein is so-called vitamin D binding protein (DBP), which was shown to bind the metabolites with high affinity in the order $25(OH)D_3$=24,25$(OH)_2D_3$>1,25$(OH)_2D_3$>vitamin D_3 (Cooke and Haddad 1989). In addition, some circulatory vitamin D metabolites are bound to albumin and lipoproteins (Fainaru and Silver 1979). Binding of vitamin D metabolites to DBP leads to their limited access to target cells, and therefore, to their longer circulating half-life (Cooke and Haddad 1989). Thus DBP appears to buffer the free levels of active vitamin D compounds, protecting against vitamin D intoxication (Bouillon et al. 1981). Only the small fraction of unbound metabolites passively enters target cells (Bikle and Gee 1989). Entry of calcidiol into the renal proximal tubule cells occurs by the megalin-mediated endocytosis (Nykjaer et al. 1999). DBP is degraded inside the cell and the released calcidiol is delivered to the 1α-hydroxylase by Intercellular vitamin D binding proteins (IDBPs) (Wu et al. 2002) or reenters the circulation bound to DBP (Dusso et al. 2005). IDBPs are homologs of heat shock protein shaperones Hsp70 (Gacad and Adams 1998). IDBP-1 and IDBP-3 directly interact with megalin (Adams et al. 2003) and 1α-hydroxylase and facilitate transport of calcidiol to the mitochondria to further hydroxylation and transport of calcitriol to the VDR (Wu et al. 2002). Stable transfection of IDBP-1 cDNA lead to up to 700-fold increase in calcitriol synthesis in cells lacking 24-hydroxylase and up to 70% in 24-hydroxylase containing cells (Brown et al. 2002).

3. MOLECULAR MECHANISMS OF VITAMIN D ACTION

3.1. Regulation of gene expression

3.1.1. VDR

The action of calcitriol is mediated primarily via binding to nuclear vitamin D receptor (VDR), which is ligand-dependent transcription factor belonging to superfamily of nuclear receptors for steroid and thyroid hormones (NR). Calcitriol binds to VDR with high affinity ($K_d = 10^{-10}$ to 10^{-11} M) (Mellon and DeLuca 1979) in the cytoplasm of target cells. OH groups in calcitriol molecule are crucial in binding to VDR (Okano et al. 1998). Thus calcidiol and 24,25$(OH)_2D_3$ bind to VDR nearly 100 times less avidly than calcitriol (Mellon and DeLuca 1979). In addition, other domains of the VDR are important for binding to VDR, as shown by their capacity to compensate for the lack of functional 1α-hydroxyl group (Peleg et al. 1996).

3.1.1.1. Structure

Human VDR is 48 kDA phosphoprotein characterized by modular structure common for the receptors of NR superfamily (Vegeto et al. 1993). VDR is encoded by a single gene in chromosome 12 of human genome and unlike other NRs is presented in a single isoform in human cells (Szpirer et al. 1991). Alhough several mRNA transcripts for VDR can be identified which differ in 5'-end sequences, the majority of them are translated into the same 427 amino acid protein (Miyamoto et al. 1997). VDR molecule can be subdivided into several functional domains (Figure 3).

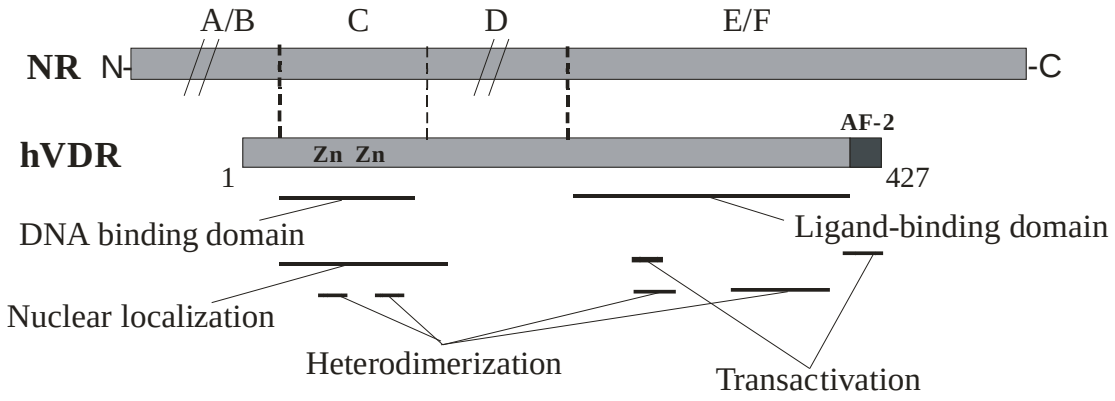

Figure 3. Schematic representation of functional domains of the human VDR molecule. Sites responsible for ligand binding, heterodimerization with RXR, nuclear localization, binding to DNA and transactivation are indicated.

A/B domain, which is highly variable among NRs and contains Activation Function-1 (AF-1) motif responsible for autonomous transactivatition is abbreviated in VDR molecule. It is short compared to other NRs and disabled (Pike and Shevde 2005). *C domain* or DNA-binding domain (DBD) is highly conservative among NRs and contains two type C2-C2 zinc fingers (Berg 1988, Berg 1989). VDR DBD binds to vitamin D responsive elements (VDRE) in DNA major groove. In addition it participates in dimerisation and blocks binding to noncorrectly spaced VDRE half sites (Umesono and Evans 1989). A role of DBD in nuclear accumulation of VDR was also suggested (Luo et al. 1994). *D domain* comprises a highly flexible hinge region which is variable in NRs (Baker et al. 1988, Burmester et al. 1988). The hinge region in VDR forms a long helix which links DNA-binding and hormone-binding components of the receptor and provides plasticity to its structure (McDonnell et al. 1989, Shaffer et al. 2005). *E/F domain* is moderately variable in sequence but highly conservative in tertiary structure between NRs. It contains ligand-binding domain (LBD) (McDonnell et al. 1989) consisting of 12 α-helixes and β-turn organised in an antiparallel sandwich. At the C-terminal of the E/F domain there is the so-called activation function-2 (AF-2) motif made of short amphipatic α helix. Importantly, this region serves as a highly complex protein-protein interface for a variety of cofactors that are integral to receptor activity (Jin and Pike 1996, Jin et al. 1996, MacDonald et al. 1995) and participates in dimerization with RXR. VDR molecule is unique among other NRs in having a long insertion between D and E/F domains endoded by a specific exon. This segment is sometimes regarded as belonging to D domain (Pike and Shevde 2005) or alternatively to E/F domain (Shaffer et al. 2005) and the role of this insertion in ligand binding and transactivation is not quite clear.

On the basis of X-ray crystallographic data (Rochel et al. 2000) it was accepted that ligand binding domain of VDR (VDR LBD) may adopt 3 different conformations dependent on ligand binding: apoVDR, holoVDR with agonist, holoVDR with antagonist (Moras and Gronemeyer 1998, Nayeri and Carlberg 1997). Upon ligand binding, helix 12 in the C-terminus of the VDR LBD, known as AF2, imparts a major conformational change in the three-dimensional structure of the VDR (Norman et al. 1999, Vaisanen et al. 2002) the same way as in the case of other NR LBDs (Renaud et al. 1995). Helix 12 covers LBD and locks the agonist in the ligand-binding pocket (Swamy et al. 2000). This transition is accompanied by structural changes in helices 3,6,11 and 12 leading to the release of corepressors and recruitment of activators

(Hashimoto and Miyachi 2005, Herdick and Carlberg 2000, Lempiainen et al. 2005, Murayama et al. 2004). This activation step appears to be required for the recruitment by the VDR of motor proteins (Racz and Barsony 1999), responsible for a rapid translocation of cytoplasmic VDR to the nucleus along microtubules (Barsony and McKoy 1992). Binding of antagonist places helix 12 into an incorrect position which hinders binding of coactivators and thus prevents transactivation. The association of VDR with RXR involves dimerization surfaces in several different domains of the VDR molecule and induces a VDR conformation that is essential for VDR transactivating function (Hsieh et al. 1995, Whitfield et al. 1996).

In the human population there are many allelic variants of the VDR gene (Koshiyama et al. 1995, Morrison et al. 1994) with considerable differences between races and ethnic groups (Nelson et al. 2000, Ojwang et al. 2001, Uitterlinden et al. 2004a). Importantly, the expression of different allelic variants was shown to be associated with decreased bone density (Eisman 1999), propensity to hyperparathyroidism (Carling et al. 1995), resistance to vitamin D therapy (Kontula et al. 1997), and susceptibility to infections, autoimmune diseases and cancer (Uitterlinden et al. 2004b). A number of functional polymorphisms were identified that may affect VDR mRNA stability and transactivation potency (Morrison et al. 1994).

3.1.1.2. Heterodimerization and nuclear translocation

The predominant form of VDR-containing transcription factor is heterodimer of VDR and retinoic X receptor (RXR). Ligand binding causes VDR hyperphosphorylation at several serine residues, which is mediated by various kinases including casein kinase II (Jurutka et al. 1993, Jurutka et al. 1996), PKC (Hsieh et al. 1991), and PKA (Hsieh et al. 1991) with diverse effects on transcriptional activity. Thus genomic action of VDR could be modulated by other hormonal systems activating protein kinase cascades and also by rapid nongenomic effects of vitamin D compounds (discussed in Section 3.2.). Hyperphosphorylation of VDR induces its heterodimerization with either α, β or χ isoforms of retinoic X receptor (RXR) (Kephart et al. 1996, Yu et al. 1991). The preferential heterodimerization partner seems to be defined by chromatin structure in the regulatory region of the target gene. In addition, the presence of VDR heterodimers with thyroid hormone receptors (TR) (Schrader et al. 1994) and retinoic A receptor (RAR) (Schrader et al. 1993) and also VDR homodimers (Cheskis and Freedman 1994, Nishikawa et al. 1994) *in vitro* were reported but the role of these dimers in transcription *in vivo* is not known. It was shown that VDR homodimer is able to regulate transcription at least *in vitro* (Kahlen and Carlberg 1994).

It was demonstrated that in the absence of ligand VDR is approximately evenly distributed between the cytoplasm and the nucleus and is constantly shuttling between them as the weak interaction between VDR and nuclear pore importin α does not provide effective VDR translocation into the nucleus. By contrast ligand-free RXR is localized mainly in the nucleus due to a high affinity of RXR to nuclear pore β importin (Yasmin et al. 2005). Conformational changes in VDR mediated by ligand binding and hyperphosphorylation open a latent nuclear localization signal in the VDR molecule, which directs massive VDR translocation into the nucleus. Translocation of VDR-RXR heterodimers is controlled primarily by vitamin D because dimerization inhibits the ability of RXR to bind importin β and heterodimer is recruited to nuclear pore via binding between liganded VDR and importin α (Yasmin et al. 2005).

3.1.2. VDRE

The dimer of VDR with RXR or other partner recognises DNA sequences named Vitamin D responsive elements (VDRE) in the regulatory regions of many genes. Most common VDRE is a direct repeat of two hexameric A/GGG/TTCA sequences intercepted by a spacer of three nucleotides (DR3) (Noda et al. 1990). This sequence directs the VDR-RXR heterodimer so that the RXR occupies the 5'-half-site and the VDR binds to 3'-half-site (Jin and Pike 1996). Other VDRE types vary in spacer length and direction of repeats so that DR4 is a direct repeat intercepted by 4 nucleotides (Yen et al. 1996), DR6 by 6 nucleotides (Kahlen and Carlberg 1994), ER6 (Thummel et al. 2001) consists of two inverted palindromic sequences separated by 6 nucleotides. Usually there are several VDREs in the regulatory regions of vitamin D target genes. For example there are four VDREs identified in the regulatory regions of CYP24 (Vaisanen et al. 2005) and cyclin C genes (Sinkkonen et al. 2005), three VDREs in the regulatory regions of IGFBP1, IGFBP3 and IGFBP5 genes (Matilainen et al. 2005) and at least three VDREs in the regulatory region of p21 gene (Saramaki et al. 2006). At least two composite VDREs were identified in the regulatory region of human TRPV6 gene encoding the epithelial calcium channel responsible for calcium uptake in the intestine (Meyer et al. 2006). Because each VDRE is able to provide transactivation by itself it was suggested that multiple VDREs increase the efficacy and the accuracy of transactivation. The level of direct transactivation by calcitriol often does not exceed 3 fold because the majority of vitamin D target genes are transcribed under the control of other transcription factors. There are a few well known exceptions, the most prominent of which is the CYP24 gene, which can be induced more than 400 fold by calcitriol (Lou et al. 2005, Sinkkonen et al. 2005). Two main reasons account for such a high efficiency of CYP24 gene transactivation. First RXR-VDR dimer is the primary transcription regulator for CYP24 gene encoding 24-hydroxylase which catalyses the key step in vitamin D inactivation loop. Another peculiarity of CYP24 gene is a very low basal transcription level compared to basal transcription levels of other vitamin D target genes such as cyclin C, PPARδ and p21 (Dunlop et al. 2005), which are maintained by the other transcription factors. The induced transcription level when measured against low basal transcription level makes a higher ratio.

Transrepression by steroid hormones has been less investigated than transactivation and the structure of negative VDRE (nVDRE) is not clear. According to one of the current models repression is mediated by binding of VDR-RXR dimer to the classical positive VDRE and recruitment of a specific corepressor complex. This hypothesis is supported by identification of several classical VDRE-like nVDRE and the finding that point mutations in nVDRE may turn it positive (Koszewski et al. 1999). The second model postulates specific nVDRE structure and is supported by the identification of nVDRE not containing classical A/GGG/TTCA consensus sequence. For example, nVDRE found in 1α-hydroxylase gene by Murayama and coauthors (Murayama et al. 2004) and thus named 1αnVDRE consists of two E-box – like motives. VDR does not directly bind 1αnVDRE and binding is mediated by a so-called VDR interacting repressor (VDIR). In the absence of calcitriol VDIR activated by protein kinase A recruits p300 family HAT coactivators to induce 1α-hydroxylase transcription. Calcitriol causes the release of coactivators and recruitment of HDAC and Sin3A corepressors leading to transrepression. Both models postulate coregulator switch as the basis for transrepression. The switch from VDR transrepression to activation of the avian PTH gene, induced by changing two bases in the 5'-element in the DR3, suggested that a changed polarity of the VDR/RXR-VDRE complex, with the

VDR occupying the 5'-half-site, may contribute to transrepression (Koszewski et al. 1999).

3.1.3. Cofactors

Chromatin remodeling complexes enable NRs to recognize hormone-responsive elements (HREs) in the regulatory regions of target genes (Dilworth and Chambon 2001). These multimeric complexes use ATP hydrolysis energy to locally disrupt nucleosome arrays (Johnson et al. 2005). Multisubunit chromatin-remodeling complex associated with VDR-mediated transactivation/transrepression was identified in 2003 and named WINAC (WSTF Including Nucleosome Assembly Complex) (Kitagawa et al. 2003). It belongs to SWI/SNF class complexes and contains SWI/SNF identical subunits BRG1 or hBRM as the ATPases. Because WINAC is recruited to both positive and negative VDRE independently of ligand binding (Kato et al. 2004) it was suggested that peculiarities of individual VDRE sequences serve as the allosteric regulators of VDR conformation leading to the recruitment of coactivators of corepressors which in its turn direct WINAC action. Two domains of VDR serve as adaptor surfaces for nuclear coregulators (Carlberg 2004). Nuclear coactivators act synergistically with the VDR to amplify its transactivating potency. Many VDR-coactivators such as SRC family proteins and CBP/p300 possessing histone acetyl transferase activity (HAT) destabilise nucleosomes by acetylation of histone N-tails lysine residues in the regulatory regions of target genes (Kim et al. 2005). This allows recruitment of a second complex of coactivators, the DRIP (VDR-interacting proteins) complex. DRIP consisting of approximately 20 proteins was identified as complex sufficient for ligand-dependent transactivation by VDR-RXR heterodimer *in vitro* (Rachez et al. 1998) and turned out to be similar/identical to SMCC/TRAP/ARC/CRSP/NAT/Mediator complexes mediating transactivation by the other NRs. DRIP205 subunit binds AF-2 motif of VDR (Rachez et al. 2000). DRIP complex serves as a bridge between the VDR and the basal transcriptional machinery of RNA polymerase II, which favours the assembly of the preinitiation complex to potentiate VDR-mediated transactivation (Jurutka et al. 2001, Rachez and Freedman 2000). In VDR-mediated transrepression binding of VDR-RXR to a negative VDRE recruits corepressors of the family of histone deacetylases (HDACs) such as NCoR-1/RIP13, NCoR-2/SMRT/TRAC2 and Hairless/TRIP8/KIAA1380, which deacetylate histone N-tails lysine residues followed by chromatin condensation and gene silencing (Banwell et al. 2003, Hsieh et al. 2003, Polly et al. 2000). Recent studies suggest a bifunctional role for the VDR comodulator NCoA62/Skip, which can promote transcriptional activation or repression mediated by VDR in a cell-specific manner, depending on the expression of coregulator molecules (Leong et al. 2004). The coactivators CBP/p300 and the corepressors NCoR and SMRT interact with the same N-terminal region of Skip molecule and the relative expression levels of these cofactors dictate whether Skip activates or represses VDR-dependent transcription.

It was hypothesized that changes in the balance of coactivators and corepressors provide epigenetic control over vitamin D target gene expression. Overexpression of corepressor genes blocks VDR-mediated transactivation and causes insensitivity to antiproliproliferative action of vitamin D. Indeed, incubation of PC-3, DU145 and LNCaP cells with combination of calcitriol and HDAC inhibitors (trichostatin A or Na butirate) remarkably increases the antiproliferative action of calcitriol (Banwell et al. 2003, Gommersall et al. 2004). Interestingly calcitriol by itself induces expression of genes of two important coregulator families, TIF2 coactivator and SMRT corepressor that suggest that vitamin D by itself could modulate the transcriptional competence of target cells (Dunlop et al. 2004).

