Published in final edited form as:

J Steroid Biochem Mol Biol. 2015 April ; 148: 156–165. doi:10.1016/j.jsbmb.2014.10.004.

Differential expression and regulation of vitamin D hydroxylases and inflammatory genes in prostate stroma and epithelium by 1,25-dihydroxyvitamin D in men with prostate cancer and an in vitro model

Angeline A. Giangreco^a, Shweta Dambal^a, Dennis Wagner^c, Theodorus Van der Kwast^e, Reinhold Vieth^c, Gail S. Prins^f, and Larisa Nonn^{a,b,*}

^a Department of Pathology, University of Illinois at Chicago, IL, USA

^b University of Illinois Cancer Center, Chicago, IL, USA

^c Department of Nutritional Sciences, Laboratory Medicine and Pathobiology, University of Toronto, ON, Canada

^e Department of Pathology, University Health Network, University of Toronto, ON, Canada

^f Department of Urology, University of Illinois at Chicago, IL, USA

Abstract

Previous work on vitamin D in the prostate has focused on the prostatic epithelium, from which prostate cancer arises. Prostatic epithelial cells are surrounded by stroma, which has well-established regulatory control over epithelial proliferation, differentiation, and the inflammatory response. Here we examined the regulation of vitamin D-related genes and inflammatory genes by 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D) in laser-capture microdissected prostate tissue from a vitamin D₃ clinical trial and in an in vitro model that facilitates stromal–epithelial crosstalk. Analysis of the trial tissues showed that VDR was present in both cell types, whereas expression of the hydroxylases was the highest in the epithelium. Examination of gene expression by prostatic (1,25(OH)₂D) concentrations showed that VDR was significantly lower in prostate tissues with the highest concentration of 1,25(OH)₂D, and down-regulation of VDR by 1,25(OH)₂D was confirmed in the primary cell cultures. Analysis of inflammatory genes in the patient tissues revealed that IL-6 expression was the highest in the prostate stroma while PTGS2 (COX2) levels were lowest in the prostate cancer tissues from men in the highest tertile of prostatic 1,25(OH)₂D. In vitro, TNF- α , IL-6 and IL-8 were suppressed by 1,25(OH)₂D in the primary epithelial cells, whereas TNF- α and PTGS2 were suppressed by 1,25(OH)₂D in the stromal cells. Importantly, the ability of 1,25(OH)₂D to alter pro-inflammatory-induced changes in epithelial cell growth were dependent on the presence of the stromal cells. In summary, whereas both stromal and epithelial

© 2014 Published by Elsevier Ltd.

* Corresponding author at: Department of Pathology, 840 S. Wood St, Room 130 CSN, Chicago, IL 60612, USA. Tel.: +1 312 996 0194; fax: +1 312 996 7586. lnonn@uic.edu, lnonn@me.com (L. Nonn)..

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jsbmb.2014.10.004>.

There are no competing financial interests in relation to this work for any of the authors.

cells of the prostate express VDR and can presumably respond to $1,25(\text{OH})_2\text{D}$, which appears to be primarily produced by the prostatic epithelium. Further, while the prostate epithelium was more responsive to the anti-inflammatory activity of $1,25(\text{OH})_2\text{D}$ than stromal cells, stroma–epithelial crosstalk enhanced the phenotypic effects of $1,25(\text{OH})_2\text{D}$ and the inflammatory process in the prostate gland.

Keywords

Prostate epithelium; Prostate stroma; Inflammation; Vitamin D

1. Introduction

The potential chemopreventive effects of the pro-hormone vitamin D on prostate tissue and cells have been shown in vitro cell culture and in vivo animal studies as well as in epidemiologic studies and clinical trials [1]. In addition to its classical function in calcium homeostasis and bone health, $1\alpha,25$ -dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) has tumor suppressive functions in cells and tissues including the prostate. In prostate cells, $1,25(\text{OH})_2\text{D}$, the active metabolite of vitamin D, is anti-proliferative [2,3], pro-apoptotic [4], pro-differentiating [5], and anti-inflammatory [5,6]. CYP2R1 and CYP27A1 are the enzymes proposed to convert cutaneously-synthesized or dietary vitamin D_3 into circulating $25(\text{OH})\text{D}$ [1]. CYP27B1 further hydroxylates $25(\text{OH})\text{D}$ to the active hormone $1,25(\text{OH})_2\text{D}$ [1]. CYP24A1 hydroxylates and inactivates $1,25(\text{OH})_2\text{D}$ into $1,24,25(\text{OH})_3\text{D}$ [1].

The anti-inflammatory activities of $1,25(\text{OH})_2\text{D}$ involve inhibition of PTGS2 (COX-2), IL-6 and IL-8 in human prostate cells [5,6]. In vivo, the $1,25(\text{OH})_2\text{D}$ analog, elocalcitol, decreased inflammatory infiltrate in the prostate, nitric oxide signaling, and cytokine production in a mouse model of prostatitis [7].

Prostate glandular epithelium is surrounded by stroma which contains smooth muscle cells, fibroblasts, myofibroblasts and resident macrophages [8]. Although prostate cancer (PCa) emerges from epithelial cells, there is evidence that a permissive stromal environment influences epithelial tumor growth. The stroma micro-environment can become “reactive” stroma around prostatic intraepithelial neoplasia (PIN) and PCa, which further evolves as carcinoma progresses [9,10]. This interplay may be especially important during the early stages of cancer when immune cells may be tumor suppressive or tumor promoting depending on the existing immune environment [11,12]. Tumor growth promoting effects of the stroma have been shown in several studies in which co-inoculation of fibroblasts with PCa cells increased xenograft growth [13–15].

In general, inflammation is a risk factor for many cancers including PCa [16,17]. A meta-analysis showed an increased risk of PCa incidence in men with a history of prostatitis (odds ratio 1.65; 95% confidence interval (CI): 1.32–2.06) or syphilis (odds ratio 1.5; 95% CI: 0.6–3.5) [18]. Proliferative inflammatory atrophy (PIA) lesions, which are associated with chronic inflammation, have similar gene expression changes (NKX3.1, PTEN, and p27) as PIN and PCa and thus may be precursors to carcinogenesis [19–21].

The expression of VDR and vitamin D metabolism genes has not previously been reported according to cell type in prostate tissue. Considering the importance of the stroma–epithelial interaction in the prostate and that both these cell types express the vitamin D receptor (VDR) [22–24], we hypothesized that stromal and epithelial cells have unique functions in mediating vitamin D actions within the prostate gland. To address the possibility, the present study examined the gene expression of vitamin D-related and inflammatory genes in laser capture-microdissected tissue from a clinical trial of men who received oral vitamin D₃ prior to radical prostatectomy. We further dissected the differential responses of prostate epithelial and stromal cells to 1,25(OH)₂D in a 3-D co-culture model of inflammation. Our findings establish a role for stromal–epithelial crosstalk for the therapeutic response to vitamin D in patients.

2. Materials and methods

2.1. Clinical trial specimens and serum samples

As previously described [25], paraffin blocks of prostatectomy specimens and serum from 45 patients (15 randomly selected patients from each treatment group) enrolled in a phase II clinical trial were obtained from University Health Network in Toronto, Canada. The randomized trial, registered with www.clinicaltrials.gov (NCT00741364), involved 66 patients given oral vitamin D₃ (cholecalciferol) at doses 400, 10,000, or 40,000 IU/day for 3–8 weeks prior to prostatectomy at which time fresh tissue was collected and cryostored. The benign and PCa areas of the blinded specimens were demarcated by a pathologist for laser-capture microdissection (LCM). LCM was used to separately collect benign epithelium, PCa epithelium and benign stroma via a procedure previously described for these samples [25]. Serum samples were obtained at entry and at the final visit before the previously described [26].

2.2. Cell culture

Primary human epithelial and stromal cells were isolated from radical prostatectomy tissue from PCa patients at the University of Illinois at Chicago Medical Center as previously described [6] based on the method developed by Peehl [27]. Fresh tissue from the peripheral zone was selected and excised with a 5 mm punch by a pathologist according to an Institutional Review Board-approved protocol. Final pathology of the tissue was determined by H&E of a thin slice of the punch and the area must be 100% benign to have that classification. Briefly, tissues were digested with collagenase and plated on collagen-coated dishes in PrEGM (Lonza, Walkersville, MD) for epithelial cell (PrE) growth and in MCDB-105/10% FBS for stromal cell (PrS) growth (Sigma–Aldrich, St. Louis, MO). Cell type was validated by qRT-PCR for the expression of known basal epithelial cell markers (CK5+, p63+, AR–) or stromal cell markers (TIMP3+, AR+). For these experiments, 6 different patient-derived benign PrE cells were used and plated at secondary passage in monolayers or in 3-D in Matrigel. To provide one consistent source of stromal cells, benign PrS cells from four patients were pooled on secondary passage and cryopreserved in multiple aliquots. This same mixed PrS cell pool was used for all experiments on the 3rd or 4th passage and plated in monolayer or on 12 mm inserts as indicated.

3. 3-D co-culture of primary human prostate epithelial and stromal cells

We adapted a previously published method [28] and seeded PrE cells 1:1 with low growth factor Matrigel (BD Biosciences, San Jose, CA) in PrEGM in a 12-well plate. Spheroid structures termed prostaspheres formed over 7–10 days of culture as previously described [28]. PrS cells were seeded 1 day prior to co-culture (10,000 cells/insert) onto 0.4 μ M transwell inserts (Corning, NY) in MCDB-105/10% FBS. At day 7 or 10, PrS cells inserts were placed in the wells with the prostaspheres and cultured in PrEGM. Co-cultures were treated according to experiments.

4. RNA isolation

For patient samples, RNA was isolated from the LCM-collected tissues using the RecoverAll kit as described previously [29]. For cell cultures, total RNA was isolated with TRIzol (Life technologies, Grand Island, NY). RNA quantity and quality were determined by 260/280 nm absorbance on the Nanodrop ND-1000.

5. Quantitative real time RT-PCR

5.1. Tissue samples

As described previously [25], 50 ng of RNA was used for cDNA synthesis (Vilo cDNA kit, Life Tech.). Preamplification (12 $\times$) with TaqMan PreAmp master mix and specific TaqMan assays was performed. TaqMan assays used; Hs0018742_m1, Hs03929097_g1*, Hs00357333_g1, Hs00985639_m1, Hs01573477_g1, Hs00989011_g1, Hs01017992_g1, Hs00168017_m1*, Hs01379776_m1*, Hs01045844_m1, Hs00152933_m1, Hs00370853_m1.

5.2. In vitro prostate cells

cDNA was generated from rv500 ng of RNA with the high capacity cDNA synthesis kit (Life Tech.). qPCR was run on cDNA using SYBR Green master mix (Life Tech.) on the Step One Plus or ViIA 7 machine using gene specific primers (Supplemental Table S1). Fold-changes were calculated using 2^{-CT} method and normalized to the expression of TBP or GAPDH.

5.3. PCR analysis

Correlation analysis showed that B2M and Actin were the best housekeeper genes for our patient tissue data. For the cell cultures, TBP was used for normalization. Relative quantity (fold-change) for each gene was calculated using 2^{-CT} method and normalized to the mean Ct of B2M and Actin. Undetectable wells (Ct > 35) were not included in analysis.

6. Vitamin D metabolite measurement in prostate tissue

Full statistical analysis of serum and tissue levels of vitamin D metabolites are reported elsewhere [26]. Vitamin D metabolites were pre-extracted from prostate tissue and 1,25(OH)₂D levels were determined by enzyme immunoassay (Immunodiagnostic Systems,

Scottsdale, AZ). [26]. Tissue vitamin D metabolites were reported as the mean of two samples of fresh frozen tissue.