3.2. Rapid nongenomic action

Vitamin D metabolites, like other steroid hormones, may induce rapid responses independent of gene expression. It was reported that within minutes calcitriol can stimulate phosphoinositide metabolism (Bourdeau et al. 1990, Lieberherr et al. 1989), cytosolic calcium levels (Hruska et al. 1988, Lieberherr 1987, Sugimoto et al. 1988), cGMP levels (Guillemant and Guillemant 1980, Vesely and Juan 1984), PKC (Sylvia et al. 1996), MAP kinases (Beno et al. 1995, Song et al. 1998), the opening of chloride channels (Zanello and Norman 1996) and some other pathways. These effects are presumably mediated via membrane vitamin D receptor whose nature, however, remains controversial. At least two distinct candidate membrane proteins have been identified, namely, rapid-response steroid-binding protein ($1,25D_3$-MARRS) from chick intestinal basolateral membranes (Nemere et al. 1994, Nemere et al. 1998) and annexin II from plasma membranes of ROS 24/1 rat osteosarcoma cells (Baran et al. 2000). The role of nongenomic actions remains unknown. Nongenomic effects, at least stimulation of protein kinases, were suggested to modulate the genomic actions of calcitriol. However, this remains controversial since there are studies demonstrating that rapid actions may not be critical for calcitriol-mediated regulation of gene expression.

4. OVERVIEW OF THE BIOLOGICAL FUNCTIONS OF VITAMIN D

Originally vitamin D refers to a substance with antirachitic properties, and the first discovered, *classic functions* of vitamin D are in maintaining calcium level in extracellular fluid for normal cellular physiology and skeletal integrity. It is now clear that vitamin D endocrine system comprises an essential component in crosstalk between the kidney, bone, parathyroid gland, and intestine maintaining calcium and phosporus homeostasis. It is accepted that the direct and primary action of calcitriol is in stimulation of active calcium uptake and mechanisms of calcium and phosphate transport in *intestine*. Calcitriol stimulates active calcium uptake and transport mechanisms primarily via the induction of gene expression of the epithelial calcium channels TRPV6 (transient receptor potential vanilloid type 6) and TRPV5 (van Abel et al. 2003). In addition, calcitriol increases the active transport of phosphate through induction of gene expression of the Na-P_i cotransporter (Tatsumi et al. 1998) and changes in the composition of the enterocyte plasma membrane (Putkey et al. 1982). The essential role of vitamin D metabolites in the regulation of growth, maturation and remodeling of bones has been questioned due to the latest results obtained in mice lacking vitamin D receptor (VDR), the major mediator of calcitriol action. It was reported that the institution of a high-calcium, high-phosphorus, lactose-supplemented diet prevented abnormalities in mineral ion homeostasis of VDR knockout mice (Li et al. 1998), suggesting that the *skeletal* effects of vitamin D metabolites are indirect and reflect their actions in the intestine. However this conclusion remains controversial since other studies showed that normalization of serum calcium cannot entirely substitute for defective calcitriol-VDR signaling in skeletal homeostasis (Panda et al. 2004). At least cartilage growth plate development and osteoclastic bone resorption demanded calcitriol action. Calcitriol-VDR system was shown to be critical for the normal coupling of bone remodeling (both osteogenesis and osteoclastogenesis) (Panda et al. 2004). The functioning of *parathyroid gland* is under the potent control of calcitriol and vitamin D deficiency leads to parathyroid hyperplasia and increased synthesis and secretion of parathyroid hormone (PTH). The effect of calcitriol is mediated primarily via direct transrepression of PTH gene (Okazaki et al. 1988, Silver

et al. 1985). In addition, calcitriol regulates the parathyroid gland response to calcium via direct transactivation of CaSR (calcium-sensing receptor) gene (Brown et al. 1996). Calcitriol-mediated inhibition of parathyroid cell growth involves the inhibition of TGFα/EGFR signaling and induction of gene expression for cyclin-dependent kinase inhibitors p21 and p27 (Cozzolino et al. 2001, Tokumoto et al. 2002). However, the functions of calcitriol in parathyroid gland seem not to be essential but cooperative with calcium action since high calcium rescue diet corrects the high PTH level in VDR knockout mice (Panda et al. 2004). Finally, calcitriol stimulates reabsorption of calcium and phosphate, and calbindin gene expression in *kidney* (Friedman and Gesek 1993). The importance of these effects also remains controversial due to the simultaneous effects of calcitriol on serum PTH and on intestine absorption of calcium and phosphorus, which potently affect the renal load of these ions.

Nonclassic actions. Since the discovery of nuclear vitamin D receptor, which could be detected in a majority of tissues of the body, a number of new "non-classic" actions of vitamin D metabolites was revealed. VDR was discovered in the tissues of pancreas (Pike et al. 1980), placenta (Pike et al. 1980), pituitary (Haussler et al. 1980), ovary (Dokoh et al. 1983), testis (Merke et al. 1983), mammary gland (Colston et al. 1980), heart (Walters et al. 1986), prostate (Miller et al. 1992), stomach (Stumpf et al. 1979), skin (Stumpf et al. 1979), and other tissues even including some neurons in the brain (Stumpf et al. 1982). Compelling genetic, epidemiological and nutritional evidence linked defects in vitamin D endocrine system with disorders unrelated to calcium homeostasis, such as hypertension (Li 2003, Li et al. 2002) and disturbed muscle function (Boland 1986, Endo et al. 2003), susceptibility to infections (Hayes et al. 2003), autoimmune diseases (Hayes et al. 2003), and cancer (Garland et al. 2006, Holick 2006). It was observed that the extensive number of cultured cell lines including cells of fibroblastic, chondrocytic, osteoblastic, myoblastic, hematopoietic, lymphopoietic origins and other cell types responded to vitamin D metabolites. Importantly calcitriol caused marked inhibition and induced terminal differentiation of VDR-expressing cells of tumorigenic origin including prostate, colon, breast, lung and melanoma via cell specific mechanisms and also induced apoptosis in a variety of cell types. This action highlights a potential therapeutic role for vitamin D metabolites and analogs in cancer treatment and in the regulation of immune system. Antiproliferative action of vitamin D compounds is currently used in the treatment of psoriasis (a hyperproliferative disorder of the skin) (Berth-Jones and Hutchinson 1992, Kragballe 1992).

5. PROSTATE AS A VITAMIN D TARGET ORGAN

5.1. Historical perspective. Epidemiological studies

It was hypothesized in 1990 that vitamin D deficiency underlies the major prostate cancer risk factors such as advanced age, black skin and residing in Northern latitudes (Schwartz and Hulka 1990), which were earlier suggested as the risk factors in epidemiological studies (Dayal et al. 1985, Gleason 1988, Ross et al. 1987, Walker 1986). All these risk factors are associated with low availability of sunshine UVB, a crucial factor determining the amount of circulating active vitamin D metabolites. The low degree of sun exposure in Northern latitudes does not provide enough UVB for the effective synthesis of vitamin D. Black skin pigment melanin competes with 7-dehydrocholesterol for UVB and thus reduces the rate of vitamin D production (Holick et al. 1981). High prevalence of vitamin D deficiency in elderly people is due to less sun exposure and thinner epidermis, which contains less 7-dehydrocholesterol.

Vitamin D deficiency is common among the elderly worldwide, especially among house-bound persons and geriatric populations (Dunnigan et al. 1986, Slovik 1983). This hypothesis is supported by the low risk of prostate cancer among Japanese living in Japan (Hirayama 1979) due to the extremely high serum calcidiol levels (Nakamura et al. 2000, Nakamura et al. 1999) which reflect the traditional diet high in oily fish.

Schwartz and Hulka suggested that vitamin D maintains the differentiated state of prostate cells and that vitamin D deficiency permits subclinical prostate cancer to progress to clinical disease (Schwartz and Hulka 1990). This hypothesis supported earlier reports on antineoplastic vitamin D action in other types of cancer such as colon and mammary gland cancer (Garland et al. 1989) and stimulated research in the field of vitamin D action in prostate. Although there are some inconsistencies in the epidemiological studies, the results of the large seroepidemiologic nested case-control studies support the hypothesis that vitamin D insufficiency is casually related to prostate cancer. In 1993 Corder and colleagues showed an association between low serum calcitriol level and incidence of palpable and anaplastic prostate tumors (Corder et al. 1993). Subsequently results of several small seroepidemiologic studies were reported which failed to find any association between prostate cancer risk and circulating vitamin D metabolites (Braun et al. 1995, Gann et al. 1996, Nomura et al. 1998). However in 2000 Ahonen et al. (Ahonen et al. 2000) in a large nested case-control study conducted on 19 000 middle-aged Finnish men among whom 149 cases of prostate cancer were diagnosed in a 13-year follow-up found that low plasma calcidiol level was associated with early incidence and fast progression of prostate cancer. Subsequently Tuohimaa et al. (Tuohimaa et al. 2004) in a large nested case-control study conducted among Nordic men (Norway, Finland and Sweden) demonstrated that both low and high serum calcidiol levels were associated with higher prostate cancer risk.

Ma et al. (Ma et al. 1998) in a large study (among 372 prostate cancer cases and 591 controls) found no association between VDR polymorphism and prostate cancer risk. However, in an analysis restricted to men with low plasma calcidiol level (plasma calcidiol levels below the median) the association was significant. Numerous other studies have shown significant associations between prostate cancer and VDR polymorphism (Correa-Cerro et al. 1999, Habuchi et al. 2000, Hamasaki et al. 2001, Hamasaki et al. 2002, Ingles et al. 1997, Ingles et al. 1998, Medeiros et al. 2002, Taylor et al. 1996, Xu et al. 2003) and a similar number of studies have reported lack of association (Blazer et al. 2000, Chokkalingam et al. 2001, Furuya et al. 1999, Gsur et al. 2002, Kibel et al. 1998, Suzuki et al. 2003, Tayeb et al. 2003, Watanabe et al. 1999). Because most studies of VDR polymorphisms have been conducted in the absence of data on serum levels of vitamin D metabolites it was suggested that VDR polymorphism may be a risk factor only in combination with low serum calcidiol level.

Several epidemiological studies have examined the risk of prostate cancer in relation to exposure to UV radiation. Importantly Bodiwala et al. showed that the exposure to UV radiation is inversely associated with a risk of prostate cancer (Bodiwala et al. 2003). John et al. also demonstrated that residence in latitudes with high UV radiation and high solar radiation in the state of birth is associated with reductions in the risk of prostate cancer (John et al. 2004).

5.2. Intraprostatic vitamin D metabolism

Expression of 1α-hydroxylase in human prostate was demonstrated in 1998 suggesting the existence of the autocrine loop of calcitriol action in prostate cells

(Schwartz et al. 1998). It was hypothesized that a balance between 1α-hydroxylase and 24-hydroxylase is crucial in prostate cancer progression and is disrupted in prostate cancer cells. Indeed, it was shown that 1α-hydroxylase expression is usually down-regulated while 24-hydroxylase expression is up-regulated in prostate cancer cells (LNCaP, DU145) compared to normal cells (PZHPV-7, PNT-2), which may lead to lack of calcitriol in cancer and take cells out of its growth control (Khorchide et al. 2005).

In addition to regulation of the activity of cytochrome P450 enzymes local calcitriol synthesis and activity are also controlled by transport proteins such as IDBP-1 (discussed in Section 2).

5.3. VDR gene expression in the prostate

The presence of VDR in human prostate was revealed in 1992 (Miller et al. 1992). Later VDR was found in various types of cultured prostate stromal and epithelial, normal, cancer and benign prostate hyperplasia (BPH) cells and different cell lines including LNCaP, PC-3, DU145 (Peehl et al. 1994, Skowronski et al. 1993). However according to the immunohistochemistry data there are significant variations in individual VDR levels in patient prostate samples (Kivineva et al. 1998). The intracellular levels of VDR in a target cell are regulated by VDR ligands and other hormones and growth factors that do not bind to the VDR. It was shown that calcitriol increases VDR protein level in virtually all tissues including prostate, probably via ligand-dependent stabilization of the VDR from proteosomal degradation (Li et al. 1999).

5.4. Action of vitamin D metabolites in prostate cancer cells

5.4.1. Antiproliferative

Vitamin D metabolites inhibit the growth of normal and malignant prostate cells as was demonstrated on primary cultures, benign prostate hyperplasia (BPH), prostate cancer cell lines, xenograph models and *in vivo* on rat prostate (Peehl et al. 1994, Skowronski et al. 1995). It was shown that systematic administration of calcitriol to rats for 3 weeks led to a 40% decrease in prostate size due to loss of both epithelial and stromal cells (Getzenberg et al. 1997, Konety et al. 1996). Calcitriol inhibits the growth of prostate cancer cells to different extent depending on the cell line. It was found that calcitriol dramatically inhibits proliferation of LNCaP cells and to a lesser extent of PC-3 cells (Skowronski et al. 1993). DU145 cells are not sensitive to the antiproliferative effect of calcitriol (Skowronski et al. 1993). Among less widely used cell lines calcitriol inhibits growth of ALVA 31 (Hedlund et al. 1996a), PPC-1 (Yu et al. 1998), MDA PCa (Zhao et al. 2000) to different extent and does not affect JCA-1 cell growth (Hedlund et al. 1996b). Molecular mechanisms of growth suppression by vitamin D compounds are cell specific. The key step in the antiproliferative action of vitamin D metabolites in a variety of cell types including LNCaP cells is cell cycle arrest followed by accumulation of cells in G0-G1 stage (Blutt et al. 1997, Zhuang and Burnstein 1998). As a rule cell cycle arrest is associated with the induction of cyclin-dependent kinase (CDK) inhibitors p21 and p27 and insulin-like growth factor binding proteins IGFBP-3 and IGFBP-5 gene expression accompanied by inhibition of cyclin-dependent kinase CDK2, CDK4 and/or CDK6 activity (Johnson et al. 2002). For example, in LNCaP cells calcitriol induces p21 gene expression leading to a decrease in CDK2 activity and subsequent retinoblastoma (Rb) protein dephosphorylation followed by transcription factor E2F repression which cause

cell cycle arrest (Zhuang and Burnstein 1998). It is noteworthy that although there are several VDREs in the regulatory region of p21 gene (Saramaki et al. 2006), calcitriol does not directly induce p21 transcription in LNCaP cells but induces it indirectly via the induction of Insulin-like growth factor binding protein-3 (IGFBP-3). IGFBP-3 neutralizing antibodies prevent induction of p21 gene expression by calcitriol and calcitriol-mediated inhibition of cell growth (Boyle et al. 2001).

5.4.2. Prodifferentiative

Although it was hypothesized as early as in 1990 that vitamin D promotes prostate cancer cell differentiation (Schwartz and Hulka 1990) nowadays there are contradictory data on the role of vitamin D compounds in prostate cell differentiation. It was shown that calcitriol does indeed induce gene expression of such prostate epithelium differentiation markers as prostate specific antigen (PSA) (Feldman et al. 1995) and E-cadherin (Campbell et al. 1997) in LNCaP and PC-3 prostate cancer cells. In rat prostate epithelial NRP-152 cells calcitriol induces gene expression for transforming growth factors (TGF-β2 and TGF-β3) followed by induction of such differentiation markers as fibronectin and trombospondin (Danielpour 1996). This hypothesis is supported by the stable and irreversible character of calcitriol-induced cell cycle arrest. However Peehl et al. reported no changes in cell morphology and cytokeratin gene expression pattern in calcitriol-treated prostate primary epithelial cells (Peehl et al. 1994).

It was shown that induction of PSA gene expression by vitamin D metabolites is mediated through activation of androgen signaling (Peehl et al. 1994). Androgens are potent differentiation agents for prostate cells (Wilson 1972). Indeed, the synergistic action of calcitriol and DHT was demonstrated on the induction of PSA gene expression which was mediated via induction of AR gene expression by calcitriol. Data supporting this hypothesis were obtained from an *in vivo* study on castrated rats, where the highest level of prostate epithelium differentiation could be seen in animals to whom a combination of calcitriol and testosterone was administered and not calcitriol or testosterone alone (Konety et al. 1996).

5.4.3. Proapoptotic

Vitamin D compounds induce apoptosis in different prostate cancer cell types (Guzey et al. 2002, Hsieh and Wu 1997). Molecular mechanisms of calcitriol–induced apoptosis are thought to be cell type specific, which explains highly variable extent of calcitriol-induced apoptosis. Induction of apoptosis by calcitriol in LNCaP cells is accompanied by inhibition of gene expression for such antiapoptotic proteins as Bcl-2 and Bcl-X_L (Blutt et al. 2000). These proteins protect cells from apoptosis by blocking cytochrome C release from mitochondria causing caspase-3 and caspase-9 activation initiating mitochondrial apoptotic reaction cascade (Tsujimoto 1998). Calcitriol does not cause apoptosis in LNCaP cells overexpressing Bcl-2 but still inhibits proliferation of these cells that further support the hypothesis of pleiotropic nature of its growth inhibitory effect (Blutt et al. 2000). Calcitriol also down-regulates gene expression of other antiapoptotic proteins such as Mcl-1, BAG1L, XIAP, cIAP1 and cIAP2 (Blutt et al. 2000).

5.4.4. Antiangiogenic and antiinvasive

Angiogenesis plays a vital role in tumor growth and progression as soon as a tumor cannot progress beyond 2-5 mm in diameter without procuring its own blood

supply (Nicholson and Theodorescu 2004, Weidner 1993). Tumor vasculature is distinct from normal vessels having structural irregularity and increased leakiness. Calcitriol inhibits tumor angiogenesis, as was demonstrated both *in vitro* and *in vivo* (Majewski et al. 1996, Mantell et al. 2000). Impaired angiogenesis leads to hypoxia of prostate cancer cells and arrest of tumor growth. Calcitriol also inhibits such cancer related processes as cell adhesion and migration and thus decreases the invasive potential of prostate cancer cells as was demonstrated in a number of *in vitro* studies (Donald et al. 1998, Schwartz et al. 1997, Sung and Feldman 2000, Tokar and Webber 2005). Recently in an *in vitro* cell invasion assay it was demonstrated that inhibition of the invasive ability of human prostate cancer cell lines, LNCaP, PC-3 and DU145 by calcitriol is accompanied by decreased activities of several major proteases that are involved in tumor invasion, namely matrix metalloproteinase-9 and cathepsins. Calcitriol did not change the activity of plasminogen activator. Simultaneously calcitriol increased the activity of protease counterparts, namely tissue inhibitors of metalloproteinase-1 (TIMP-1) and cathepsin inhibitors. Enhancement of TIMP-1 activity by calcitriol resulted in inhibition of activity of metalloproteinases and suppression of tumor invasion (Bao et al. 2006). Inhibition of matrix metalloproteinase activity (MMP-9 and MMP-2) with concomitant decrease in invasion by vitamin D_3 was also demonstrated in RWPE-1 and RWPE2-W99 prostate cancer cells, which express 25-hydroxylase (CYP27A1) and are therefore able to convert vitamin D_3 to calcitriol (Tokar and Webber 2005).