6.1. Prostasphere count and size

For prostasphere counting, 15,000 cells/well of PrE cells were plated in Matrigel. PrS cells were plated on inserts and placed in the wells with the PrE cells. Media containing ethanol or 50 nM 1,25(OH)₂D ± TI (10 ng/mL TNFα and 5 ng/mL IL-1β) was replaced every 3 days. Dispace was used to release the prostaspheres from Matrigel for counting and size measurement. The EVOS microscope (Advanced Microscopy Group, Life Tech.) was used to capture pictures and count the total number of prostaspheres/well. PS size was measured in Matlab software as described previously [30].

6.2. Statistical analysis

RT-qPCR gene expression data were analyzed by Wilcoxon signed-rank test or Kruskal–Wallis test as the data were not distributed normally. ELISA and cell counts were analyzed with student's *t*-test. All data are shown as the mean ± SEM of replicate experiments (or as described in the figure legend).

7. Results

7.1. Prostatic expression of VDR and vitamin D metabolism genes in the clinical trial specimens

Cell-specific expression of VDR and vitamin D metabolizing hydroxylases was measured in prostate epithelium and stroma tissues from PCa patients from a Phase II Clinical Trial of vitamin D₃. As previously described [26], patients were randomized into three treatment groups; 1 = 400 IU/day, 2 = 10,000 IU/day, 3 = 40,000 IU/day of oral vitamin D₃ for 3–8 weeks prior to surgery (PIs: RV and TvdK). We previously reported that patients in this trial had no adverse effects and there were significant changes in their vitamin D status from the short-term supplementation [26]. In the high dose vitamin D group, serum 25(OH)D levels increased 300% over baseline and 1,25(OH)₂D increased 80% over baseline. The prostate tissue 25(OH)D levels were 30% higher and 1,25(OH)₂D 10% higher in the high dose group compared to the 400IU group [26].

In the present study tumor and benign epithelium and stroma (surrounding benign tissue only) were collected by LCM from 15 patients per treatment group as shown (Fig. 1A). Areas of PCa epithelium were AMACR positive and stromal tissues were NKX3.1 negative, confirming proper sampling of cell types (Fig. 1B and C). VDR, CYP2R1, CYP27A1, CYP27B1, and CYP24A1 were quantified by RT-qPCR in LCM-collected tissues from 15 patients from each of the treatment groups.

Cell-type specific expression was initially established in the reference group (400 IU/day, *N* = 15). As shown in Table 1, VDR was expressed at similar levels in the benign epithelium, PCa epithelium and in stroma implicating all as potential targets of 1,25(OH)₂D actions. CYP27A1, CYP27B1 and CYP2R1 levels were lower or absent in the stroma compared to benign epithelium of these control patients (Table 1). In contrast, CYP24A1, which

inactivates 1,25(OH)₂D was expressed in all cell types. There were not significant changes in any of the vitamin D-related genes when analyzed by vitamin D treatment group (Supplemental Table S2).

The levels of intra-prostate 1,25(OH)₂D were determined for 37 men in this study and they were included in a previous report [26]. Gene expression was analyzed by tertile of prostatic 1,25(OH)₂D and showed that the stroma had the most differences in that the VDR was lower in the higher tertiles and CYP2R1 was positively related to 1,25(OH)₂D levels. Please note that although all samples were subjected to RNA isolation and RT-qPCR, the N is variable based on detection. CYP27A1 was lower in the highest tertile for the benign epithelium (Fig. 2A–C). Analyses were also done stratified by serum 25(OH)D levels and findings were similar, but without statistical significance (data not shown).

Together these patient data suggest that while all cells of the prostate are receptor positive targets of 1,25(OH)₂D, activation of circulating vitamin D occurs primarily in the epithelium.

7.2. In vitro cell type-specific expression of vitamin D metabolism genes in primary human prostate cells

Human primary prostatic epithelial (PrE) and stromal (PrS) cells derived from benign prostate were used to further interrogate cell type-specific expression of the vitamin D metabolism genes. Cells from multiple patients (not part of the clinical trial) were grown, treated with 50 nM 1,25(OH)₂D, and gene expression measured by RT-qPCR. 1,25(OH)₂D was used to circumvent patient-to-patient differences in 25(OH)D metabolism. VDR was expressed in all cell types and was decreased by 1,25(OH)₂D treatment, validating the clinical trial findings (Fig. 3A). Also similar to the clinical trial specimens, CYP2R1 and CYP27B1 were expressed at low levels in the PrS cells (Fig. 3B and C). Treatment with 1,25(OH)₂D did not change PrS expression of CYP2R1 which differs from the increase in CYP2R1 expression observed in the highest tertile in the trial patients. CYP24A1 was highly induced by 1,25(OH)₂D in all cell types, but present at a lower level in the PrS cells under control conditions (Fig. 3D). CYP27A1 was not detectable in any of the cell cultures (data not shown).

7.3. Inflammatory genes are differentially expressed in benign and PCa epithelium, and stroma in clinical trial prostate tissues

Based on prior reports of their regulation by 1,25(OH)₂D in prostate cells [5,6], the expression of PTGS2, IL-6 and IL-8 was measured in the clinical trial specimens. In the reference group (400 IU/day), cell-type specific expression was established with PTGS2 levels highest in the epithelium compared to stroma (Table 1). In contrast, IL-6 was highest in the stromal compartment compared to epithelium (Table 1), with a similar trend for IL-8.

As observed with the vitamin D-related genes, intention to treat analysis by group did not reveal significant differences by intervention dose in the trial specimens (Supplemental Table S2). When the expression was analyzed by tertile of prostatic 1,25(OH)₂D, PTGS2 was significantly lower in the highest tertile in PCa epithelium (Fig. 4A–C). We also analyzed by serum 25-hydroxyvitamin D levels and found no statistical significance for any

of the genes (data not shown). Overall, these data demonstrate cell-specific expression and responses to $1,25(\text{OH})_2$ in prostatic inflammation-associated genes.

7.4. In vitro cell type-specific expression of inflammatory genes and regulation by $1,25(\text{OH})_2$ in primary human prostate cells

Because the paracrine interaction between the epithelium and stroma is important in PCa initiation, development, and metastasis [31] and because the primary PrE and PrS cells differentially secrete pro-inflammatory cytokines (Supplemental Fig.1), we examined the effect of $1,25(\text{OH})_2$ on co-cultured PrE and PrS cells. To create an in vitro model of a pro-inflammatory state, we adapted a 3-D co-culture model (Fig. 5A) of PrE and PrS cells [28]. In this model PrE cells were grown as spheroid structures (prostaspheres) in Matrigel (1:1) over a 7–10 day period to mimic the cell-to-cell contact of acini in prostate tissues. PrS cells were added to the culture on inserts to facilitate crosstalk via secreted factors to further mimic whole tissue. For continuity, the same mix PrS cells was used for all experiments (see Section 2). Human PrE and PrS cells derived from benign prostate were used in all experiments. Co-culture with the PrS cells increased the expression of all the inflammatory genes in the PrE cells (Fig. 4B), demonstrating that PrS-secreted factors alter gene expression in PrE cells.

Next, to simulate the pro-inflammatory state found during PCa progression [32], $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ (termed TI) were added to the culture medium. The concentrations of TI were optimized to 10 ng/mL $\text{TNF}\alpha$ and 5 ng/mL $\text{IL-1}\beta$ which had maximal cytokine response (data not shown) similar to our previous studies [6,33]. Optimal time course for inflammatory gene response was determined to be 3 h (data not shown).

Using this model system, we next examined the effect of $1,25(\text{OH})_2\text{D}$ on the inflammatory genes expressed by the separate PrE and PrS cell populations. Co-cultures were pretreated overnight (~16 h) with vehicle (0.1% ethanol) or 50 nM $1,25(\text{OH})_2\text{D}$, a dose that stimulates the VDR (as shown by CYP24A1 induction in Fig. 3D) and does not alter cell proliferation of PrE cells [25]. $1,25(\text{OH})_2\text{D}$ pretreatment reduced TI-stimulated $\text{TNF}\alpha$, IL-6 and IL-8 expression in PrE cells at 3 h (Fig. 5C). In the PrS cells, which express higher levels of IL-6 compared to PrE, $1,25(\text{OH})_2\text{D}$ did not influence expression levels (Fig. 5D). $\text{TNF}\alpha$ and PTGS2 levels were significantly suppressed by $1,25(\text{OH})_2\text{D}$ in the PrS cells (Fig. 5D).

These in vitro studies demonstrate that co-culture alters inflammatory gene expression in epithelial cells and that the cell types of the prostate respond differently to the anti-inflammatory actions of $1,25(\text{OH})_2\text{D}$.

7.5. Co-culture with prostate stroma is required for proliferative response of epithelium to pro-inflammatory stimulus

In prostate tissues, pro-inflammatory cytokine release from immune cells regulates proliferation of prostate epithelial cells (reviewed in [34]). Thus, we tested whether the stromal–epithelial crosstalk in our co-culture model altered cytokine-induced changes in the visible phenotype of the prostaspheres. The cultures were set up and placed into media containing $1,25(\text{OH})_2\text{D}$ and/or TI on day 1, with or without PrS cell inserts and on day 7, prostatesphere number and size were measured. In the presence of PrS cells, TI treatment

increased prostasphere number by 30% (mean of four different patient-derived PrE cells) and this effect was reversed by 1,25(OH)₂D (Fig. 6). In contrast, without PrS co-culture, TI slightly inhibited prostasphere formation and 1,25(OH)₂D had no effect (Fig. 6). Heterogeneity between patients alters the inherent sphere number, thus representative data from 1 patient is shown in Fig. 6. These experiments were performed in four different patient-derived PrE cells (using the same source of PrS for all replicates) and all showed similar responses to the co-culture and treatments. Together, these data demonstrate that crosstalk from the stromal cells alters the phenotypic response of the epithelial cells in the pro-inflammatory environment and that 1,25(OH)₂D inhibits this phenotype in stromal cell-dependent manner.

8. Discussion

The prostate stroma has well-characterized regulatory control over differentiation, proliferation and inflammatory response of the glandular epithelium [35]. Much of this regulation is mediated by hormones and the cell type-specific expression and roles of other hormone receptors, such as those for estrogen and androgen, have been defined in the prostate [36,37]. We sought to better understand differences in the vitamin D system in epithelial and stromal prostate cell types. LCM-collected prostate tissues from a vitamin D₃ clinical trial and primary human prostatic cell cultures provided unique samples and a model system for us to examine these cell-specific effects. Herein, for the first time we report cell-type specific expression of vitamin D-related and inflammatory genes in prostate tissue from a clinical trial.

Previously we reported the prostate vitamin D metabolite levels and regulation of microRNAs in the prostate epithelium in this vitamin D₃ clinical trial [25,26]. In the present study we examined the vitamin D pathway components and showed that while VDR was expressed in both stromal and epithelial cell types, there was differential expression of the vitamin D hydroxylases between cell types. VDR was lower with high 1,25(OH)₂D in the trial stromal tissues and this was validated in both epithelial and stromal cell type in vitro. Consistent with previous reports [38], we observed lower VDR expression in stromal cells compared to epithelial cells in vitro, but this was not observed in stromal patient tissues. In the trial specimens CYP2R1 and CYP27A1, the two putative 25-hydroxylases, and CYP27B1, the 1 α -hydroxylase, were present at lower levels in prostate stroma compared to epithelium. The in vitro studies with the primary prostate cells supported these differences in expression of the vitamin D metabolism genes. Furthermore, the stromal expression of both VDR and CYP2R1 varied by intra-prostatic 1,25(OH)₂D levels, demonstrating that the stroma responded to the cholecalciferol intervention despite low levels of the hydroxylases. Therefore, although both stromal and epithelial cells can respond to 1,25(OH)₂D via VDR, the epithelium is the major source of prostatic 1,25(OH)₂D and likely regulates local production of the hormone.