5.5. Crosstalk between vitamin D and androgens

In 1941, Huggins and Hodges discovered that prostate development and disease was driven by androgens. Androgens are essential for the development of the prostate and for the maintenance of normal growth and differentiation of the adult prostate (Desjardins 1978, Dohle et al. 2003). Androgens are also required for the initiation and progression of prostate cancer, and androgen deprivation therapy has long been the primary mode of treatment for advanced prostate cancer. Whilst a direct role for androgens in the stimulation of cancer growth is recognized, their indirect action via growth factors and cytokines is an emerging field. Androgen action is mediated via binding to androgen receptor (AR) which is expressed mostly in differentiated secretory cells of the normal and benign prostate epithelium and in cancer cells (Aumuller et al. 1998, Cooke et al. 1991, Prins et al. 1991, Sweat et al. 1999). Prostate stromal cells also produce AR, though at a lower level (Olapade-Olaopa et al. 1999). Stromal cells stimulated by androgens release cytokines that promote growth and differentiation of epithelial cells. Interestingly it was shown that 5α-dihydrotestosterone stimulates LNCaP cell growth in physiological concentrations (0.01-1 nM) but may also suppress cell growth when used in high concentrations (over 10 nM) (Lee et al. 1995).

Because LNCaP cells are highly sensitive to growth inhibition by calcitriol and express androgen receptor (AR) while ALVA31, CWR22R and some other prostate cancer cell lines lack AR and are much less sensitive to growth inhibition by calcitriol, it was suggested the antiproliferative effects of 1,25-$(OH)_2$ D require androgen (Bao et al. 2004, Zhao et al. 1997). However, this suggestion seems not to be completely true. Indeed, the induction of gene expression for prostate-specific antigen (PSA) and the repression of gene expression for fatty acid synthase (FAS) by calcitriol in androgen sensitive prostate cancer cells could be seen only in the presence of androgens in culture medium and are blocked by antiandrogen Casodex (Qiao et al. 2003, Zhao et al. 1997, Zhao et al. 2000). It was also shown that blocking of AR signaling by antiandrogens (Qiao et al. 2003, Zhao et al. 1997), AR RNA interference (Bao et al.

2004), or targeted disruption of AR (Bao et al. 2004) led to the reduction in calcitriol-mediated inhibition of cell growth. These data suggest that the androgen/AR signaling plays an important role in the antiproliferative effects of calcitriol. On the other hand, several androgen independent prostate cancer cell lines were reported to show sensitivity to growth-inhibitory action of calcitriol. Chen et al. reported that calcitriol potently suppressed the growth of AR-negative primary cultures of PC-3 cells stably transfected with VDR expressing construct (Chen et al. 2000). Zhao et al. showed that calcitriol-mediated growth inhibition of MDA PCa human prostate cancer cells does not appear to involve androgen as the addition of Casodex has no effect (Zhao et al. 2000). These cell lines express mutated AR with very poor affinity for androgen (Zhao et al. 1999b). LNCaP-104R1, an AR-expressing but androgen independent variant of LNCaP cells, also remained responsive to growth inhibition by calcitriol (Yang et al. 2002). Calcitriol-mediated LNCaP-104R1 cell growth suppression was accompanied by the induction of p27 gene expression, activation of CDK2 and subsequent G0/G1 accumulation and was not blocked by Casodex. Similarly, the introduction of AR into a VDR-expressing but vitamin D-resistant prostate cancer cell line, ALVA 31, did not restore sensitivity to calcitriol (Yang et al. 2002). Thus there seems to be no correlation between sensitivity to androgens and sensitivity to the antiproliferative action of vitamin D compounds among prostate cancer cell lines. A wide variation in the pattern of vitamin D and androgen-regulated genes was reported on the basis of cDNA microarray data. One of the appealing examples is IGFBP-3 gene, which is up-regulated by calcitriol and is regarded as an important mechanism of vitamin D antiproliferative action (Boyle et al. 2001, Krishnan et al. 2003b). DHT was reported to repress IGFBP-3 gene expression in LNCaP cells (Arnold et al. 2005) as well as in human prostate primary stromal cells (Le et al. 2006). Overall these data suggest that vitamin D has roles that are both androgen dependent and androgen independent, possibly providing potential sites of intervention in androgen independent prostate cancer.

Molecular mechanisms underlying the interaction between vitamin D and androgens signaling pathways are not clear. Several mechanisms are implicated in the interaction between various nuclear receptor ligands, such as competition for cofactors, regulation for receptors gene expression and metabolic enzymes gene expression. Many of the effects of the vitamin D on androgen-responsive prostate cancer cells appear to be mediated through the effects of vitamin D on the expression of AR gene. Treatment of LNCaP cells with calcitriol led to induction of AR gene expression, increased nuclear AR localization, and increased ligand binding (Hsieh and Wu 1997, Hsieh et al. 1996). However, vitamin D does not directly induce AR gene transcription (Zhao et al. 1999a) and the transcription factors mediating this induction are not known. A study on ovarian cancer cells demonstrated that 5α-dihydrotestosterone in its turn may induce expression of VDR gene (Ahonen et al. 2000). cDNA microarray screening revealed a number of vitamin D regulated genes which are also androgen target genes (Krishnan et al. 2003a, Krishnan et al. 2004, Qiao et al. 2003). In particular, both calcitriol and androgens inhibited growth, increased expression of PSA gene and decreased expression of FAS gene. These responses to calcitriol appear to be mediated, at least in part, by androgen action via AR.

5.6. Clinical trials

Encouraging results were obtained recently from the studies on the antineoplastic activity of vitamin D compounds in several pre-clinical models and in a few first clinical trials on patients with recurrent prostate carcinoma. Because calcitriol by itself is not suitable as a chemopreventive agent due to the risk of hypercalcemia,

calcidiol was suggested to be a good candidate for human clinical trials on prostate cancer (Chen et al. 2000). It was postulated that calcitriol produced from calcidiol within prostate cell exerts its biological effects within the cell and is not released into the systemic circulation, thus greatly reducing the problem of hypercalcemia. The second way to solve the hypercalcemia problem is the intermittent administration of high doses of calcitriol to patients (Beer 2003). A third way is using low-calcemic vitamin D analogs as chemopreventive agents. In the last decade more than 2000 vitamin D analogs were synthesized for this purpose. Among the most attractive and best investigated are EB1089 (Chen et al. 2003, Crescioli et al. 2004, Hansen and Mäenpää 1997) and BXL-628 (Marchiani et al. 2006) analogs, which suppressed prostate cell growth in preclinical models without significant hypercalcemia. Finally preclinical models revealed that vitamin D remarkably potentiates the action of other antineoplastic medicines such as dexamethasone (Flaig et al. 2006, Trump et al. 2006) and several classes of cytotoxic agents (Beer et al. 2005). Such combinatorial treatment allows significant antiproliferative action with lower concentrations of calcitriol. Currently combinations of calcitriol with docetaxel and estramustine are concerned as one of the most promising agents in prostate cancer treatment (Tiffany et al. 2005).

6. TRANSFORMING GROWTH FACTOR-β SUPERFAMILY IN PROSTATE CANCER. REGULATION BY VITAMIN D

6.1. TGF-β superfamily growth factors in normal and malignant prostate. Prostate-derived factor

TGF-β superfamily in humans includes at least 29 and probably up to 42 structurally related growth factors, which are subdivided into TGF-βs, activins, inhibins, bone morphogenic proteins (BMPs) and smaller subfamilies (Feng and Derynck 2005). TGF-β family members are expressed in most cell types. TGF-β family members control cell proliferation, adhesion, differentiation and survival and thus play key roles in tissue morphogenesis underlying wound healing and tissue repair (Grose and Werner 2004), immune function (Kriegel et al. 2006, Li et al. 2006), regulation of angiogenesis and carcinogenesis (Govinden and Bhoola 2003). TGF-βs (Cardillo et al. 2000, Merz et al. 1994, Parada et al. 2004, Story et al. 1996), activins and inhibins (Ying et al. 1997), and BMPs (Bobinac et al. 2005, Harris et al. 1994) are expressed at high levels in normal prostate and BPH acting through local (autocrine or paracrine) mechanisms, rather than through endocrine systems with some exception for inhibin which is supplied to prostate epithelium from testis Sertoli cells via circulation (De Jong 1988, Kumanov et al. 2005).

TGF-βs, their receptors and the signaling proteins comprise a tumor-suppressor pathway in early carcinogenesis by its antiproliferarive activity, its ability to induce apoptosis and to promote genomic stability. However their role in late tumorigenesis is complicated. In advanced stages of tumor development, TGF-βs act as promoters of tumor growth and metastasis, stimulating the epithelial to mesenchymal transition, matrix metalloproteinase expression and angiogenesis, and inhibiting immunosurveillance. Thus the biological action of the TGF-β family is clearly complex, depending on the cell context and physiological environment and their role in tumorigenesis is regarded as dual. In human prostate TGF-β1 is expressed mostly in stromal cells, is secreted and exerts its action primarily on epithelial cells inhibiting their growth and inducing apoptosis (Story et al. 1996). By contrast, TGF-β2 (Story et al. 1996) is mostly expressed in prostate epithelium. The development of resistance to

TGF-β by tumor cells represents a key event in the progression of malignancy. Down-regulation of gene expression for TGF-β receptors, mainly that of TβRII, and the up-regulation of gene expression for TGF-βs is typically associated with the high-grade invasive, hormone-refractory forms of prostate cancer (Guo et al. 1997, Lee et al. 1999, Zeng et al. 2004). Recently Tu et al. claimed that the loss of TGF-β signaling promotes prostate cancer metastasis. They bred transgenic mice expressing the tumorigenic SV40 large T antigen in the prostate with transgenic mice expressing a dominant negative TβRII mutant in the prostate. Although the neoplastic prostates were not enlarged, in mice expressing both transgenes the amounts of metastasis were increased compared to mice expressing only the large T antigen transgene (Tu et al. 2003).

Activins and *inhibins* have a primary role in embryogenesis and in reproductive functions (de Kretser et al. 2004, Hurwitz and Santoro 2004, Lambert-Messerlian et al. 2004). In human prostate activin subunits are expressed mainly in epithelial cells (Thomas et al. 1997) while the predominant inhibitory binding protein of activins, follistatin, is produced mostly by the basal epithelial and stromal cells. Activin subunits are expressed in all grades of cancer in the prostate epithelial cells though in the progression to malignancy, activin and follistatin gene expression colocalize in the tumor cells, implying that resistance to the growth inhibitory effects of activin may be conferred by follistatins (Thomas et al. 1997). Loss of inhibin subunits in high-grade prostate cancer was demonstrated (Mellor et al. 1998).

Primary function of *BMPs* are in bone remodeling (Lee 1997, Sykaras and Opperman 2003). In human prostate BMPs are expressed mostly in the epithelium with a predominance of BMP-2/4 and BMP-7 (Bobinac et al. 2005). In prostate cancer tissues, a variable expression of all BMPs was reported. BMP-2/4 was predominantly expressed in prostate carcinoma, whereas the expression of BMP-7 in carcinoma was significantly lower than in the normal prostate (Bobinac et al. 2005). In contrast to the tumor-suppressor role of other members of TGF-β superfamily, BMPs are critical promoters of the formation of the osteoblastic lesions associated with prostate cancer metastases (Dai et al. 2005, Feeley et al. 2005).

Prostate-derived factor, a distant TGF-β family member sometimes classified as BMP, is known as PDF (Paralkar et al. 1998), placental bone morphogenic protein (PLAB) (Hromas et al. 1997), placental transforming growth factor-β (PTGF-β) (Lawton et al. 1997, Yokoyama-Kobayashi et al. 1997), macrophage inhibitory cytokine-1 (MIC-1) (Bootcov et al. 1997), growth/differentiation factor-15 (GDF-15) (Bottner et al. 1999, Strelau et al. 2000) and nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1) (Baek et al. 2001a). Compared to other TGF-β superfamily growth factors, PDF expression is more tissue-specific, with the highest expression level in the placenta and the prostate and also in macrophages (Bottner et al. 1999, Lawton et al. 1997, Paralkar et al. 1998). In prostate PDF is abundantly expressed mostly in the epithelial cells under the control of androgens (Paralkar et al. 1998, Thomas et al. 2001, Uchida et al. 2003). PDF is expressed in various human prostate cancer cell lines, being most highly expressed in LNCaP and PC-3 cells, which were shown to secrete a large amount of biologically active PDF (Karan et al. 2003, Liu et al. 2003, Uchida et al. 2003). Similar to the other TGF-β family growth factors, PDF exerts various biological effects in an autocrine and paracrine manner and its effects are heavily dependent on the cellular context. PDF was reported to have potent proapoptotic, prodifferentiative and antiproliferative properties toward different cell types (Baek et al. 2001b, Li et al. 2000b, Liu et al. 2003). The major function of PDF and also the role of PDF in tumorigenesis are not known. The ability of PDF to

suppress tumor cell growth was demonstrated on colorectal carcinoma (Baek et al. 2001a) and osteosarcoma (Tan et al. 2000) cells and also on glioblastoma cells growth in culture or transplanted into a nude mice (Albertoni et al. 2002). PDF is often overexpressed in cancer (Koopmann et al. 2004, Nakamura et al. 2003, Welsh et al. 2001, Welsh et al. 2003) and is up-regulated during the transition of human prostate cancer LNCaP cells to the androgen independent state (Karan et al. 2002, Karan et al. 2003). Studies on gastric cancer cells also suggest the involvement of PDF in tumor progression (Lee et al. 2003). *In situ* hybridization studies demonstrated the high level of PDF in normal prostate with considerable decrease during the progression of cancer at the primary site and reexpression in the osseous metastatic lesions (Thomas et al. 2001). In this sense, PDF shares the main characteristics of growth factors belonging to the TGF-β superfamily, which act both positively and negatively on tumorigenesis depending on the molecular and cellular context.

6.2. TGF-β family growth factor signaling pathway

6.2.1. Activation and receptors

TGF-β superfamily growth factors are disulfide-linked dimers. The majority of them are secreted as precursors with a large propepetide and a C-terminal mature polypeptide that form dimers via a disulfide bond and are proteolytically cloven from the precursors (Annes et al. 2003). The propeptide remains associated with the dimer and maintains it in the inactive complexes targeted to the extracellular matrix (ECM) via latent TGF-β binding protein. TGF-β family members are activated by proteolitic cleavage of prosegment, which causes growth factor release from extracellular matrix. The availability of TGF-β growth factors is effectively regulated by secreted and matrix-associated proteins that sequester the ligands from binding to their receptors (Annes et al. 2003).

TGF-β-related growth factors act by binding to two structurally related transmembrane heteromeric serin/threonin kinase receptor complexes (Feng and Derynck 2005). The characteristic structural features of the receptors are a three-finger toxin fold (Greenwald et al. 1999) in the ligand binding extracellular domain, a single transmembrane domain, and an intracellular serine kinase domain. Type I receptors are distinct from type II receptors in having the Gly-Ser-rich juxtamembrane activation domain. In spite of the structural similarity, the two types of receptors play different roles in signaling. In the absence of ligands type I and type II receptors exist as homodimers at the cell surface. After ligand is bound concurrently to both of the receptor types, the constitutively active kinase domain of the type II receptor phosphorylates the type I receptor on multiple sites in its GS activation domain (Wrana et al. 1994). The activated type I receptor then initiates the downstream signaling process by phosphorylating SMAD proteins in the cytoplasm (Massague et al. 2005).