We examined several genes involved in inflammation that had been previously reported to be regulated by 1,25(OH)₂D in prostate cells [5,6]. IL-6 and PTGS2 were differentially expressed between cell types with IL-6 being 18-fold higher in stroma compared to epithelium and PTGS2 being lowest in the stroma. This high expression of IL-6 mRNA in

the stroma indicates that the stromal cells are the main producers of IL-6 in the prostate. These findings complement other studies that found IL-6 protein was primarily located in PCa regions while IL-6 receptor protein was present in all the cell types of the prostate [39,40]. IL-6 is produced by multiple other cell types including macrophages and lymphocytes, leading to enhanced proliferation [41]. Another study showed that serum levels of IL-6 were elevated in patients with metastatic PCa [42], even though IL-6 protein was not detectable in the cancer regions of patient samples in that study. Similar to the vitamin D genes, there were no differences in inflammatory genes by treatment group. PTGS2 was the only inflammatory gene significantly different in the clinical trial and it was lowest in the highest tertile of 1,25(OH)₂D in the PCa tissue. This provides translational relevance to previous in vitro studies that found PTGS2 to be reduced by 1,25(OH)₂D in prostate cancer cell lines [5].

We used a co-culture model to examine inflammatory gene regulation in a physiologically relevant in vitro model. In the co-culture, cytokine stimulation initiated an epithelial proliferative response, similar to intact human prostate, which did not occur in 3-D culture without PrS cells. Similar to other in vitro findings [5,6], 1,25(OH)₂D had anti-inflammatory activity in the model as it suppressed this proliferative phenotype and decreased the expression of several inflammatory genes. Interestingly, 1,25(OH)₂D suppressed the target genes differently in the PrE cells and in the PrS cells. PTGS2, which was associated with 1,25(OH)₂D in the clinical trial in PCa tissues, was suppressed by 1,25(OH)₂D only the PrS cells in vitro.

There were two expected findings that we did not observe in these clinical trial samples; (1) lower CYP27B1 in PCa and (2) higher CYP24A1 in the highest 1,25(OH)₂D tertile. Prior in vitro studies have demonstrated diminished CYP27B1 expression and activity in primary PCa cells and PCa cell lines compared to normal prostate cells [43,44]. As well, CYP24A1 is a well-characterized target of VDR and 1,25(OH)₂D and contains two VDREs [45]. There are several potential reasons why we did not observe these changes. First, this is a relatively small study ($N = 15$ each group). Second, the intervention may have been too short (3–8 weeks). Lastly, there was inherent patient heterogeneity which could not be controlled for in this trial since it was an endpoint study without pre- and post-sampling of prostate tissue. This latter point also likely contributes to the fact that we did not detect differences in any of the endpoints between the treatment groups, whereas there were differences when the actual prostatic concentration of 1,25(OH)₂D was considered. One example of heterogeneity is the presence of single nucleotide polymorphisms (SNPs) in VDR and all the vitamin D metabolism genes as well as vitamin D binding protein (VDBP) [46-48]. Presently, there is limited published data on these endpoints in intact tissues for comparison to our results. The most comparable is a study by Healy et al. [49] in the murine kidney which showed that treatment with 1,25(OH)₂D increased VDR and CYP24A1 expression in a calcium-dependent manner. However, the kidney has a vital role in the tightly controlled regulation of circulating 1,25(OH)₂D and calcium homeostasis, and its response to vitamin D is likely to be very different from other solid tissues.

In summary, we show that prostate stroma responds to 1,25(OH)₂D and is essential in mediating the vitamin D response in the prostate epithelium. Specifically, some vitamin D

metabolism enzymes were not expressed in prostate stroma whereas IL-6 was expressed at high levels in the stroma. These current findings improve our understanding of the prostate stroma and build on previous studies which focused on the anti-inflammatory regulation of 1,25(OH)₂D in epithelial cells. Future clinical studies of longer duration may further clarify the anti-inflammatory activities of vitamin D in the prostate stromal and epithelial cells and how this action may contribute to cancer preventive actions of this essential pro-hormone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the NIH grant K22CA133105 (Nonn), R03CA172827 (Nonn), American Institute for Cancer Research Postdoctoral Scholar Grant (Giangreco), the Canadian Cancer Society (Vieth), and by a studentship from Canadian Institutes for Health Research (Wagner). We thank the prostate cancer patients who donated their tissue for primary cell cultures and who participated in the clinical trial. We thank Dr. Michael Abern, Dr. Leslie Deane, Dr. Klara Valyi-Nagy and Ra-el Henderson for their roles in tissue collection from UIC Urology patients for cell culture; Drs. Antonio Finelli and Neil Fleshner (Surgical Oncology University Health Network, Canada) for their roles in the clinical trial, Dr. Ming Jin for the CRP measurement, Lindsay Gallagher for sectioning of the clinical trial specimens, Avani Vaishnav for help with LCM, Dr. Wen Hu for help with prostasphere cultures, and Andrew Fairchild for assisting with the real time PCR analysis.