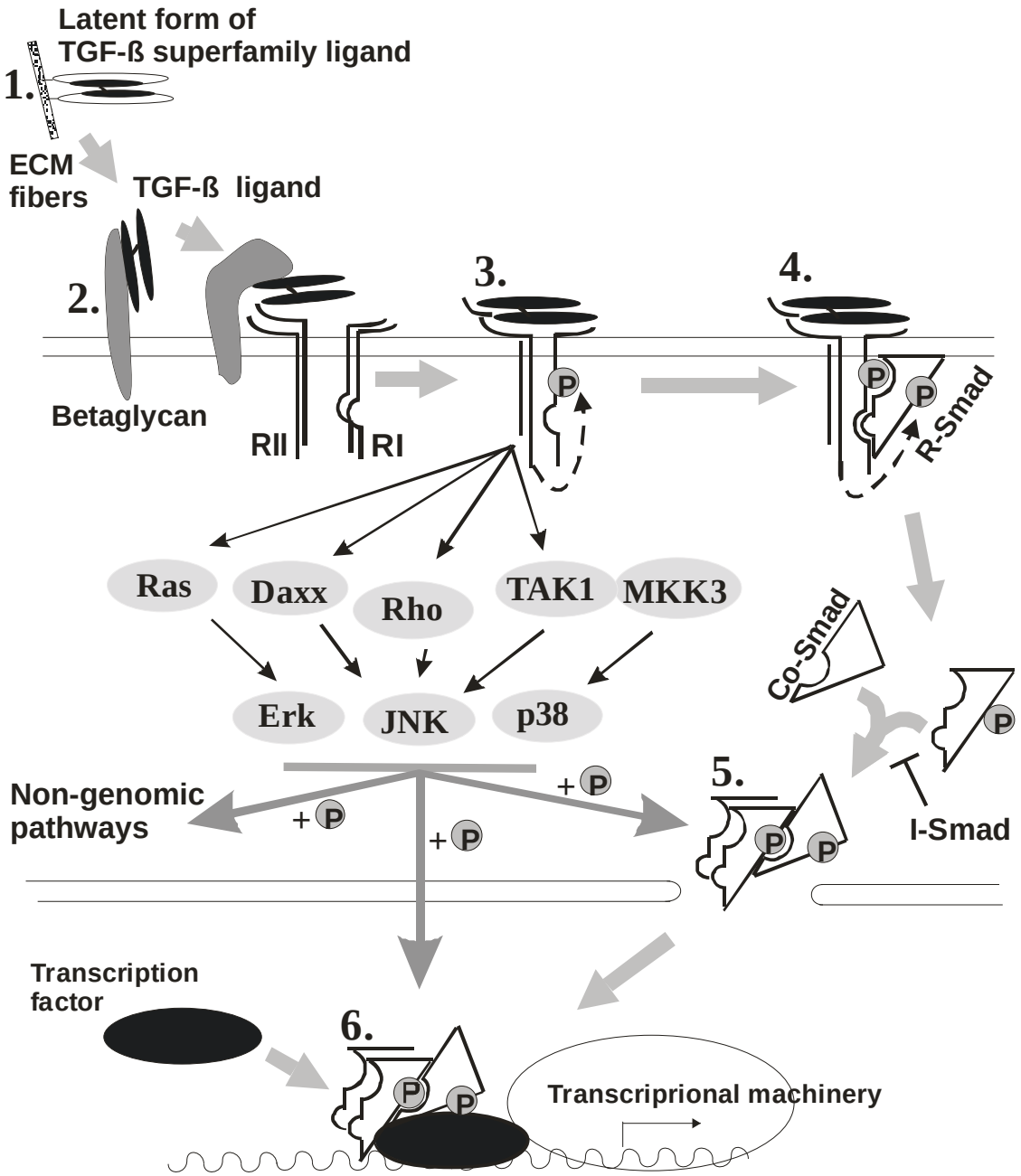

Figure 3. General model of TGF-β superfamily ligands action. 1. Latent complex of TGF-β superfamily growth factor assembled on the extracellular matrix (ECM) fibers via latent TGF-β binding protein (LTBP); growth factors are released from the ECM during events including proteolysis; 2. Activation of growth factors on cell surface. Betaglycan (type III receptor) presents growth factors to type I (RI) or type II (RII) receptors; 3. Binding of active growth factor to its specific cell surface receptors, leading to dimerization of type I and type II receptors and activation of type I receptors via phosphorylation by type II receptor; 4. Recruitment of R-SMADs to receptor complex and their activation by phosphorylation on SHS domain; 5. Assembly of R-SMAD-Co-SMAD complex and its translocation into the nucleus; 6. Action of SMAD-complex as a transcriptional activation signal on target genes. Known interactions between non-SMAD specific signaling proteins (in grey circles) and TβRs are indicated.

There is a variety of type I and type II receptors. As a rule ligands show high affinity binding only to one type of receptor (either type I or type II) and the binding to high-affinity receptor is required for the other receptor type to bind ligand. It was found for TGF-β1, TGF-β3 and activins that they bind with high affinity to their correspondent type II receptors, TβRII and ActRII with no need for type I receptor (Boesen et al. 2002, Greenwald et al. 2004, Hart et al. 2002). In contrast, BMP-2 and -4 bind with moderately higher affinity to their type I (BMPRIA/ALK3 and BMPRIB/ALK6) than to their type II receptor (BMPRII). Moreover, for high affinity binding BMPs require the heteromeric complex (Keller et al. 2004, Kirsch et al. 2000). However, the homologous ligand, BMP7 (60% identical to BMP2) retains the TGF-β/activin-like preference for its type II receptor (Greenwald et al. 2003). Accessory cell surface proteins such as betaglycan (type III receptor, TbetaRIII) and endoglin further define the binding efficiency and specificity of the ligand to the receptor complex (Lebrin et al. 2004, Lopez-Casillas et al. 1993). Moreover, TGF-β2, unlike TGF-β1 and TGF-β3, appears to require betaglycan for high affinity binding and signaling. In the absence of type III receptor, the complexed TβRII and TβRI is needed to bind TGF-β2 (del Re et al. 2004). Moreover, a number of receptors have multiple specificities. For instance ActRII has a particularly broad specificity, making a link between subfamilies by binding to both activin and BMP ligands. Interestingly, although it is a high-affinity receptor for activin and BMP7, ActRII, like BMPRII, is the lower-affinity receptor for BMP2. A broad specificity also exists in the type I receptor, ALK2, transmitting signals from both BMP7 (Macias-Silva et al. 1998) and the distantly related Müllerian inhibiting substance (MIS) (Clarke et al. 2001, Visser et al. 2001). In addition to binding related ligands to the same receptor complex, a single ligand often activates several type II-type I receptor combinations. Thus the signaling responses are defined by the composition of the receptor complex (Feng and Derynck 2005). The molecular mechanisms that underlie cooperative receptor assembly and the origin of multiple specificity and variable affinity in the TGF-β superfamily are largely unknown.

6.2.2. SMADs

SMADs are central signal transducers in signaling of TGF-β superfamily growth factors, which transfer the signals from ligand-receptor complexes at the cell surface to gene transcription in the nucleus. There are three subgroups of SMADs: R-SMADs, the ligand-specific, receptor-activated SMADs (SMAD1,2,3,5 and 8 in mammals), a common SMAD (SMAD4 in mammals), and inhibitory SMADs (SMAD6 and 7 in mammals). R-SMADs and SMAD4 contain two conserved polypeptide segments, the MH1 (N-terminal) and MH2 (C-terminal) domains linked by a less conserved linker region. The MH1 domain has DNA-binding activity wheareas the MH2 domain has protein-binding properties. R-SMADs directly interact with, and are phosphorylated on their SXS motif by activated type I receptor. SMAD1,5 and 8 are specific substrates of the BMP receptors (BMP-RIA, BMP-RIB, ALK-1, and ALK-2 (Feng and Derynck 2005, Itoh et al. 2000)), whereas SMAD2 and 3 are activated by TGF-β and activin receptors (TβRI and ActRIB respectively). Upon phosphorylation R-SMADs dissociate from the receptors and form the heteromeric complexes with SMAD4, consisting of two R-SMADs and one SMAD4, as was suggested by the latest biochemical studies (Chacko et al. 2004). These heterotrimers translocate into the nucleus, where the SMADs bind specific DNA sequences and direct the assembly of a large nucleoprotein complex which provide transactivation/transrepression thus acting as ligand-induced transcription factors. Inhibitory SMADs control SMAD signaling by preventing phosphorylation and/or

nuclear translocation of R-SMADs and by inducing proteosomal degradation of the receptor complex through the recruitment of ubiquitin-ligases. There are several other proteins, which modulate SMAD binding to, and phosphorylation by the type I receptors such as SARA (Tsukazaki et al. 1998, Wu et al. 2000) and Disabled-2 (Dab-2) (Hocevar et al. 2001).

SMADs are weak DNA-binding proteins and naturally function by cooperating with a large number of high-affinity sequence-specific DNA-binding transcription factors and thus may be considered as the coactivators for select transcription factors. SMAD complex associates with the DNA-binding transcription factors and binds to an adjacent DNA sequence. SMAD3 and SMAD4 contact DNA selectively in the major groove, with 5'-GTCTAGAC-3' as the optimal sequence for their binding (Shi et al. 1998, Zawel et al. 1998). SMAD3 also binds a GGCGGG sequence in the c-myc promoter, which is required for the transcriptional repression of c-myc by TGF-β signaling (Frederick et al. 2004). In contrast to other R-SMADs, SMAD2 is unable to bind DNA (Shi et al. 1998). It is thought that SMAD2/4 complexes bind DNA through SMAD4. SMAD4 also stabilizes the interaction of activated R-SMADs with CBP/p300. SMADs cooperate through physical interactions with a remarkable diversity of DNA sequence–binding transcription factors (Feng and Derynck 2005) determining signaling specificity. A higher level of complexity is reached by the interaction of the SMAD complex with several various DNA-binding transcription factors simultaneously. This complex scenario of transcriptional control with multiple SMAD complexes or a larger complex was suggested, for example, for the regulation of SMAD7 transcription, through interactions of SMADs with TFE3, AP-1, and Sp1 (Brodin et al. 2000, Hua et al. 2000); or the germ line IgCα promoter, through interactions of SMADs with CREB and Runx proteins (Zhang and Derynck 2000). In addition, SMADs can recruit various coactivators and corepressors into the transcription machinery (Feng and Derynck 2005). Interaction of R-SMADs with the CBP/p300 further enhances the inherent transcription activity of the interacting transcription factors (Pouponnot et al. 1998).

Overall, the TGF-β signaling responses are effectively regulated and finely tuned by differential type I-type II receptor interactions, SMAD complex formation, receptor and SMAD interactions with accessory proteins and crosstalk of the SMADs with other signaling pathways.

6.2.3. SMAD-independent pathways

TGF-β responses are not solely the result of SMADs activation, but are dependent upon interactions of SMAD signaling with a variety of other intracellular signaling pathways, initiated or not by TGF-β itself, that may either potentiate, synergize, or counteract a SMAD-mediated pathway. Moreover, there are instances where SMAD signaling may even be dispensable for some of the TGF-β responses, as exemplified by SMAD-independent activation of the cyclin kinase inhibitors p15 and p21 in HaCaT keratinocytes (Hu et al. 1999), or transcriptional activation of the fibronectin promoter via mitogen-activated protein kinase (MAPK)-dependent mechanism (Hocevar et al. 1999).

Besides the activation of SMADs, TGF-β activates other signaling pathways including MAPK signaling cascades of Erk, JNK and p38, which either regulate SMAD activation or induce SMAD-independent responses (Javelaud and Mauviel 2005). Activation of MAPKs by TGF-β has been described to occur either with slow

kinetics, possibly resulting from SMAD-dependent transcription responses, or with rapid kinetics (5-15 min), suggesting direct activation of MAPK kinase kinases (MAPKKKs) and independence from transcription (Derynck and Zhang 2003).

It was demonstrated in SMAD4-deficient cells and in cells overexpressing dominant-negative SMADs that TGF-β is able to activate JNK pathway in these models despite the deficient SMAD cascade (Engel et al. 1999). It was also shown that mutated TβRI, defective in SMAD activation, activate p38 signaling in response to TGF-β (Yu et al. 2002). The molecular mechanisms as well as the physiological role of TGF-β-induced MAPKs activation have been poorly investigated. It was suggested that rapid activation of Ras by TGF-β in epithelial cells may implicate Ras in TGF-β-induced Erk signaling (Yue and Mulder 2000). It was shown that TGF-β-induced Ras/Erk signaling can regulate SMAD activation and induce TGF-β1 expression, thereby amplifying the TGF-β response and inducing secondary TGF-β responses (Yue and Mulder 2000). Both TGF-β and BMP-4 can activate TGF-β-activated kinase 1 (TAK1), a MAPKKK family member (Yamaguchi et al. 1999). MAPKKKs are known to activate JNK and p38 signaling. TAK1 was shown to activate NF-κB signaling thus providing a mechanism for TGF-β/BMP induced NF-κB. TGF-β-induced SMAD and MAPK pathways might converge through the effects on SMAD-interacting transcription factors. The convergence of TGF-β-induced SMAD and MAPK pathways often result in cooperativity. However, these pathways may counteract each other. For example, SMAD6 can bind TAK1 and down-regulate its activity (Kimura et al. 2000), whereas SMAD7 can enhance and sustain JNK activation (Mazars et al. 2001). Inhibition of SMAD2 signaling by c-Jun through association with SMAD corepressors is also regulated by JNK signaling (Pessah et al. 2002). Thus, the balance between activation of SMADs and MAPKs might define physiological responses to TGF-β.

Besides MAPKs, TGF-β can induce other signaling pathways including Rho-like GTPases, PP2A and PI3K (Derynck and Zhang 2003, Javelaud and Mauviel 2005).

Activation of R-SMADs is also regulated by various cytoplasmic kinases dependent on cell type. These include cyclin-dependent kinases (CDKs) (Matsuura et al. 2004), PKC (Yakymovych et al. 2001), CaMKII (Abdel-Wahab et al. 2002), Casein kinase I (Waddell et al. 2004) and Akt (protein kinase B) (Conery et al. 2004, Remy et al. 2004), which phosphorylate SMADs linker region on different sites. The combination of phosphorylation events contribute greatly to the final gene responses to SMAD signaling.

6.3. Regulation of TGF-β signaling by vitamin D

Regulation of TGF-β superfamily growth factors signaling by vitamin D metabolites is cell type specific and is not completely understood. So far these studies have concentrated on the interaction between signaling pathways of vitamin D metabolites and TGF-βs and only a few reports have considered crosstalk with other TGF-β subfamilies. Studies on the interaction between signaling pathways of vitamin D metabolites or their analogs and TGF-βs performed in human osteoblasts, keratinocytes, mammary cancer, leukemia, and prostate cancer cells revealed a number of diverse mechamisms of crosstalk (listed in Table 1). It was demonstrated that vitamin D metabolites and their analogs may either potentiate or suppress the activities of TGF-βs via transcriptional regulation of TGF-βs, their receptors or latent binding

proteins or via non-transcriptional pathways. It was found that crosstalk between calcitriol and TGF-βs in the regulation of gene expression might be mediated via SMAD3 and SMAD7 acting as VDR cofactors (Yanagi et al. 1999, Yanagisawa et al. 1999). This finding may shed light on the nature of the synergistic effect between calcitriol and TGF-β superfamily growth factors in regulation of the expression of certain genes (Kassem et al. 2000, Kveiborg et al. 2002, Liu et al. 1999, Pavasant et al. 2003). However, other mechamisms may also be considered.

Table 1. Regulation of TGF-βs signaling by vitamin D. VD = vitamin D

Cell line	VD metabolite or analog	VD effect on TGF-β signaling pathway	Blocking of antiproliferative action of VD with TGF-β neutralizing antibody	Reference
MC 903 murine epidermal keratinocytes	calcitriol	↑TGF-β1 expression and secretion ↑TGF-β2 expression ↑ proportion of bioactive TGF-β1	+	(Koli and Keski-Oja 1993)
Human osteoblasts	calcitriol	↓ TGF-β2 expression		(Iimura et al. 1994)
BT-20 human mammary cancer	calcitriol	↑TGF-β1 expression and secretion ↑TGF-β2 expression ↑latent TGF-β1 binding protein secretion	+	(Koli and Keski-Oja 1995)
NRP-152 rat prostate epithelial cells	calcitriol	↑TGF-β2 and β3 expression		(Danielpour 1996)
MCF-7 human mammary cancer	Calcitriol and KH1060	↑TGF-β1 expression and secretion	+	(Mercier et al. 1996)
Human osteoblasts	calcitriol	↑TGF-β2 expression and secretion ↑TβRI and TβRII expression	partially	Wu, 1997 #735
MCT mouse renal cells	calcitriol	↑TGF-β1 secretion	-	(Weinreich et al. 1999)
HL60 human leukemia	EB1089	↑TGF-β1 expression ↑TβRI and TβRII expression	partially	(Jung et al. 1999)
Primary cultures of mouse and rat renal cells	calcitriol	↓ bioactive TGF-β1 level and signaling		(Aschenbrenner et al. 2001)
Caco-2 human colon cancer	calcitriol	↑TGF-β1 expression ↑activation of latent TGF-β1 via ↑ expression of IGF-II receptors ↑TβRI expression	+	(Chen et al. 2002)
Human osteoblasts	calcitriol	↑TGF-β1 secretion and binding ↑TβRI and TβRII expression		(Nagel and Kumar 2002)
RM4 human mammary cancer	calcitriol	↑TGF-β1 secretion		(Bizzarri et al. 2003)
PC-3 human prostate cancer	calcitriol	↑TGF-β1 expression and signaling ↑TβRI and TβRII expression	+	(Murthy and Weigel 2004)

Koli et al. showed that calcitriol and several vitamin D3 analogs (EB1089, MC903, and KH1060) that are known to be potent inhibitors of mammary cancer cell growth both *in vitro* and *in vivo* significantly increased TGF-β1 mRNA level in BT-20 human breast carcinoma cells (Koli and Keski-Oja 1995). They also provided evidence for the coordinated regulation of latent TGF-β binding protein and TGF-β1 in calcitriol-treated BT-20 cells showing that the enhancement of TGF-β1 mRNA level, protein secretion and activity by calcitriol was accompanied by an increase of the amount of latent TGF-β binding protein in culture medium which leads to sequestration of TGF-β1. These data indicated that vitamin D and its analogs potently induce both active and latent forms of TGF-β1 in BT-20 cells. Nagel et al. demonstrated that growth-inhibitory effect of calcitriol on cultured human osteoblasts is mediated by TGF-β receptors by showing that osteoblasts which express dominant negative, kinase-deficient TβRII, fail to respond to the growth-inhibitory effect of calcitriol (Nagel and Kumar 2002). Calcitriol increased mRNA levels and the amount of TβRI and TβRII on the cell surface. However, these effects were not due to changes in receptor gene transcription. Chen et al. reported that calcitriol sensitized Caco-2 human colon cancer cells to the growth-inhibitory effect of TGF-β1 via two independent pathways – induction of the expression of insulin-like growth factor 2 (IGF-2) receptors, which facilitated activation of latent TGF-β1 activated TGF-β signaling in Caco-2 cells, and induction of TβRI expression (Chen et al. 2002). They also showed that calcitriol increased the amount of active TGF-β1 in conditioned medium from Caco-2 cells. Murthy and Weigel found that the growth-inhibitory action of calcitriol in prostate cancer PC-3 cells is mediated through at least two distinct pathways, the TGF-β pathway and IGFBP-3 pathway, each of them being insufficient to inhibit the growth of PC-3 cells (Murthy and Weigel 2004). They showed that in PC-3 cells calcitriol increased TGF-β1 production and signaling. In its turn TGF-β1 may induce VDR gene expression, as was shown in human acute myeloid leukemia HL-60 cells, suggesting another mechanism for the synergistic action of TGF-β1 and vitamin D (Jung et al. 1999).

A crosstalk between calcitriol and activin A was demonstrated in murine erythroleukemia (MEL) cells (Nagasaki et al. 1997, Waki et al. 2001). In this study it was shown that calcitriol enhances the inhibitory effect of activin A on cell proliferation and differentiation in a dose-dependent manner. It was also shown in human leukemia HL-60 cells that combinations of calcitriol with TGF-β1, TGF-β2 or activin A acted synergistically in inhibiting cell proliferation and inducing monocytic differentiation (Okabe-Kado et al. 1991).

7. PLATELET-DERIVED GROWTH FACTOR SUPERFAMILY IN PROSTATE CANCER.

7.1. PDGF superfamily growth factors in normal and malignant prostate.

To date the superfamily of platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) includes at least nine closely related growth factors. Four different PDGF polypeptides have been identified (PDGF-A, PDGF-B, PDGF-C and PDGF-D). The VEGF family is further subdivided into VEGFs (VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E and placental growth factors (PIGFs). The PDGF superfamily members are expressed in a variety of normal human tissues and in cancers including glioblastoma, ovarian cancer and prostate cancer. They have a potent

mitogenic effect on many cell types including prostate BPH cells (Vlahos et al. 1993), stimulate cell motility, maintain cell survival and promote angiogenesis. The primary role of PDGF family members in neovascularisation is the most widely studied. The selective blockade of both PDGF and VEGF receptors inhibits tumor angiogenesis (Haluska and Adjei 2001, Lu et al. 2005, Shibuya 2003). VEGFs serve as potent mitogens for endothelial cells (Asahara et al. 1999, Li and Keller 2000). Nowadays there is an increasing body of evidence implicating the PDGF superfamily members in the development of solid tumors.

PDGFs are able to induce malignant transformation and also serve as the potent mitogenic factors for malignant cells. Coexpression of PDGF and its receptors in tumor cells or cells supporting tumor growth suggests both autocrine and paracrine mechanisms for PDGF-mediated tumor growth. The first indication that autocrine PDGF receptor signaling may lead to malignant transformation came from the discovery of the homology between the PDGF B-chain and the simian sarcoma viral oncogene product v-Sis. This suggestion has gained support from the studies demonstrating the transforming ability of human PDGF-B cDNA (Johnsson et al. 1984) and similar studies showing that the expression of PDGF-AA, -CC or -DD in NIH3T3 fibroblasts also leads to a transformed phenotype (Fry et al. 1986, Greco et al. 1998). It was demonstrated that PDGF and PDGF receptors are often co-expressed in malignancies derived from PDGF-responding cells like gliomas (Maxwell et al. 1990, van der Valk et al. 1997) and soft-tissue sarcomas (Reis et al. 2005). Moreover, different malignancies have been shown to be associated with mutational activation of PDGF or PDGF receptors. It was demonstrated that cells overexpressing PDGF-B can induce tumor formation when transplanted into nude mice (Forsberg et al. 1993). Overall these data suggest that PDGF superfamily members may promote tumor growth by autocrine stimulation of malignant cells, by overexpression or overactivation of these growth factors, or by stimulation of angiogenesis within the tumor.

The expression of PDGF isoforms and the role of PDGF in the control of prostate cell growth are not well characterized. It was found that receptors for PDGF are often overexpressed in primary prostate cancer and in prostate metastases to the bone (Chott et al. 1999, Fudge et al. 1994) leading to the suggestion that PDGF plays a role in the progression of prostate cancer in bone. Indeed human prostate cancer PC-3MM2 cells grown in the tibia of nude mice show high level of activated PDGFs and their receptors (Uehara et al. 2003). The blockade of PDGF action with PDGF receptor inhibitors led to lower tumor incidence, smaller tumors and fewer lymph node metastases that were associated with a significant reduction in activated PDGF receptors on tumor cells, less tumor cell proliferation and increased apoptosis. A potential role in prostate cancer progression was suggested for PDGF-BB and PDGF-DD isoforms acting via PDGFRβ, which is highly expressed in human BPH cells (Ustach et al. 2004, Vlahos et al. 1993). A mitogenic effect of PDGF-BB on cultured human BPH cells was shown (Vlahos et al. 1993) that led to the suggestion that PDGF-BB might be involved in the development of BPH but the data are not consistent. It was also suggested that PDGF-A signaling via PDGFRα might play the major role in the development of BPH (Fudge et al. 1994). Selective inhibitors of PDGFR tyrosine kinase slow down prostate cancer progression in animal studies and also in clinical trials (van der Poel 2004). VEGF expression in prostate decreases significantly in response to androgen ablation (Jain et al. 1998).

7.2. PDGF superfamily growth factors signaling pathway

7.2.1. Activation and receptors. PDGFRβ

PDGF superfamily growth factors act as homo- or heterodimers which bind to transmembrane tyrosine kinase receptors. Ligand binding induces receptor dimerization and activation. The complete set of events leading to receptor activation has not been elucidated although several critical events are known. These include autophosphorylation of the receptor in the activation loop that initiates signaling cascade and suppression of intrinsic receptor phosphatase activity what most probably serves to amplify signaling (Shimizu et al. 2001).

So far five PDGF dimers have been described: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (Yu et al. 2003). The majority of PDGFs are secreted in active form with the exception of PDGF-CC and PDGF-DD. The secreted form of PDGF-CC and PDGF-DD cannot activate the PDGFR until they are proteolitically cloven (Bergsten et al. 2001, Kazlauskas 2000, LaRochelle et al. 2001). PDGFs acts by both paracrine and autocrine mechanisms via binding to two structurally related tyrosine kinase receptors, PDGFRα and PDGFRβ (Matsui et al. 1989) so that PDGFRαα can be activated by PDGF-AA, PDGF-AB and PDGF-BB and PDGF-CC, whilst PDGFRββ is activated by PDGF-BB and PDGF-DD (Bergsten et al. 2001, Claesson-Welsh 1994, Claesson-Welsh et al. 1988, Gilbertson et al. 2001, LaRochelle et al. 2001, Li et al. 2000c, Matsui et al. 1989). A heterodimeric PDGFRαβ complex has also been identified which can be activated by PDGF-AB, PDGF-BB and PDGF-CC. Receptor availability is one of the primary factors in PDGF action control and is regulated by various factors including TGF-β1 (Gronwald et al. 1989, Zhou et al. 2003), retinoids (Liebeskind et al. 2000), estrogens (Gray et al. 1995), TGF-α (Li and Tseng 1996) and also such pathological processes as cancer (Aoyagi et al. 1993, Fudge et al. 1994, Langerak et al. 1996, Pistritto et al. 1994), AIDS (Langerak et al. 1996) and inflammation (Fukuoka et al. 1999, Okazaki et al. 1992, Tsukamoto et al. 1991).

The difference in signaling pathways mediated by PDGFRα and PDGFRβ is not clear. The tissue culture-based *in vitro* systems have revealed that PDGFs trigger a similar set of cellular responses in cells expressing either PDGFRα or PDGFRβ although a number of differences have been reported (Rosenkranz and Kazlauskas 1999). In contrast, a crucial difference was found in the phenotype of mice that lack either PDGFRα or PDGFRβ leading to the conclusion that PDGFRα and PDGFRβ engage signaling events that have fundamentally distinct consequences during development (Soriano 1994, Soriano 1997). The similarity in receptor-proximal events and sellular responses induced by PDGFRα and PDGFRβ and the receptor-specific phenotypes of the knock-out mice may be explained by the fact that these two receptors are expressed at non-identical times and locations during development.

7.2.2. Downstream effectors

Within minutes of PDGF receptor autophosphorylation many downstream signaling systems are engaged (Tallquist and Kazlauskas 2004). Activated receptor dimer recruits several signal transduction proteins containing intrinsic SH2 domain and activates signaling enzymes of which the most studied are Src, PI3K, phospholipase Cγ1 (PLCγ), and Ras. While phosphorylation of the PDGFR within the kinase domain is essential for the activation of the receptor's kinase activity, tyrosine phosphorylation of the PDGFR at the appropriate tyrosine residues outside of kinase domain completes a high-affinity binding site for each of these signaling enzymes. The translocation of

PI3K and PLCγ to the plasma membrane increases their access to substrates in the membrane. PDGF-stimulated cells do not enter S phase when Ras, Src family kinases, PI3K, PLCγ and the phosphotyrosine phosphatase SHP-2 are blocked (Claesson-Welsh 1994, Kazlauskas 1994, Roche et al. 1996). The complete picture for the intracellular events that drive PDGF-mediated cell proliferation is not clear. The involvement of different signaling enzymes actions in cell cycle program regulation has been poorly studied. Within minutes of adding PDGF, signaling systems such as PI3K and PLCγ/PKC direct cell migration (Kundra et al. 1994). At later times these same signaling enzymes engage intracellular cascades that drive cell cycle progression (Jones and Kazlauskas 2000).

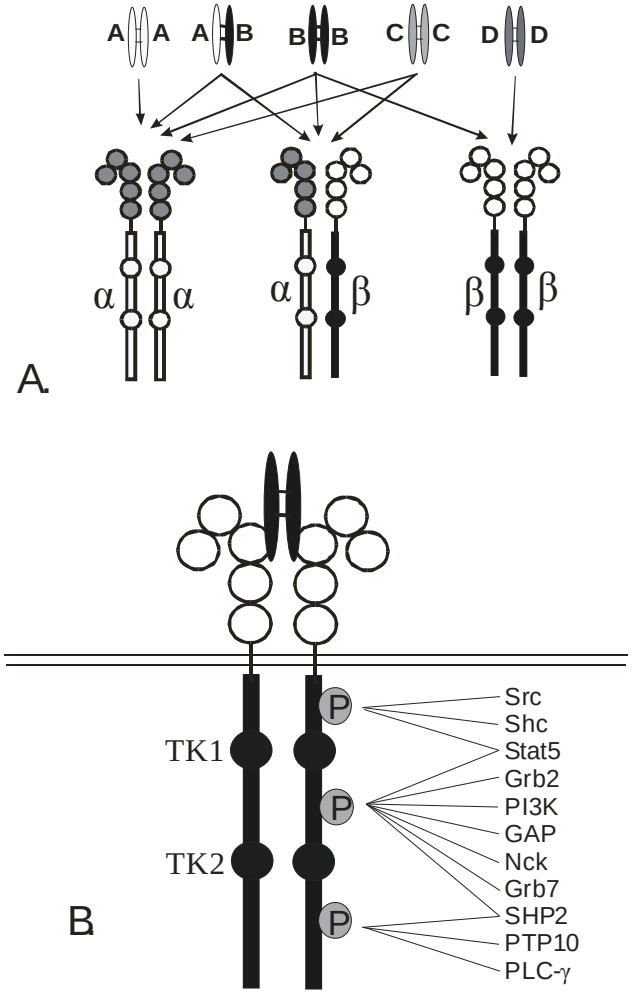

Figure 4. Schematic representation of interaction between PDGF isoforms and PDGF receptors (A) and signal transduction by PDGF receptors. A. Illustrated are the combinations of five different PDGF isoforms with the receptors they activate. B. Illustrated is the structure of PDGFRβ. Extracellular immunoglobulin-like domains are shown as empty circles. A bipartite tyrosine-kinase domain (TK1 and TK2) are shown as black circles. Upon binding of PDGF ligand dimers, specific tyrosine residues (group of several different tyrosine residues which are phosphorylated in one domein is indicated as P in the gray circle) within the cytoplasmic domain are autophosphorylated. Known interactions between specific signaling proteins and phosphotyrosines are indicated. (Modified from Jones and Cross 2004).

7.2.3. Interaction between PDGFRs and other tyrosine kinase receptor signaling pathways

Multiple lines of evidence indicate that sensitivity to PDGF is modulated by various factors, including peptide growth factors (Gronwald et al. 1989, He et al. 2001, Novosyadlyy et al. 2006, Thommes et al. 1996, Zhou et al. 2003). Moreover, the positive crosstalk with EGF receptor was found to be essential for PDGF-mediated activation of p21-activated kinase (PAK) family kinases and PDGF-mediated stimulation of fibroblasts migration (He et al. 2001). This crosstalk was shown to occur via transactivation of EGF receptor (ErbB1) by PDGF (Li et al. 2000a).

AIMS OF THE STUDY

The present study was undertaken to characterize growth factor signaling pathways mediating the antiproliferative action of calcitriol in human prostate cells.

The specific aims of the study were as follows:

1. to study the regulation of gene expression of growth factors and their receptors by calcitriol in human prostate cancer LNCaP cells and human prostate primary stromal cells

2. to analyze the biological significance of regulation of chosen growth factor and their receptor genes by calcitriol

MATERIALS AND METHODS

1. REAGENTS (I, II, III, IV)

Calcitriol was generously donated by Leo Pharmaceuticals (Ballerup, Denmark). Casodex was a gift of AstraZeneca (London, U.K.). DHT was obtained from Merck (Darmstadt, Germany). Recombinant human PDF (GDF-15/MIC-1) and human TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA), recombinant human PDGF, BB isoform, from Sigma-Aldrich Laboratories (Saint Louis, MO, USA) and recombinant human EGF from Oncogene Research products, Calbiochem-Novabiochem GmbH (Ober der Roeth, Germany). COT-1 DNA, PolyA and yeast tRNA were purchased from GibcoBRL (Grand Island, NY, USA). Cy3-dUTP, Cy5-dUTP, dNTP and Oligo(dT)$_{(12-18)}$ primers were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). RPMI-1640 and DMEM/F12 media, pifithrin-α, actinomycin D and sodium orthovanadate were obtained from Sigma-Aldrich Laboratories (Steinheim, Germany), FBS and penicillin-streptomycin from GibcoBRL (Groningen, The Netherlands). Amphothericin B and insulin were purchased from Sigma-Aldrich (St.-Louis, MO, USA), BSA from Roche (Mannheim, Germany). Cycloheximide was from Sigma (St. Louis, MO, USA), hydroxyflutamide from Shering-Plough Avondale (Rathdrum, Ireland) and Lipofectamine™ 2000 from Invitrogen (Paisley, U.K.). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Forster City, CA, USA). M-PER mammalian protein extraction reagent and BCA protein assay kit were purchased from Pierce (Rockford, IL). Complete Mini Protease inhibitor was obtained from Roche (Mannheim, Germany). Protein molecular weight standards were obtained from Bio-Rad (Richmond, CA, USA). Oligonucleotide primers for RT-PCR were ordered from TAG Copenhagen A/S (Copenhagen, Denmark). Laminin was from Sigma (St. Louis, MO, USA) and collagen I was kindly provided by Riina Sarkanen (University of Tampere Medical School, Department of Cell Biology, Tampere, Finland).

2. CELLS

2.1. Cell culture (I, II, III, IV)

Human prostate cancer LNCaP and PC-3 cell lines were obtained from American Type Culture Collection (Rockville, MD). Human prostate primary stromal cells P29SN and P32S were obtained and characterized in our laboratory as previously described (Lou et al. 2004). LNCaP cells were routinely cultured in phenol-red free RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. PC-3 cells were cultured in phenol-red DMEM/F12 medium supplemented with 5% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. P29SN and P32S cells were cultured in phenol-red free DMEM/F12 medium supplemented with 5% FBS, 5 μg/ml insulin, 2.5 μg/ml amphotericin B, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were routinely cultured in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2. Culture media were renewed every 48 h. Cells were subcultured when grown to 70 – 90% confluency.

2.2. Treatment with hormones and growth factors (I, II, III, IV)

Cells grown to subconfluency were treated with calcitriol, DHT, growth factors or other substances. The concentrations of ethanol or other vehicles were adjusted in control and treated samples.

2.3. Transient transfection (III)

PC3 cells (6 x 10^5) were transfected with a wild-type human AR cDNA expression vector (pSG5-hAR) or the mutant AR of LNCaP cells (pARL) in 25 cm^2 flasks. Each flask received 1 μg of DNA and 2.5 μl of Lipofectamine™ 2000 in 1.5 ml of serum-free RPMI-1640 medium for 4 h following the manufacturer's instructions. After transfection, the cells were incubated in RPMI-1640 medium supplemented with 10% DCC-FBS for 44 h to allow the expression of the transfected AR.

3. RNA ISOLATION (I, II, III, IV)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells grown in 25 cm^2 area were lysed in 1 ml TRIzol reagent, phases were separated by centrifugation and total RNA was precipitated from the RNA-containing phase with isopropanol. The pellets were washed once with 75% ethanol, dried in air and diluted in DEPC-treated water. RNA concentration and purity were assessed by A_{260}/A_{280} absorption using GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Uppsala, Sweden) and RNA quality was assessed by agarose gel electrophoresis. The total RNA was immediately converted to cDNA or stored at -70°C for not longer than 1 month.

4. cDNA MICROARRAY AND DATA ANALYSIS (I)

4.1. cDNA microarray

cDNA microarray was performed according to the manufacturer's instructions. 20 μg of the total RNA were labeled with Cy5-dUTP by reverse transcription under Oligo$(dT)_{(12-18)}$ primer direction. The RNA labeling was performed at 42°C for 80 min. After the labeling reaction, cDNA was separated from RNA by the addition of a small amount of NaOH solution (1 M) followed by neutralization with Tris-HCl (1 M, pH 7.5). Cy3-cDNA and Cy5-cDNA samples were combined in a Microcon Column (Millipor, Bedford, MA) and washed 4 times in TE buffer (pH 7.4) by centrifugation. In the final washing step, COT-1 DNA, PolyA and yeast tRNA were added to the washing buffer and centrifuged to make the final volume of the labeled cDNA mixture less than 10 μl. A glass chip containing 12,000 cDNA probes (Biomedicum Biochip Center, Helsinki, Finland) was pretreated with 0.1% SDS, sterile H_2O and 95% ethanol and air-dried. The labeled cDNA mixture was hybridized with the chip in a humid chamber at 65°C overnight. After hybridization, the chip was washed 4 times in sterile water with slight shaking and spun-dry by centrifugation.

4.2. Data analysis

The chip was scanned in ScanArray 4000 Series (Packard BioScience, Billerica, MA, USA), the hybridization images were analyzed using QuantArray Microarray Analysis Software (Packard BioScience) and the data were finally normalized to median using Excel Data Normalization Macro. Microarray analysis was performed in duplicates and the data are presented as the average of 2 independent experiments.