Abbreviations

1α,25(OH)₂D	1 α ,25-dihydroxyvitamin D ₃
PCa	prostate cancer
VDR	vitamin D receptor
CYP	cytochrome P450
LCM	laser capture microdissection
RT	reverse transcription
qPCR	quantitative polymerase chain reaction
PTGS2 or COX2	prostaglandin synthase 2
IL	interleukin
TNF	tumor necrosis factor
PrE	primary prostatic epithelial cells
PrS	primary prostatic stromal cells

References

- [1]. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. *Nat. Rev. Cancer.* 2014; 14:342–357. [PubMed: 24705652]
- [2]. Krishnan AV, Peehl DM, Feldman D. Inhibition of prostate cancer growth by vitamin D: regulation of target gene expression. *J. Cell. Biochem.* 2003; 88:363–371. [PubMed: 12520538]

- [3]. Rigby WF, Noelle RJ, Krause K, Fanger MW. The effects of 1,25-dihydroxyvitamin D3 on human T lymphocyte activation and proliferation: a cell cycle analysis. *J. Immunol.* 1985; 135:2279–2286. [PubMed: 2993410]
- [4]. Blutt SE, McDonnell TJ, Polek TC, Weigel NL. Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. *Endocrinology.* 2000; 141:10–17. [PubMed: 10614618]
- [5]. Moreno J, Krishnan AV, Feldman D. Molecular mechanisms mediating the anti-proliferative effects of vitamin D in prostate cancer. *J. Steroid Biochem. Mol. Biol.* 2005; 97:31–36. [PubMed: 16024246]
- [6]. Nonn L, Peng L, Feldman D, Peehl DM. Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. *Cancer Res.* 2006; 66:4516–4524. [PubMed: 16618780]
- [7]. Penna G, Amuchastegui S, Cossetti C, Aquilano F, Mariani R, Sanvito F, Doglioni C, Adorini L. Treatment of experimental autoimmune prostatitis in nonobese diabetic mice by the vitamin D receptor agonist elocalcitol. *J. Immunol.* 2006; 177:8504–8511. [PubMed: 17142748]
- [8]. Cunha GR, Cooke PS, Kurita T. Role of stromal-epithelial interactions in hormonal responses. *Arch. Histol. Cytol.* 2004; 67:417–434. [PubMed: 15781983]
- [9]. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. *J. Urol.* 2001; 166:2472–2483. [PubMed: 11696814]
- [10]. Barron DA, Rowley DR. The reactive stroma microenvironment and prostate cancer progression. *Endocr. Relat. Cancer.* 2012; 19:R187–R204. [PubMed: 22930558]
- [11]. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. *Curr. Opin. Genet. Dev.* 2008; 18:11–18. [PubMed: 18308558]
- [12]. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat. Immunol.* 2011; 12:1035–1044. [PubMed: 22012443]
- [13]. Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res.* 1991; 51:3753–3761. [PubMed: 1712249]
- [14]. Camps JL, Chang SM, Hsu TC, Freeman MR, Hong SJ, Zhau HE, von Eschenbach AC, Chung LW. Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 1990; 87:75–79. [PubMed: 2296606]
- [15]. Chung LW, Davies R. Prostate epithelial differentiation is dictated by its surrounding stroma. *Mol. Biol. Rep.* 1996; 23:13–19. [PubMed: 8983015]
- [16]. Nakai Y, Nonomura N. Inflammation and prostate carcinogenesis. *Int. J. Urol.* 2013; 20:150–160. [PubMed: 22852773]
- [17]. Sfanos KS, De Marzo AM. Prostate cancer and inflammation: the evidence. *Histopathology.* 2012; 60:199–215. [PubMed: 22212087]
- [18]. Dennis LK, Lynch CF, Torner JC. Epidemiologic association between prostatitis and prostate cancer. *Urology.* 2002; 60:78–83. [PubMed: 12100928]
- [19]. De Marzo AM, Marchi VL, Epstein JI, Nelson WG. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. *Am. J. Pathol.* 1999; 155:1985–1992. [PubMed: 10595928]
- [20]. Bethel CR, Faith D, Li X, Guan B, Hicks JL, Lan F, Jenkins RB, Bieberich CJ, M. De Marzo A. Decreased NKX3. 1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion. *Cancer Res.* 2006; 66:10683–10690. [PubMed: 17108105]
- [21]. De Marzo AM, Nakai Y, Nelson WG. Inflammation, atrophy, and prostate carcinogenesis. *Urol. Oncol.* 2007; 25:398–400. [PubMed: 17826659]
- [22]. Hidalgo AA, Paredes R, Garcia VM, Flynn G, Johnson CS, Trump DL, Onate SA. Altered VDR-mediated transcriptional activity in prostate cancer stroma. *J. Steroid Biochem. Mol. Biol.* 2007; 103:731–736. [PubMed: 17368189]