5. REAL-TIME RT-PCR (I, II, III, IV)

5.1. Reverse transcription

Reverse transcription was performed in GeneAmp PCR System 2400 (Perkin Elmer, Oak Brook, IL, USA) using high-capacity cDNA archive kit (Applied Biosystems, Forster City, CA, USA) according to the manufacturer's instructions. 8 μg of total RNA were taken in one reaction. Reaction conditions were as follows: MultiScribe reverse transcriptase activation at 25°C for 10 min, reverse transcription at 37°C for 2 h and enzyme inactivation at 95°C for 5 min. cDNA was stored at -20°C.

5.2. Primer design

Specific oligonucleotide primers for real-time PCR were designed for each gene of interest using PrimerExpress software (Applied Biosystems) according to the recommendations of ABI Prism 2000 Sequence Detection System manufacturer (Applied Biosystems). On-line BLAST search was performed to confirm the specificity of the primer sequences. Primers were ordered from TAG Copenhagen (Copenhagen, Denmark). All primers were designed to be intron-spanning to preclude amplification of genomic DNA.

Gene name	Gene bank account number		Primer sequence
PDF	NM_004864	Forward	5'-CCCGGGACCCTAGAGTT-3'
		Reverse	5'-CAGGTCCTCGTAGCGTTTCC-3'
PDGFRα	NM_006206	Forward	5'-CACCCTGCGTTCTGAACTCA-3'
		Reverse	5'-TTTCTGTTTCCAAATGACAACCA-3'
PDGFRβ	NM_002609	Forward	5'-AGCGCTGGCGAAATCG-3'
		Reverse	5'-TTCACGCGAACCAGTGTCA-3'
PDGF-B	NM_002608	Forward	5'-CGATCCGCTCCTTTGATGAT-3'
		Reverse	5'-TCCAACTCGGCCCCATCT-3'
RPLP0	NM_001002	Forward	5'-AATCTCCAGGGGCACCATT-3',
		Reverse	5'-CGCTGGCTCCCACTTTGT-3'
CYP24	NM_000782	Forward	5'-GCCCAGCCGGGAACTC-3'
		Reverse	5'-AAATACCACCATCTGAGGCGTATT-3'
VDR	NM_000376	Forward	5'-CCTTCACCATGGACGACATG-3'
		Reverse	5'-CGGCTTTGGTCACGTCACT-3'
AR	NM_000044	Forward	5'-TGTCAACTCCAGGATGCTCTACTT-3'
		Reverse	5'-ATTCGGACACACTGGCTGTACA-3'

5.2.2. PCR amplification

Real time quantitative PCR was performed in ABI Prism 2000 Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix reagents (Applied Biosystems). Polymerase chain reaction conditions were as follows: AmpliTaq Gold polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. To verify the specific products, dissociation curves were analyzed after 45 cycles.

The data were quantified by the standard curve method using ABI Prism SDS Data Analysis software (Applied Biosystems). Standard curves were generated on the basis of serial dilutions of cDNA from the control sample. The relative expression ratio of a target gene was calculated based on the reaction efficiency value and the Ct deviation of an unknown sample *versus* a control and expressed in comparison to a reference gene as previously described (Pfaffl 2001). Reference gene (acidic ribosomal phosphoprotein large P0 subunit, RPLP0 gene) was used to normalize the amount of cDNA in the reaction mix.

6. RNA STABILITY ASSAY (I, III)

Cells were pre-treated with with 10 nM calcitriol or vehicle (0.2% ethanol) for 24 h prior to addition of 5 μg/ml actinomycin D followed by incubation during increasing periods of time (1 - 5 h). After 0, 1, 2.5 and 5 h of incubation total RNA was isolated and amount of specific RNA was analyzed by real-time RT-PCR as described in Section 5.

7. WESTERN BLOTTING ANALYSIS (I, II, III)

7.1. Protein extraction

Cells were washed in ice-cold PBS and lysed according to the manufacturer's instructions in M-PER mammalian protein extraction reagent supplemented with protease inhibitors (Complete Mini Protease inhibitor cocktail, 1 tablet per 10 ml buffer) and sodium orthovanadate (1 mM) activated according to the manufacturer's instructions. In brief, 400 μl of modified M-PER reagent were added to each 25 cm^2 flask and left at RT for 5 min with mild agitation. Lysates were collected into Eppendorf tubes, left on ice for 5-10 min and microcentrifuged at 12,000*g* for 10 min to remove cell debris. Supernatant was collected and immediately analyzed in Western blot or stored at -70°C for not longer than 1 month. The total protein concentration in the lysate was measured using BCA protein assay kit.

7.2. Antibodies

PDF-specific (anti-NAG-1/PTGF-β) rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Antiphospho-SMAD1/5/8 and antiphospho-SMAD2 rabbit polyclonal antibody were from Cell Signaling Technology (Beverly, MA). Anti-ERK1/2 and antiphospho-ERK1/2 rabbit polyclonal antibody were obtained from New England BioLabs (Beverly, MA, USA). Mouse monoclonal anti-β-actin antibody was obtained from Sigma (St. Louis, MO, USA). Anti-p53 antibody (DO-7) mouse monoclonal was purchased from Novocastra Laboratories (Newcastle upon Tyne, U.K.). Polyclonal rabbit antibody AR70 was produces against synthetic polypeptides corresponding to the N-terminal amino acids 1-17 of human AR (Tahka et al. 1997).

7.3. SDS-PAGE and Western blotting

Total cell lysate samples containing the same amount of proteins (25 μg) were mixed with 2% SDS Laemmli buffer, heated at 95°C for 5 min and analyzed by electrophoresis in polyacrylamide gel (SDS-PAGE). Proteins separated by SDS-PAGE were electrophoretically transferred to the Protran nitrocellulose membrane (Schleider and Schuell, Dassel, Germany) for 1 h at RT using transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. Membranes were incubated at 37°C

for 1 h in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0) containing 5% nonfat dry milk powder to saturate the nonspecific protein binding sites and incubated with specific primary antibody diluted in either 5% nonfat milk-TBST or 5% BSA-TBST at 4°C overnight with gentle agitation. The membranes were washed 3 times for 10 min with TBST and incubated for 1 h with secondary HRP-conjugated antibody in 1% nonfat milk-TBST with gentle agitation. The membranes were washed 3 times for 10 min with TBST and subjected to ECL detection according to the manufacturer's instructions. The intensities of scanned bands were measured by ImageJ software.

8. CELL GROWTH ASSAYS (I, II)

8.1. Crystal violet staining assay

Relative cell number was quantified at 0, 2, 4 and 6 days as previously described (Kueng et al. 1989). Cells were fixed with 11% glutaraldehyde, washed with deionized water, air-dried and stained with 0.1% crystal violet. After washing with deionized water and air-drying 10% acetic acid was added into wells and absorbance at a wavelength of 590 nm, which represents relative cell number, was measured using Wallac Victor 1420 Multilabel counter (Wallac Oy, Turku, Finland). Two to three independent experiments were performed in which six determinations were used for each sample.

8.2. Cell number counting assay

Cells were harvested with trypsin, pelleted and counted in a Burker chamber (Assistent, Sondheim, Germany). To evaluate a number of viable cells cells were stained with trypan blue as was described previously (Freshney 1987). 0.4% trypan blue dye was added to cell suspension in the ratio 1:5. The plates were left for 5 min. at RT so that non-viable cells absorbed the dye. The number of viable cells (which excluded the stain) was counted in a Burker chamber. Three independent experiments were performed in which three determinations were used for each sample.

9. ADHESION ASSAY

96-well plates were pre-coated with laminin or collagen I in the following procedure. 50 µl of 15 µg/ml laminin or 15 µg/ml collagen I were added to each well. The plates were incubated for 2 h at 37°C followed by washing with PBS containing 0.1% BSA. Nonspecific binding sites were blocked with 0.5% BSA in PBS during incubation for 30 min at 37°C followed by washing with PBS containing 0.1% BSA. The plates were used immediately or kept at 4°C for several days.

Cells were harvested with trypsin, resuspended in growth medium and counted in a Burker chamber using trypan blue dye as described in Section 8.2. Cell suspension diluted to a final count 4×10^5 cells/ml growth medium was plated into 96-well plates pre-coated with laminin or collagen I and incubated for 2 h at 37°C. After incubation the wells were gently rinsed with PBS and fixed with glutaraldehyde. Attached cells were counted in a crystal violet staining assay described in Section 8.1.

10. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (III)

LNCaP cells (3 X 10^5) cultured in 25 cm^2 flasks in the growth medium containing 10% DCC-FBS for three days were pre-treated with 10 nM 1α,25-$(OH)_2D_3$ in the absence or presence of 10 nM DHT for 48 h and then incubated with a physiological concentration of calcitriol for 16 h. The media and cells were collected for the assay of the unmetabolized calcitriol. Radioactive 1α,25-dihydroxy (26,27 methyl-^{3}H) vitamin D_3 TRK 656 5 uCi, Amersham) was added and the samples were pre-purified using the acetonitrile-C18 Sep-Pak Cartridge (Waters, Ireland) (Turnbull et al. 1982) followed by separation of the metabolites by high performance liquid chromatography (HPLC, Pharmacia LKB HPLC pump 2248, VWM 2141, Uppsala, Sweden). The mobile phase solvent system was hexane:dichloromethane:methanol:isopropanol (75:12:6:7). The concentrations of calcitriol were quantified by radioreceptor assay (Reinhardt et al. 1984). The corresponding protein concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL, USA). Enzymatic activity was expressed as fmol/mg protein/h. Data are expressed as means (± SD) of five repeats. Statistical significance was determined by Student's *t*-test.

11. STATISTICS (I, II, III, IV)

Statistical significance was analyzed using Wilcoxon test (Conover 1998, Wilcoxon 1945), if not otherwise stated.

RESULTS

1. cDNA MICROARRAY SCREENING FOR CALCITRIOL-REGULATED GENES IN HUMAN PROSTATE CANCER LNCaP CELLS (I)

A search for calcitriol-regulated genes in human prostate cancer LNCaP cells was performed using cDNA microarray. In the screening commercial cDNA chips were used made for simultaneous analysis of 100.000 human genes. LNCaP cells were incubated with 10 nM calcitriol for 24 h and subjected to RNA isolation followed by reverse transcription. Further analysis made on cDNA microchips revealed calcitriol-induced increase in mRNA levels of approximately 2000 genes and decrease in mRNA levels of approximately 200 genes (1.8-fold change was used as a threshold level). Further study was narrowed to genes encoding for the components of growth factor signaling pathways mainly of transforming growth factor-β (TGF-β) and platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) superfamilies and the regulation of their expression by calcitriol.

Among these candidates the most prominent increase in mRNA level (2.5 fold) was revealed for prostate derived factor (PDF), known as potent antimitogenic, prodifferentiative and proapoptotic factor. mRNA levels for other TGF-β superfamily growth factors, receptors and binding proteins were not significantly changed.

Among those genes encoding for PDGF/VEGF superfamily growth factors and receptors, cDNA microarray revealed significant decrease (50%) in mRNA level for platelet-derived growth factor receptor β (PDGFRβ), which mediates diverse PDGF actions including a potent mitogenic effect on prostate cancer cells.

On the basis of cDNA microarray data and literature search we chose PDF and PDGFRβ genes as promising calcitriol-regulated genes for further investigation.

2. INDUCTION OF PROSTATE-DERIVED FACTOR (PDF) EXPRESSION BY CALCITRIOL (I)

2.1. Basal expression of PDF gene in prostate cancer epithelial cells

The expression of PDF gene in LNCaP cells was further studied with real-time RT-PCR and Western blotting assays. A comparative analysis of PDF mRNA levels in two prostate cancer epithelial cells lines, LNCaP and PC-3, was performed. The results of RT-PCR showed that LNCaP cells contain about 5 times more PDF mRNA compared to PC-3 cells. At Western blot of total protein lysates from LNCaP cells incubated with PDF-specific antibodies a band was revealed, which corresponded to PDF in molecular weight (MW 35 kDa). These data suggest that LNCaP cells produce detectable level of PDF.

2.2. Induction of PDF gene expression by calcitriol

The initial cDNA microarray data on the induction of PDF gene expression by calcitriol were confirmed and quantified by real-time RT-PCR and Western blotting. Incubation of LNCaP cells with calcitriol led to a significant increase in PDF mRNA level, which could be seen after 4 h of incubation with 10 nM calcitriol and reached

2.5-3 fold after 24 h of incubation. Induction was dose-dependent in the concentration range of 0.1 – 100 nM calcitriol.

RNA stability assay with actinomycin D showed that induction of PDF mRNA level by calcitriol was not accompanied by increase in PDF mRNA stability, which led to the conclusion that calcitriol-mediated induction of PDF gene expression is mediated via transcriptional rather than posttranscriptional mechanism. Further support for the direct transcriptional mechanism of calcitriol-induced PDF was obtained in the assay with translation inhibitor cycloheximide, which demonstrated that cycloheximide did not prevent calcitriol-induced increase in PDF mRNA level (Figure 5).

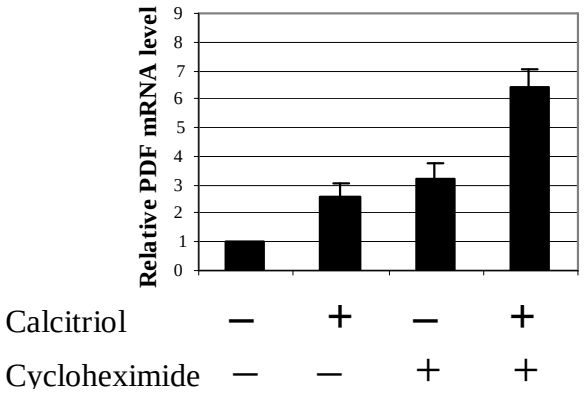

Figure 5. Regulation of PDF mRNA level with calcitriol and cycloheximide. LNCaP cells were incubated with 10 nM calcitriol, 10 μg/ml cycloheximide or both substances for 24 h. RNA was isolated and analyzed by quantitative RT-PCR. The relative expression ratio of the target gene was calculated based on reaction efficiency value and the C_T deviation of the unknown sample versus the control and expressed in comparison to the reference gene (RPLP0).

For the analysis of the regulation of PDF protein level by calcitriol LNCaP cells were incubated with calcitriol and subjected to total protein extraction followed by SDS-PAGE, transfer to nitrocellulose membrane and incubation with PDF-specific antibody. Western blotting revealed significant increase in PDF protein level in calcitriol-treated cells, which could be seen after 24 h of incubation with 10 nM calcitriol and reached approximately 3-4 fold after 72 h of incubation and which was dose-dependent within concentration range of 0.1 – 100 nM calcitriol.

2.3. Pifithrin-α does not prevent calcitriol-mediated increase in PDF mRNA level

To check if tumor suppressor p53 is involved in calcitriol-mediated induction of PDF gene expression selective p53 inhibitor pifithrin-α was used. LNCaP cells were pre-treated with 30 μM pifithrin-α or vehicle (DMSO) for 24 h followed by incubation with 10 nM calcitriol, 30 μM pifithrin-α or both substances for 16 h. RNA was isolated and analyzed by quantitative RT-PCR with PDF-specific primers. Pifithrin-α caused increase in PDF mRNA level and had no significant effect on calcitriol-mediated elevation of PDF mRNA level (Figure 6).

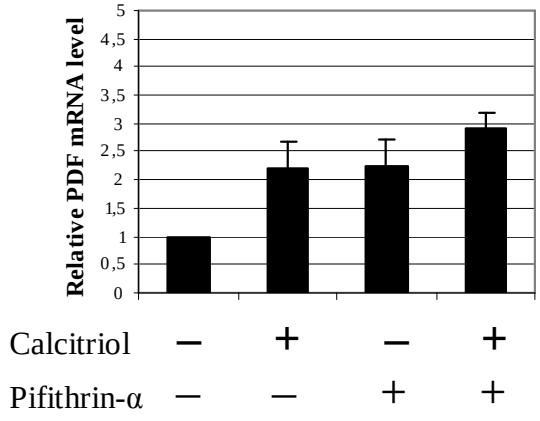

Figure 6. Regulation of PDF mRNA level with calcitriol and pifithrin-α. LNCaP cells pre-treated with pifithrin-α were incubated with 10 nM calcitriol, 30 μM pifithrin-α or both substances for 16 h. RNA was isolated and analysed by quantitative RT-PCR. The relative expression ratio of the target gene was calculated as in Figure 5.

2.4. PDF action in LNCaP cells and its regulation by calcitriol

2.4.1. Suppression of cell growth with PDF

To evaluate the biological activity of PDF in LNCaP cells human recombinant PDF (hrPDF) was used in cell growth assay. The results obtained using crystal violet cell growth assay demonstrated that incubation of LNCaP cells with 50 – 250 ng/ml hrPDF for 6 days led to a significant decrease in cell number (Figure 7). Inhibition of LNCaP cell growth with hrPDF was not dose-dependent and was about the same in the concentration range used.

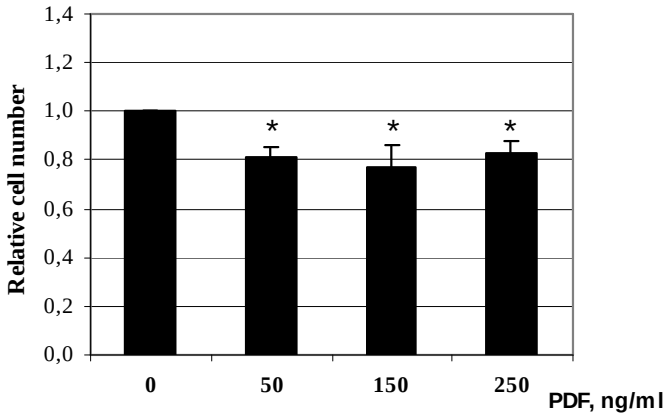

Figure 7. Inhibition of LNCaP cell growth by PDF. Cells were treated with 50, 150 and 250 ng/ml hrPDF for 6 days. Relative cell number was quantified using crystal violet assay. Relative cell number counted as absorbance at 590 nm normalized to vehicle-treated sample. Asterisk, significantly different from control at $p < 0.05$

2.4.2. Lack of the effect of PDF on the adhesion of LNCaP cells to basement membrane proteins.

To study the effect of PDF on the adhesive properties of LNCaP cells 96-well plates were covered with laminin or collagen I, the basement membrane proteins. Cells were incubated with 50 ng/ml hrPDF for 24 h and their adhesion to pre-coated 96-well plates was assayed. No significant change in the number of cells attached to the laminin- or collagen I-coated plates could be seen for cells incubated with hrPDF (Figure 8) suggesting that this growth factor does not affect the ability of LNCaP cells to adhere to common basement membrane proteins.