- [23]. Barreto AM, Schwartz GG, Woodruff R, Cramer SD. 25-Hydroxyvitamin D3, the prohormone of 1,25-dihydroxyvitamin D3, inhibits the proliferation of primary prostatic epithelial cells. *Cancer Epidemiol. Biomarkers Prev.* 2000; 9:265–270. [PubMed: 10750664]
- [24]. Krill D, DeFlavia P, Dhir R, Luo Becich MJ, Lehman E, Getzenberg RH. Expression patterns of vitamin D receptor in human prostate. *J. Cell. Biochem.* 2001; 82:566–572. [PubMed: 11500934]
- [25]. Giangreco AA, Vaishnav A, Wagner D, Finelli A, Fleshner N, Van der Kwast T, Vieth R, Nonn L. Tumor suppressor microRNAs, miR-100 and -125b, are regulated by 1,25-dihydroxyvitamin D in primary prostate cells and in patient tissue. *Cancer Prev. Res.* 2013
- [26]. Wagner D, Trudel D, Van der Kwast T, Nonn L, Giangreco AA, Li D, Dias A, Cardoza M, Laszlo S, Hersey K, Klotz L, Finelli A, Fleshner N, Vieth R. Randomized clinical trial of vitamin d3 doses on prostatic vitamin d metabolite levels and ki67 labeling in prostate cancer patients. *J. Clin. Endocrinol. Metab.* 2013; 98:1498–1507. [PubMed: 23463655]
- [27]. Peehl DM. Are primary cultures realistic models of prostate cancer? *J. Cell. Biochem.* 2004; 91:185–195. [PubMed: 14689590]
- [28]. Hu WY, Shi GB, Lam HM, Hu DP, Ho SM, Madueke IC, Kajdacsy-Balla A, Prins GS. Estrogen-initiated transformation of prostate epithelium derived from normal human prostate stem-progenitor cells. *Endocrinology.* 2011; 152:2150–2163. [PubMed: 21427218]
- [29]. Nonn L, Vaishnav A, Gallagher L, Gann PH. mRNA and micro-RNA expression analysis in laser-capture microdissected prostate biopsies: valuable tool for risk assessment and prevention trials. *Exper. Mol. Pathol.* 2010; 88:45–51. [PubMed: 19874819]
- [30]. Prins HW, Shi GS, Hu GB, Majumdar DP, Li S, Nelles G, Ho J, Walker SM, Kajdacsy-Balla CL, vanBreeman AR. Bisphenol A promotes human prostate stem-progenitor cell self-renewal and increases in vivo carcinogenesis in human prostate epithelium. *Endocrinology.* 2013 in press.
- [31]. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat. Rev. Cancer.* 2006; 6:392–401. [PubMed: 16572188]
- [32]. Haverkamp J, Charbonneau B, Ratliff TL. Prostate inflammation and its potential impact on prostate cancer: a current review. *J. Cell. Biochem.* 2008:1344–1353. [PubMed: 17955503]
- [33]. Nonn L, Duong D, Peehl DM. Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. *Carcinogenesis.* 2007; 28:1188–1196. [PubMed: 17151092]
- [34]. Bostanci Y, Kazzazi A, Momtahn S, Laze J, Djavan B. Correlation between benign prostatic hyperplasia and inflammation. *Curr. Opin. Urol.* 2013; 23:5–10. [PubMed: 23159991]
- [35]. Hayward SW, Rosen MA, Cunha GR. Stromal-epithelial interactions in the normal and neoplastic prostate. *Br. J. Urol.* 1997; 79(Suppl. 2):18–26. [PubMed: 9126066]
- [36]. Ricke EA, Williams K, Lee YF, Couto S, Wang Y, Hayward SW, Cunha GR, Ricke WA. Androgen hormone action in prostatic carcinogenesis: stromal androgen receptors mediate prostate cancer progression, malignant transformation and metastasis. *Carcinogenesis.* 2012; 33:1391–1398. [PubMed: 22535887]
- [37]. Risbridger G, Wang H, Young P, Kurita T, Wang YZ, Lubahn D, Gustafsson JA, Cunha G. Evidence that epithelial and mesenchymal estrogen receptor- α mediates effects of estrogen on prostatic epithelium. *Dev. Biol.* 2001; 229:432–442. [PubMed: 11150243]
- [38]. Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. *Cancer Res.* 1994; 54:805–810. [PubMed: 7508338]
- [39]. Hobisch A, Rogatsch H, Hittmair A, Fuchs D, Bartsch G Jr. Klocker H, Bartsch G, Culig Z. Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue. *J. Pathol.* 2000; 191:239–244. [PubMed: 10878544]
- [40]. Cardillo MR, Ippoliti F. IL-6, IL-10 and HSP-90 expression in tissue microarrays from human prostate cancer assessed by computer-assisted image analysis. *Anticancer Res.* 2006; 26:3409–3416. [PubMed: 17094460]
- [41]. Nguyen DP, Li J, Tewari AK. Inflammation and prostate cancer: the role of interleukin-6. *BJU Int.* 2013; 113:986–992. [PubMed: 24053309]

- [42]. Michalaki V, Syrigos K, Charles P, Waxman J. Serum levels of IL-6 and TNF- α correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br. J. Cancer*. 2004; 90:2312–2316. [PubMed: 15150588]
- [43]. Whitlatch LW, Young MV, Schwartz GG, Flanagan JN, Burnstein KL, Lokeshwar BL, Rich ES, Holick MF, Chen TC. 25-Hydroxyvitamin D-1 α -hydroxylase activity is diminished in human prostate cancer cells and is enhanced by gene transfer. *J. Steroid Biochem. Mol. Biol.* 2002; 81:135–140. [PubMed: 12137802]
- [44]. Hsu JY, Feldman D, McNeal JE, Peehl DM. Reduced 1 α -hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D₃-induced growth inhibition. *Cancer Res.* 2001; 61:2852–2856. [PubMed: 11306457]
- [45]. Kumar R, Iachini DN, Neilsen PM, Kaplan J, Michalakis J, Anderson PH, May BK, Morris HA, Callen DF. Systematic characterisation of the rat and human CYP24A1 promoter. *Mol. Cell. Endocrinol.* 2010; 325:46–53. [PubMed: 20450955]
- [46]. Gozdzik A, Zhu J, Wong BY, Fu L, Cole DE, Parra EJ. Association of vitamin D binding protein (VDBP) polymorphisms and serum 25(OH)D concentrations in a sample of young Canadian adults of different ancestry. *J. Steroid Biochem. Mol. Biol.* 2011; 127:405–412. [PubMed: 21684333]
- [47]. McGrath JJ, Saha S, Burne TH, Eyles DW. A systematic review of the association between common single nucleotide polymorphisms and 25-hydroxyvitamin D concentrations. *J Steroid Biochem. Mol. Biol.* 2010; 121:471–477. [PubMed: 20363324]
- [48]. Muindi JR, Adjei AA, Wu ZR, Olson I, Huang H, Groman A, Tian L, Singh PK, Sucheston LE, Johnson CS, Trump DL, Fakih MG. Serum vitamin D metabolites in colorectal cancer patients receiving cholecalciferol supplementation: correlation with polymorphisms in the vitamin D genes. *Hormones Cancer*. 2013; 4:242–250. [PubMed: 23456391]
- [49]. Healy KD, Zella JB, Prah JM, DeLuca HF. Regulation of the murine renal vitamin D receptor by 1,25-dihydroxyvitamin D₃ and calcium. *Pro. Natl. Acad. Sci. U. S. A.* 2003; 100:9733–9737.