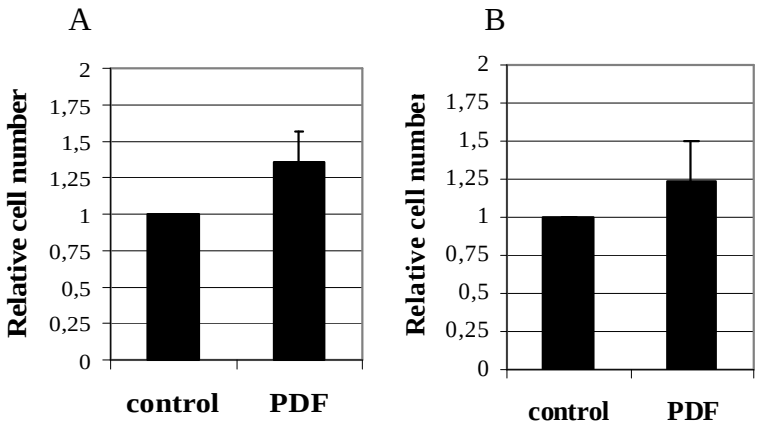

Figure 8. Adhesion of LNCaP cells to basement memebrane proteins. Cells were incubated with 50 ng/ml hrPDF for 24 h and plated into 96-well plates pre-coated with laminin (A) or collagen I (B). Relative cell number quantified and counted as in Figure 7.

2.4.3. Induction of MAP kinase phosphorylation by PDF

To identify the pathways mediating PDF action in LNCaP cells we studied the activation of two main potential candidate mediators – SMAD proteins and ERK MAP kinases by hrPDF, which were earlier reported to be involved in PDF signaling.

To study SMAD phosphorylation by PDF LNCaP cells incubated with 50 ng/ml hrPDF for 15 – 90 min. were subjected to total protein extraction followed by SDS-PAGE and Western blotting. After incubation with antibodies specifically binding to phosphorylated forms of SMAD1/5/8 (P-SMAD1/5/8) and phosphorylated forms of SMAD2/3 (P-SMAD2/3) no effect of hrPDF on SMAD phosphorylation within the time-range studied could be seen. Taking into account the impaired action of TGF-β1 in LNCaP cells (due to the lack of TβRII) we suggested that SMAD-mediated pathway of PDF signaling does not act as well and the antiproliferative activity of PDF is mediated via alternative pathways.

To study ERK MAP kinase phosphorylation by PDF LNCaP cells incubated with 50 ng/ml hrPDF for 2 - 30 min. were subjected to total protein extraction followed by SDS-PAGE and Western blotting. Incubation with antibodies specifically binding to phosphorylated forms of ERK1/2 (P-ERK1/2) revealed a significant increase in both P-ERK1 and P-ERK2 levels, which came early and was transient (the effect could be seen after 2 min incubation with hrPDF and was spontaneously reversed after 10 min. of incubation).

2.4.4. Restoration of calcitriol-suppressed cell growth with PDF-specific antibody

To evaluate the biological significance of basal and calcitriol-induced PDF PDF-specific antibody was used to neutralize the growth factor secreted into the culture medium. The growth curve made with LNCaP cells incubated with pre-tested dilutions of PDF-specific antibodies demonstrated no effect of the antibody on cell growth. These data suggest that the constitutively expressed PDF gene did not significantly contribute to LNCaP cell growth, probably due to the relatively low basal expression level.

However, the growth curve made with LNCaP cells incubated with 10 nM calcitriol in the presence of pre-tested dilutions of PDF-specific antibodies demonstrated that the number of LNCaP cells incubated with a combination of calcitriol and anti-PDF for 4 days was increased 1.3-1.5 fold compared to the number of cells incubated with calcitriol alone. These data show that PDF-specific antibodies partially reverse calcitriol-mediated suppression of LNCaP cell growth and suggest that calcitriol-induced PDF contributes to LNCaP cell growth.

3. REPRESSION OF PLATELET-DERIVED GROWTH FACTOR-BB (PDGF-BB) SIGNALING BY CALCITRIOL (II)

3.1. Differential expression of PDGF receptor genes in prostate epithelial and stromal cells

To study the expression of PDGFRβ gene in prostate epithelial and stromal cells the examination of basal level of the expression of this gene was performed using real-time RT-PCR and Western blotting assays. A similar analysis of PDGFRα gene

expression was conducted to obtain complete information on the expression of PDGF receptor genes in these cells. A comparative analysis of PDGFRα and PDGFRβ mRNA levels in two prostate cancer epithelial cells lines, LNCaP and PC-3, was performed. The results of RT-PCR showed that LNCaP cells contain approximately the same amount of PDGFRα and PDGFRβ mRNA as PC-3 cells do. However, on a Western blot of total protein lysates from LNCaP as well as from PC-3 cells incubated with antibodies specific for PDGFRα or PDGFRβ no band was revealed, which might correspond to PDGFRα and PDGFRβ in molecular weight (MW 190 kDa). To exclude the possibility that the antibodies failed to bind PDGFRs, Western blotting analysis of PDGFRs in control cells (prostate primary stromal cells) was performed, which obtained positive results on the PDGFRα and PDGFRβ proteins content in stromal cells. As was demonstrated by RT-PCR stromal P29SN and P32S cells contained remarkably higher levels of PDGFRs mRNA than LNCaP and PC-3 cells. In prostate primary stromal cells amplification of PDGFRα mRNA was detected at C_T (Cycle at threshold) 20-22 compared to C_T 25-27 in PC-3 cells and C_T 31-33 in LNCaP cells. The amplification of PDGFRβ mRNA was detected at C_T 20-22 in primary stromal cells compared to C_T 26-28 in LNCaP and PC-3 cells. These data suggest that the basal expression levels of both PDGFRα and PDGFRβ genes are low in prostate epithelial cells, LNCaP and PC-3. Interestingly, the expression pattern of PDGF-B gene in prostate epithelial and stromal cells was found to be the opposite, showing high expression level in LNCaP and PC-3 cells (amplification was detected at C_T 22-25) and low in P29SN and P32S cells (amplification was detected at C_T 30-32). These findings lead to the suggestion that in prostate PDGF-BB signaling is mediated by paracrine rather than autocrine mechanism so that PDGF-BB produced by epithelial cells acts primarily on stromal cells.

However, taking into account the positive crosstalk between PDGF and EGF and other peptide growth factors, it was suggested that low level of PDGFRs gene expression may not reflect the condition present in many cancers such as overexpression of EGF or its hyperactivation as well as similar cancer-specific activation of other peptide growth factors. To see if EGF would regulate the expression of PDGF receptor genes LNCaP cells were incubated with 30 ng/ml human recombinant EGF (hrEGF) for 24 h and subjected to RNA isolation and real-time RT-PCR. The RT-PCR results demonstrated that EGF caused a 3-fold increase in PDGFRβ mRNA level. Western blotting results confirmed RT-PCR data revealing the band corresponding to PDGFRβ in molecular weight in sample treated with 30 ng/ml EGF for 48 h. No effect of EGF on the level of PDGFRα gene expression was found (data not shown).

3.2. Repression of PDGFRβ gene expression by calcitriol

The initial cDNA microarray data on the suppression of PDGFRβ gene expression by calcitriol in LNCaP cells were confirmed and quantified by real-time RT-PCR. The results of RT-PCR demonstrated that calcitriol caused a significant decrease in PDGFRβ mRNA level although the effect was late (50% decrease could bee seen only after 24 h of incubation with 10 nM calcitriol) suggesting the indirect mechanism of regulation. The effect was dose-dependent in the range 1 – 100 nM calcitriol. Incubation of LNCaP cells with 10 nM calcitriol in the presence of 30 ng/ml EGF for 24 h led to a 50-60% decrease in PDGFRβ mRNA level compared to that in LNCaP cells treated with EGF alone. Such a partial reversal of EGF-induced PDGFRβ mRNA level was confirmed on protein level by Western blotting assay with antibody specific for PDGFRβ. Western blotting revealed the band corresponding to PDGFRβ

protein only in LNCaP cells incubated with EGF alone but not in LNCaP cells incubated with combination of EGF and calcitriol.

Incubation of prostate primary stromal P29SN cells with calcitriol led to a significant decrease in PDGFRβ mRNA level, which was dose-dependent in the range 1 – 100 nM calcitriol, as was demonstrated by RT-PCR. The effect was also late, and a significant 20% decrease could be seen only after 24 h of incubation with 10 nM calcitriol.

3.3. Lack of the effect of calcitriol on PDGFRα and PDGF-B mRNA levels

Calcitriol had no significant effect on the mRNA levels of PDGF receptor α and PDGF-B, PDGF subunit, which binds uniquely PDGFRβ (Figure 9). This was demonstrated by quantitative RT-PCR analysis of LNCaP cells incubated with 10 nM calcitriol for the increasing periods of time (4 – 48 h).

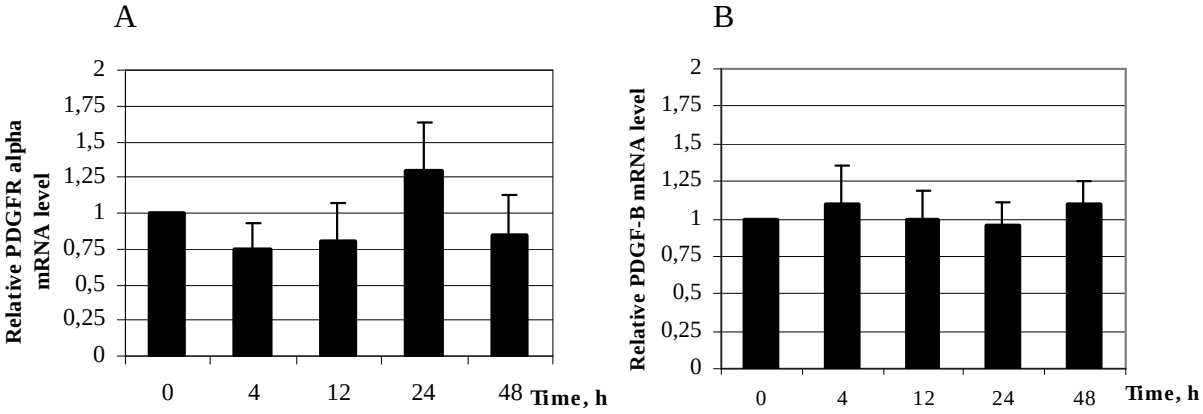

Figure 9. mRNA levels of PDGFRα (A) and PDGF-B (B) in LNCaP cells treated with calcitriol. LNCaP cells were incubated with 10 nM calcitriol for the increasing periods of time (4 - 48 h). Total RNA was isolated and analyzed by quantitative RT-PCR. The relative expression ratio of the target gene was calculated as in Figure 5.

3.4. Regulation of prostate epithelial cell growth by PDGF-BB and EGF

Although PDGF is concerned as a powerful mitogen for human prostate cancer cells we found that it did not control LNCaP cell growth due to low levels of PDGFRα and β. It was suggested that EGF-mediated induction of PDGFRβ gene expression might induce sensitivity of LNCaP cells to growth-promoting effect of PDGF. The cell counting method was used to evaluate the effect of human recombinant PDGF, BB isoform (hrPDGF-BB) and human recombinant EGF (hrEGF) on the growth of LNCaP cells. LNCaP cells were incubated with a combination of 30 ng/ml hrPDGF-BB and 30 ng/ml hrEGF for 6 days. As expected, hrEGF induced some growth response to hrPDGF showing a 1.2-fold increase in cell number compared to cells incubated with EGF alone. These data suggest that the suppression of PDGFRβ gene expression by calcitriol may contribute to its antiproliferative action in EGF-induced LNCaP cells.

3.5. Suppression of PDGF-BB-induced human prostate primary stromal cell growth by calcitriol

The crystal violet staining assay was used to evaluate the effect of hrPDGF-BB on the growth of prostate primary stromal P29SN cells. P29SN cells showed the remarkable growth response to PDGF, its growth being increased 6 fold after 6 days of incubation with 25 ng/ml hrPDGF-BB. Calcitriol suppressed PDGF-induced P29SN cell growth by 20%.

4. CROSSTALK BETWEEN CALCITRIOL AND 5α-DIHYDROTESTOSTERONE IN REGULATING 24-HYDROXYLASE AND PDF GENE EXPRESSION (III, IV)

4.1. Inhibition of calcitriol-induced 24-hydroxylase gene expression and activity by DHT

4.1.1. Inhibition of calcitriol-induced expression of 24-hydroxylase gene by DHT

To investigate the effect of DHT on the action of calcitriol in LNCaP cells the expression of 24-hydroxylase was used as an indicator. It was shown earlier that 10 nM calcitriol at 24 h cause the maximal induction of 24-hydroxylase mRNA level in LNCaP cells (Lou et al. 2004). 24-hydroxylase mRNA levels in LNCaP cells treated with calcitriol, DHT or their combination were evaluated by real-time RT-PCR. The results of RT-PCR demonstrated that 10 nM calcitriol increased the level of 24-hydroxylase mRNA approximately 30,000 fold, whereas DHT alone in the concentration range 0.01-100 nM had no significant effect. The calcitriol-induced level of 24-hydroxylase was strongly decreased by DHT in the concentration range 1-100 nM (by 50-97%) and slightly increased by DHT in the concentration range 0.01-0.1 nM (by 27-37%). As expected, hydroxyflutamide (an agonist for the mutated AR of LNCaP cells) in 1 μM concentration also significantly decreased calcitriol-induced 24-hydroxylase mRNA level and antiandrogen, Casodex, in the same concentration partially antagonized the DHT effect. RNA stability assay with actinomycin D showed that inhibition of 24-hydroxylase mRNA level by DHT was not accompanied by decrease in mRNA stability of 24-hydroxylase. These data are the first indication that DHT reduces the ability of calcitriol to induce the expression of 24-hydroxylase gene.

4.1.2. Suppression of catabolism of calcitriol by DHT

High performance liquid chromatography (HPLC) measurement of calcitriol concentration in culture medium after exposure of cells to 10 nM calcitriol for 16 h was used to evaluate the rate of calcitriol catabolism. Pre-treatment with 10 nM calcitriol for 48 h significantly enhanced the degradation of calcitriol. Pre-incubation with 10 nM calcitriol and 10 nM DHT did not cause a significant change (compared to untreated control) in calcitriol concentration, indicating that 10 nM DHT protects calcitriol from being catabolized by 24-hydroxylase. These data indicate that the biological consequence of DHT-mediated inhibition of 24-hydroxylase gene expression is the protection of calcitriol from inactivation.

4.1.3. Lack of the effect of DHT on 24-hydroxylase mRNA level in androgen independent prostate cancer cells transfected with AR

To evaluate the role of AR in the inhibition of calcitriol-induced level of 24-hydroxylase mRNA, androgen independent prostate cancer PC-3 cells were transfected with wild-type AR or mutated AR containing the same mutation as in LNCaP cells. Transfection efficiency was verified in RT-PCR and Western blotting assays. AR mRNA and protein amounts in AR-transfected PC-3 cells were increased to the levels similar to that in LNCaP cells. However, no effect of DHT on calcitriol-induced 24-hydroxylase gene expression was detected in these cells, suggesting that other factors than AR are necessary for DHT action on 24-hydroxylase gene expression.

4.2. Lack of the effect of DHT on calcitriol-induced PDF gene expression

The study on crosstalk between calcitriol and androgens in LNCaP cells was continued by analyzing the effect of androgens on calcitriol-induced expression of PDF gene. The results of RT-PCR demonstrated that DHT significantly induced PDF mRNA level in LNCaP cells, which could be seen after 24 h of incubation with 10 nM DHT and reached maximum (2 fold) after 48 h of incubation. To see if the induction of PDF gene expression by calcitriol is mediated via an androgen dependent pathway LNCaP cells were incubated with calcitriol in the presence or absence of 10 nM DHT or 50 μM antiandrogen Casodex. Contrary to expectations, Casodex did not prevent calcitriol-induced elevation of PDF mRNA level. Incubation of LNCaP cells with a combination of calcitriol and DHT likewise showed no synergistic effect of PTGF-β mRNA level. These data demonstrate an androgen independent mechanism of calcitriol-induced PDF. Comparison of time curves for PDF mRNA induction with 10 nM calcitriol and 10 nM DHT demonstrated that induction with DHT is slower compared to calcitriol-mediated induction (which could be seen after 2 h of incubation) supporting the androgen independent mechanism of calcitriol action on PDF gene expression.

DISCUSSION

1. INDUCTION OF PDF EXPRESSION BY CALCITRIOL

The study demonstrates for the first time that calcitriol, a hormonally active metabolite of vitamin D, induces gene expression of prostate-derived factor (PDF), a distant member of TGF-β superfamily, in human prostate cancer LNCaP cells. Regulation of PDF by vitamin D metabolites has not been reported earlier. However, induction of this growth factor by various chemopreventive drugs for prostate cancer including retinoids (Newman et al. 2003), genistein (Wilson et al. 2003) and NSAIDs (Baek et al. 2001b, Bottone et al. 2002) has been recently shown. PDF gene was also shown to be a direct target for transactivation by p53 tumor suppressor (Bottone et al. 2002, Li et al. 2000b, Tan et al. 2000). Taking into account that PDF exerts antiproliferative and proapoptotic action towards a variety of malignant cell types, induction of PDF gene expression may comprise a novel potential mechanism of antineoplastic action of vitamin D in prostate cancer.