Fig. 1.

Collection of benign epithelium, PCa epithelium and stroma tissue from FFPE prostate samples by laser capture microdissection (LCM). (A) Representative image of areas selected by pathologist and isolated by LCM on an adjacent H&E slide prostate tissue. Areas of benign epithelium (blue line), benign stroma (dashed blue line) and PCa epithelium (black line) were collected by LCM within the marked areas. Gene expression shown as relative quantity (RQ) normalized to GAPDH and TBP of (B) AMACR, and (C) NKX3.1 by RT-qPCR in control tissues to demonstrate appropriate molecular markers in the prostate cell types. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Gene expression of vitamin D-related genes in prostate tissues from the clinical trial by tertile of prostatic 1,25-dihydroxyvitamin D. Tertiles: 15–24 ($N = 13$), 25–33 ($N = 12$), 34–79 ($N = 12$) pmol/kg. RT-qPCR analysis of VDR, CYP2R1, CYP27A1, CYP27B1 and CYP24A1 in (A) benign epithelium, (B) Pca epithelium, and (C) stroma. $*p < 0.05$ by Kruskal–Wallis test and Dunn’s multiple comparison posthoc test. N for each group in parentheses.

Fig. 3.

Expression of vitamin D-related genes in primary prostate human epithelial (PrE) and stromal (PrS) cells. RT-qPCR analysis of (A) VDR, (B), CYP2R1, (C), CYP27B1, and (D) CYP24A1 in PrE cells from three individuals and PrS cells from two individuals (CYP27A1 undetectable in all cells). Cells were vehicle-treated (EtOH, open bars) or treated with 50 nM 1,25D (dark bars) for 24 h. Error bars represent standard deviation of technical replicates. Difference between EtOH and 1,25D across biological replicates by Wilcoxon signed-rank test * $p < 0.05$ and ** $p < 0.01$.

Fig. 4.

Gene expression of PTGS2 (COX2) in prostate tissues from the clinical trial by tertile of prostatic 1,25-dihydroxyvitamin D. RT-qPCR analysis of PTGS2 in (A) benign epithelium, (B) PCa epithelium, and (C) stroma. * $p < 0.05$ by Kruskal–Wallis test and Dunn’s multiple comparison posthoc test.

Fig. 5. PrE and PrS cells differentially express cytokines and respond to 1,25-dihydroxyvitamin D (1,25D) in a 3-D co-culture model. (A) Diagram of 3-D co-culture model. (B) The effect of 48 h co-culture on the gene expression of $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , IL-8 and PTGS2 determined by RT-qPCR in PrE (E) and PrS (S) grown as solo monolayer or in the co-culture. Mean data are shown for at least three independent experiments $\pm$ SEM, a $p < 0.05$ compared to PrE monolayer, b $p < 0.05$ compared to PrS monolayer, c $p < 0.05$ compared to PrE co-culture by Wilcoxon signed-rank test. (C and D) The effect of pretreatment with 50 nM 1,25D on pro-inflammatory-induced gene expression (fold-change over untreated cells) by RT-qPCR. Mean gene expression of $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , IL-8 and PTGS2 was measured after 3 h $\text{TNF}\alpha$ and $\text{IL-1}\beta$ (TI) in cells from 3 patients (experiments 1, 2, and 3 on x -axis) in (C) in epithelial (PrE) cells and (D) stromal (PrS) cells of the co-culture. Difference between EtOH and 1,25D across biological triplicates by Wilcoxon signed-rank test $*p < 0.05$ and $**p < 0.01$.

Fig. 6.

Co-culture with stromal cells alters response of epithelial prostasphere to pro-inflammatory stimulus and 1,25D. The number of prostaspheres was counted $\pm$ 50 nM 1,25D $\pm$ TI for 7 days $\pm$ co-culture with PrS cells. Representative results are shown for 1 patient-derived PrE cell line. PS count was performed 3 times independently, ^a $p < 0.01$ compared to EtOH control ($-TI$, $+PrS$), ^b $p < 0.01$ compared to EtOH $+TI$ $+PrS$ control, ^c $p < 0.01$ compared to EtOH control ($-TI$, $-PrS$).

Table 1

gene expression of inflammatory genes and vitamin D-related genes in LCMcollected prostate stroma, benign epithelium and PCa epithelium.

Gene group	mRNA		Tissue			KW ^a p value
			Benign Epi	PCa Epi	Stroma	
Vitamin D	VDR	Mean	1.43	2.44	2.64	0.3088
		SE	0.28	0.80	2.10	
		N	10	9	7	
	CYP2R1	Mean	2.13 ^a	2.59 ^b	0.64 ^{a,b}	0.0007
		SE	0.25	0.67	0.34	
		N	14	14	10	
	CYP27A1	Mean	4.14 ^{a,b}	2.02 ^b	0.34 ^a	<0.0001
		SE	0.74	0.83	0.05	
		N	14	15	11	
	CYP27B1	Mean	1.84	4.14	0.07	0.3133
		SE	0.55	2.10	0.00	
		N	10	10	1	
	CYP24A1	Mean	8.48	1.26	1.25	0.0462
		SE	6.28	0.37	0.81	
		N	13	8	7	
Inflammation	PTGS2	Mean	3.84 ^a	1.18	0.52 ^a	0.0003
		SE	1.16	0.21	0.26	
		N ^b	14	11	11	
	IL6	Mean	0.61 ^a	0.59 ^b	18.81 ^{a,b}	0.0005
		SE	0.18	0.16	8.35	
		N	8	3	10	
	IL8	Mean	1.76	6.49	6.27	0.1061
		SE	0.71	2.52	2.48	
		N	13	14	11	

^{a,b} Difference in rank sum $p < 0.05$ for Dunn's multiple comparison post test indicated by same letter.

^a Kruskal–Wallis test (unpaired).

^b N = number detectable samples out of the 15 patients examined.