The present study demonstrates that human recombinant PDF added into the culture medium suppresses the growth of LNCaP cells. LNCaP cells represent an early, androgen dependent stage of prostate cancer and the ability of PDF to inhibit their growth further supports the tumor suppressor activity of TGF-β superfamily growth factors in prostate cancer. The ability of PDF to suppress tumor cell growth was demonstrated earlier *in vitro* and *in vivo* on a number of malignant cell types including colorectal carcinoma and glioblastoma cells (Albertoni et al. 2002, Baek et al. 2001b, Tan et al. 2000), and prostate cancer cells (Lambert et al. 2006); however the data on the role of PDF in prostate cancer cells growth are somewhat controversial. Liu et al. reported no effect of PDF on the proliferation of human prostate cancer LNCaP and DU145 cells as they found by BrdU incorporation assay (Liu et al. 2003). However, in the same study the role of PDF was suggested in decreasing cell adhesion leading to secondary apoptosis. In support of this suggestion the expression of several genes encoding proteins involved in cell adhesion such as RhoE and catenin delta 1 genes was found to be regulated by PDF (Liu et al. 2003). The effect of PDF on adhesion and invasiveness of gastric cells was also found to be mediated by the induction of the urokinase-type plasminogen activator system (Lee et al. 2003). The pathways underlying the growth-inhibitory effect of PDF remain unclear. A role for PDF in apoptosis, differentiation and migration of malignant cells has also been suggested. Importantly, it was found that the apoptosis induced by such antitumorigenic agents as NSAIDs and resveratrol is due to the induction of PDF gene expression in human colorectal cancer cells (Baek et al. 2002, Baek et al. 2001b, Bottone et al. 2002). cDNA microarray demonstrated down-regulation of the antiapoptotic gene metallothionein 1E by PDF in human prostate cancer DU145 cells (Liu et al. 2003). In the present study no effect of the recombinant PDF on LNCaP cells adhesion was found. Also no adhesion-related genes which are regarded as potential targets for PDF action were regulated by calcitriol according to cDNA microarray results. Further investigation of specific pathways potentially mediating the inhibitory effect of PDF on LNCaP cell growth such as induction of apoptosis and differentiation are needed to clarify the mechanism of PDF action in prostate cancer cells.

Calcitriol does not induce PDF gene expression in human prostate cancer PC-3 cells as the study shows, although these cells are responsive to growth-inhibitory effect of calcitriol (but to a lesser extent than LNCaP,(Skowronski et al. 1993). Analysis of

the expression of PDF gene in LNCaP and PC-3 cells shows the higher level of PDF gene expression in LNCaP cells compared to PC-3. These results are in agreement with the literature. In a comparative study of human prostate cancer LNCaP, PC-3, and DU145 cells it has been shown that LNCaP cells produced the highest levels of active PDF protein of the cell lines studied (Liu et al. 2003). The results on the lack of calcitriol-mediated induction of PDF gene expression in PC-3 cells suggest that this induction might be mediated via pathways which are not functional in PC-3 cells. We tested two potential pathways – mediated via androgen receptor (AR) and tumor suppressor p53. Both AR and p53 are known to mediate induction of PDF gene expression and these pathways are disabled in PC-3 cells (p53-null, androgen insensitive cell line). The blockade of AR signaling with antiandrogen Casodex did not prevent induction of PDF gene expression by calcitriol in LNCaP cells. Together with the early response to calcitriol (increase in RNA level after 2 h of treatment), independence from protein synthesis and lack of effect of calcitriol on PDF mRNA stability these data suggest a direct mechanism of regulation of PDF gene expression. However, whether PDF gene is a direct target for transactivation by VDR remains to be investigated in transcription assay *in vitro*.

In LNCaP cells p53 inhibitor pifithrin α, which had been shown to block p53 signaling in a variety of cell types, including human prostate cancer cells (Jiang et al. 2004, Mohapatra et al. 2005) did not prevent calcitriol-mediated increase in PDF mRNA. In opposite, pifithrin α caused significant increase in PDF mRNA level. However, the conclusion that calcitriol-mediated induction of PDF gene goes via p53-independent mechanism would not be consistent with recently reported finding of Lambert et al. who clearly demonstrated that the induction of PDF gene by calcitriol in nine prostate cancer cell lines including LNCaP is dependent on functional p53 (Lambert et al. 2006). In addition, they demonstrated the requirement for functional p53 for the ability of VDR to induce PDF gene expression in prostate cancer ALVA-31 cells (p53-null) transfected with p53 expression plasmid, and in LNCaP cells expressing dominant negative p53 (Lambert et al. 2006). The discrepancy between our results and those obtained by Lambert et al. may be due to different methods used to study impact of p53 in calcitriol-mediated PDF induction. Recently it was reported that in fact pifithrin-α can not be regarded as a ubiquitous inhibitor of p53 function as it did not inhibit p53 function in two wild-type p53 human tumor cell lines (Walton et al. 2005). It was also shown that pifithrin-α is unstable in culture medium and easily precipitates (Walton et al. 2005). Moreover, this substance may potentiate p53 activity (Kaji et al. 2003). These features of pifithrin-α may likely account for its inability to prevent calcitriol-induced expression of PDF gene in LNCaP cells demonstrated in the present study.

The molecular mechanism of PDF action in prostate cancer cells and the nature of PDF cell surface receptor remain unclear. Tan et al. reported that PDF may act through classical TGF-β pathway mediated by activation of TβRI and TβRII and subsequent activation of SMAD4 to suppress growth of malignant cells (Tan et al. 2000). We could not detect the effect of recombinant PDF on the phosphorylation of R-SMAD proteins, neither of SMAD2/3, the primary intracellular mediators of TGF-β signals, nor of SMAD1/5/8, that mediate BMP signals. The lack of effect of PDF on SMAD phosphorylation in LNCaP cells may be explained by the impaired classical TGF-β signaling pathway due to the lack of functional TβRI in these cells suggested in some studies (Kim et al. 1996, Song et al. 2006, van der Poel 2005). The lack of TβRI in LNCaP cell seems to be due to a mutation in TβRI gene resulting in rapidly degraded TβRI mRNA (Kim et al. 1996, Song et al. 2006). However, data on the

responiveness of LNCaP cells to growth inhibitory action of TGF-β are not consistent (Chipuk et al. 2002). In the present study it was demonstrated that human recombinant TGF-β1 had no effect on LNCaP cell growth, supporting the suggestion that the TGF-β signaling pathway is not active in these cells.

In a search for other possible mediators of PDF signaling we found a report on a strong phosphorylation of mitogen-activated protein kinase kinases 1 and 2 (MAPKK1/2) and extracellular regulated kinases 1 and 2 (ERK1/2) by PDF in human gastric cancer cells, which was independent on the activation of TGF-β receptors, led to activation of the urokinase-type plasminogen activator system, and enhanced invasiveness of these cells (Lee et al. 2003). Indeed, we could see a fast and transient increase in the phosphorylation of MAP kinases (ERK1 and ERK2) after exposure of LNCaP cells to growth-inhibitory concentrations of recombinant PDF. These data give rise to the possibility that the effect of PDF in LNCaP cells may be mediated through a pathway alternative to the classical TGF-β signaling pathway, such as the MAPKKK/ERK cascade. ERKs are traditionally viewed as growth-promoting and survival factors (MacCorkle and Tan 2005, Torii et al. 2006). However, some recent reports have suggested that the ERK pathway may induce events both associated with cell proliferation and cell cycle arrest, differentiation and apoptosis dependent on the intracellular substrates (Peyssonnaux and Eychene 2001). Recently it has been shown that MAP kinases may phosphorylate and activate p53, leading to p53-mediated cellular responses (Wu 2004). It has been also found that constitutive MAPKKK activity may contribute to the phosphorylation of p53 and p53-dependent apoptosis. Blocking of the activity of this kinase with a specific inhibitor resulted in decreased ERK1/2 activation accompanied by a change of the cellular reaction from apoptosis to cell cycle arrest (Brown and Benchimol 2006). The biological consequences of PDF-induced ERK activation remain to be solved.

As was demonstrated in the present study, PDF-specific antibodies partially reversed the growth-inhibitory effect of calcitriol in LNCaP cells further supporting the suggestion that PDF at least partially mediates the growth-inhibitory effect of calcitriol in these cells. The expression of genes encoding for other proteins involved in TGF-β superfamily growth factors signaling was not regulated by calcitriol in LNCaP cells according to our cDNA microarray results, although many of them, such as TGF-β1, TGF-β2, TGF-β3, latent TGF-β binding protein, TβRI, and TβRII genes, are known to be regulated by vitamin D compounds in other cell types, whose growth is inhibited by this steroids. These data further support the cell type specific character of growth-inhibitory action of calcitriol. The question that remains to be solved is whether induction of PDF by calcitriol represents a specific feature of LNCaP cells or a more general mechanism in calcitriol-mediated suppression of cancer cells growth.

2. INTERACTION BETWEEN DHT AND CALCITRIOL IN REGULATING 24-HYDROXYLASE AND PDF GENE EXPRESSION

In prostate the action of vitamin D metabolites is closely connected to the action of androgens, which are the primary regulatory hormones governing the development and the maintenance of normal prostate and prostate cancer progression. To date data on the requirement for androgen signaling for calcitriol-mediated growth inhibition of LNCaP cells remain controversial (Bao et al. 2004, Qiao et al. 2003, Yang et al. 2002, Zhao et al. 1997, Zhao et al. 1999a, Zhao et al. 2000). In the present study the expression of 24-hydroxylase gene was used as an indicator for the effect of

androgens on calcitriol action. The study demonstrates that DHT regulates calcitriol-induced gene expression of 24-hydroxylase, a key enzyme in the inactivation of calcitriol, in LNCaP cells in a concentration-dependent manner. The basic level of 24-hydroxylase gene expression in LNCaP cells is low and calcitriol causes a remarkable increase in 24-hydroxylase mRNA level. DHT at low, growth-stimulating concentrations (0.01-0.1 nM) slightly increases the levels of the induced 24-hydroxylase mRNA and at higher, growth-inhibitory concentration (1-100 nM) causes up to 99% suppression. The results of HPLC analysis show that 10 nM DHT maintains higher concentration of calcitriol in cell culture medium confirming that DHT indeed reduces catabolism of calcitriol. Using AR antagonist Casodex and AR agonist hydroxyflutamide (acts as AR agonist due to the mutant AR in LNCaP cells) and also androgen-resistant cells, it is demonstrated that AR is required for the effect of DHT. Indeed, the induction of 24-hydroxylase expression by calcitriol is strongly inhibited by hydroxyflutamide, while Casodex partially antagonizes the action of DHT. The partial action of Casodex is likely due to the dose (100-fold excess) used in the study, which provides only partial inhibition of DHT binding. These data are the first indication that DHT reduces the ability of calcitriol to induce the expression of 24-hydroxylase gene and not only support the earlier finding of a crosstalk between androgen and vitamin D metabolites in human prostate cancer cells but also provide a possible mechanism how androgen and vitamin D signaling pathways may interact.

Meanwhile the induction of PDF gene expression by calcitriol is found to occur via an androgen independent mechanism. It is shown that Casodex in 1 μM concentration (5000-fold excess), which is high enough to block androgen action, does not prevent the induction of PDF gene expression by calcitriol and, although growth-inhibitory concentrations of DHT potently induce the expression of this gene, no synergistic effect of calcitriol and DHT on PDF gene expression can be seen. The androgen independent activities of vitamin D metabolites in prostate cancer cells are important issues because they represent potential activities of vitamin D compounds in hormone-refractory high-grade prostate cancer. The ability of calcitriol to inhibit growth of hormone-refractory prostate cancer cells has been found earlier, and this finding suggests that in these cells calcitriol acts via an androgen independent mechanism (Zhao et al. 2000). It was also suggested that even in hormone sensitive prostate cancer cells such as LNCaP some of the vitamin D activities are androgen independent. Our data on the induction of PDF gene expression by calcitriol support the latter suggestion.

3. SUPPRESSION OF PDGFRβ GENE EXPRESSION BY CALCITRIOL

The next finding of the study is calcitriol-mediated inhibition of platelet-derived growth factor-BB (PDGF-BB) signaling in LNCaP cells resulting from suppression of platelet-derived growth factor receptor β (PDGFRβ) gene expression. The expression of PDGFRα and PDGF-B genes was not regulated by calcitriol in LNCaP cells as was demonstrated by real-time RT-PCR method. The expression pattern of genes encoding components of PDGF signaling pathways in human prostate has not been fully characterized before. In the present study it is shown that the basal expression levels for PDGF receptors (both PDGFRα and PDGFRβ) are low in human prostate cancer epithelial cells LNCaP and PC-3. However, it is demonstrated that in the specific inducing conditions such as in the presence of elevated level of EGF in culture medium calcitriol-mediated suppression of PDGFRβ is biologically significant.

The regulation of PDGF signaling by vitamin D compounds has been poorly studied although this pathway has been implicated in prostate cancer. Three subunits of PDGF, PDGF-A, PDGF-B and PDGF-D are regarded as potentially involved in prostate cancer progression (Fudge et al. 1994, Ustach et al. 2004, Vlahos et al. 1993). PDGF-BB isoform is used in the present study as the most common isoform acting uniquely through PDGFRβ homodimer. Earlier reports have demonstrated a mitogenic effect of PDGF-BB on cultured human benign prostate hyperplasia (BPH) cells and have shown that its mitogenic signal was mediated via high affinity PDGFRβ in these cells (Vlahos et al. 1993). The present study demonstrates that LNCaP cells are resistant to mitogenic action of PDGF-BB due to the low level of receptor expression. The study also demonstrates that human recombinant EGF added into the culture medium induces responsiveness of these cells to the mitogenic action of PDGF-BB via induction of PDGFRβ gene expression. Although recombinant EGF induces only a moderate (3-fold) increase in PDGFRβ mRNA level it results in the synthesis of PDGFRβ protein detectable in Western blotting. In this context calcitriol prevents EGF-induced PDGFRβ gene expression and the growth-promoting effect of PDGF in LNCaP cells. Overall these results suggest that the potency of calcitriol action in the control of PDGF-BB signaling in LNCaP cells is limited to specific conditions such as, for example, elevated EGF signaling, which, however, commonly occurs in prostate cancer.

Comparative study of mRNA levels of PDGF receptors and PDGF-B subunit in LNCaP and PC-3 cell lines, which are both epithelial, and human prostate primary stromal cells P29SN and P32S demonstrates the opposite pattern of gene expression for PDGF receptors and PDGF-B ligand in epithelial and stromal cells. Both stromal cell lines are characterized by high levels of PDGFRα and PDGFRβ mRNA and protein and low level of PDGF-B mRNA, while both epithelial cell lines are characterized by high level of PDGF-B mRNA and low levels of PDGFRα and PDGFRβ gene expression. These data permit speculation of a primary role for prostate epithelial cells in the production of PDGF-BB, which mostly act in a paracrine manner on the stromal cells of the prostate. Indeed, the difference in sensitivity to the mitogenic effect of the recombinant PDGF-BB correlates well with the levels of PDGFRα and PDGFRβ expression in stromal and epithelial cells. The present study shows that calcitriol suppresses the expression of PDGFRβ gene in P29SN cells and reduces the mitogenic effect of the recombinant PDGF-BB in these cells.

Nowadays specific inhibitors of tyrosine kinase receptor pathways such as the PDGF receptor tyrosine kinase are regarded as some of the most attractive agents in the treatment of many types of malignancies including prostate cancer. It was recently demonstrated that such inhibitors as SU101 and imatinib mesylate (STI571) showed a positive effect in prostate cancer prevention in animal studies and clinical trials (George 2003, Ko et al. 2001, Uehara et al. 2003). However, these inhibitors are regarded mostly in their relation to the inhibition of cancer angiogenesis, which is a limiting step in tumor progression. The understanding of a link between PDGF and EGF is one of the issues addressed in this study, which is important in our understanding of the mechanisms of action of selective tyrosine kinase inhibitors and their use in cancer therapy.

The data obtained in the present study support the role of vitamin D compounds in the regulation of PDGF-BB signaling in human prostate and suggest a new potential mechanism of calcitriol-mediated inhibition of prostate cancer LNCaP cell growth.

SUMMARY AND CONCLUSION

This study demonstrates the induction of gene expression of prostate-derived factor (PDF), a growth factor belonging to the transforming growth factor-β superfamily, by calcitriol in androgen sensitive human prostate cancer LNCaP cells.

In cell growth assay, PDF demonstrates a moderate antiproliferative effect in LNCaP cells, suggesting that regulation of its expression may be an important factor in LNCaP cell growth control.

Calcitriol induces PDF gene expression via androgen independent pathway supporting the hypothesis that even in androgen sensitive prostate cancer cells, such as the LNCaP cells, the antiproliferative effect of vitamin D compounds is at least partially independent of androgen receptor.

On the other hand, the study shows that androgens may increase the availability of calcitriol through regulation of calcitriol-induced gene expression of 24-hydroxylase, the key enzyme in calcitriol inactivation. Interaction of vitamin D compounds and androgens in the regulation of vitamin D catabolism indicates a new level of crosstalk between these steroids in hormone dependent prostate cancer.

Calcitriol suppresses the signaling of platelet-derived growth factor-BB (PDGF-BB) in LNCaP cells stimulated with epidermal growth factor and in prostate primary stromal cells via inhibition of gene expression for platelet-derived growth factor receptor β.

The two pathways involved in the regulation of growth of androgen sensitive human prostate cancer LNCaP, mediated by PDF and PDGF-BB, are important mediators of the growth-inhibitory effect of calcitriol in these cells, as was found in this study. However, the regulation of these specific pathways and its impact on the calcitriol-mediated growth suppression of other cell types need further characterization.

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APPENDIX (original communications)

I. Reprinted from Int J Cancer, 112(6), Nazarova N, Qiao S, Golovko O, Lou YR, Tuohimaa P "Calcitriol-induced prostate-derived factor: autocrine control of prostate cancer cell growth", pp. 951-958, Copyright © 2004, with permission from JOHN WILEY & SONS

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ERRATUM

There is an error in the original communication I:
Page 956, caption to Figure 6, “Inhibition of LNCaP cell growth by calcitriol and hrPDF. Cells were treated with 10 nM calcitriol or 50 ng/ml hr PDF or vehicle (0.1% ethanol)” should read “Inhibition of LNCaP cell growth by human recombinant TGF-β1 (TGFβ1) and human recombinant PDF (PDF). Cells were treated with 10 ng/ml TGFβ1 or 50 ng/ml PDF or vehicle (4 mM HCl)”.