TITLE: To investigate the Therapeutic Efforts of the COX-2 Inhibitor NS-398 as a Single Agent, and in Combination with Vitamin D, in Vitro and in Vivo

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**ABSTRACT**

The incidence of prostate cancer has increased rapidly and much effort is needed towards understanding the mechanisms involved in development and progression of prostate cancer and developing new strategies for its prevention and treatment. Recent studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs), such as COX-2 inhibitor act as chemopreventative agents. Prostate Tissue Microarray analyses found that COX-2 expression in prostate cancer was correlated with cancer progression. Treatment of prostate cancer cells with a selective COX-2 inhibitor, NS-398, induces VDR expression, and thus might result in increasing the vitamin D sensitivity. In return, treatment of prostate cancer cells with 1,25-VD results in reduction of COX-2 expression. Based on the bi-directional regulation involving vitamin D and the COX-2 inhibitor, we hypothesize that combining vitamin D and a COX-2 inhibitor in the treatment of prostate cancer will be beneficial to the treatment of prostate cancer. Four aims are proposed. 1: Evaluation of the molecular mechanism of COX-2 inhibitor NS-398 action on the growth of prostate cancer cells. 2: Evaluation of the effects of 1,25-VD and its analogs in combination with COX-2 inhibitor on progression of prostate cancer cells. 3: Evaluation of the underlying mechanism of the bi-directional regulatory pathways between the COX-2 inhibitor NS-398 and vitamin D. 4: Evaluation of the effects of COX-2 inhibitor, administered in combination with vitamin D compounds, on prostate cancer progression and invasion in vivo.
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TO INVESTIGATE THE THERAPEUTIC EFFECTS OF THE COX-2 INHIBITOR NS-398 AS A SINGLE AGENT, AND IN COMBINATION WITH VITAMIN D, IN VITRO AND IN VIVO

Introduction and Body:

A summary of this proposal was the follow statement

The incidence of prostate cancer has increased rapidly during the past decades and it has now become the most common malignancy of men in many Western nations. In the USA, prostate cancer represents the second leading cause of cancer death in men. Most prostate cancer initially responds to androgen ablation treatment, however, eventually it relapses to an androgen-independent state, leading to tumor outgrowth. Therefore, much effort is needed towards understanding the mechanisms involved in development and progression of prostate cancer and developing new strategies for its prevention and treatment.

Results of recent epidemiologic and animal model studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs), which prevent biosynthesis of prostaglandins, biosynthesis through inhibition of COX activity, act as chemopreventative agents. We examined the expression of COX-2 in prostate cancer tissue arrays, which consist of normal, BPH, PIN, and low- and high-grade prostate tumor samples. In our preliminary study we found that COX-2 expression is significantly higher in cancer than in normal or BPH prostate samples and the tendency of COX-2 expression in prostate cancer cells is consistent with prostate cancer cell lines, implying COX-2 expression in prostate cancer might be up-regulated during cancer progression. Treatment of prostate cancer cells with a selective COX-2 inhibitor, NS-398, induces VDR expression, and thus might result in increasing the vitamin D sensitivity of such cells. In return, treatment of prostate cancer cells with 1,25-VD results in reduction of COX-2 mRNA expression, but not COX-1 expression.

Based on the bi-directional regulation involving vitamin D and the COX-2 inhibitor, we hypothesize that combining vitamin D and a COX-2 inhibitor in the treatment of prostate cancer will be beneficial to the treatment of prostate cancer.

In this proposal, we will examine the effects of combination of 1,25-VD, its analog (EB1089), and a COX-2 inhibitor NS-398, compared with single agent treatment, on prostate cancer growth, apoptosis, invasion, angiogenesis, and neuroendocrine differentiation in vitro and in vivo. Four Specific Aims are proposed.

AIM 1: Evaluation of the molecular mechanism of COX-2 inhibitor NS-398 action on the growth of prostate cancer cells.

AIM 2: Evaluation of the effects of 1,25-VD and its analogs in combination with COX-2 inhibitor on progression of prostate cancer cells.

AIM 3: Evaluation of the underlying mechanism of the bi-directional regulatory pathways between the COX-2 inhibitor NS-398 and vitamin D.

AIM 4: Evaluation of the effects of COX-2 inhibitor, administered in combination with vitamin D compounds, on prostate cancer progression and invasion in vivo.

Our proposed schedule for completion of this proposal included the following tasks during the first 12 month.

Task 1: Determine whether the inhibition of growth mediated by NS-398 is a COX-2-dependent pathway. (Month 1-6)

Measure the COX-2 protein level and prostaglandin secretion level

Restore NS-398 effect by adding prostaglandin

Task 2: Determine the NS-398 mediated anti-tumor pathways. (Month 1-12)

Anti-proliferation

Anti-invasion

Anti-tumorigenesis

Anti-angiogenesis

Task 3: Determine the molecules that are responsible for NS-398 anti-tumor action. (Month
RT-PCR, and real-time PCR analysis of expression of known genes involved in NS-398 action DNA array analysis

Key Research Accomplishments and Reportable Outcomes:
Our progresses and competition of Task 1, 2 and 3 have generated two publications (please see the appendix). These two publications cover the roles of 1,25-vitamin D3/VDR cross-talk with androgen/androgen receptor, the key factor control the prostate cancer growth, as well as how 1,25-Vitamin D3 inhibit prostate cancer invasion via modulate several proteinase activities. Both of publication can serve as the base for investigation of combination effects of 1,25-vitamin D3 and COX-2 inhibitors for inhibition prostate cancer progression. We have also tested the COX-2 inhibitor NS-398 effects on VDR/1,25-Vitamin D3 down-stream targeted genes CYP24 expression by Real-time quantification PCR. As shown in Figure 1, in both PC3, and LNCaP cells, applying NS-398 into the cells reduced the VDR mRNA expression as well as VDR/1,25-Vitamin D3 targeted genes CYP24 expression significantly.

In addition to these two publications, COX-2 RNAi has generated to confirm the specificity of COX-2 action in prostate. We will use COX-2 RNAi for further analyses.

Most importantly, we have started animal studies to examine the combination effects of COX-2 inhibitor NS-398 and 1,25-Vitamin D3. Human prostate cancer xenograft in an athymic mouse model, which mimics prostate cancer progression in vivo has established. Young adult male mice, at age of 6-8 weeks will be subcutaneously injected with 2.5 x 10^6 LNCaP cells. Tumors will be allowed to grow, measured weekly with calipers, and tumor volumes will be calculated using the formula 0.52 x r_1^2 x r_2 (r_1 < r_2). Once tumors reach a volume of 0.5-1.0 cm^3, animals will be randomly grouped in two categories: (1) castration alone, or (2) castration plus EB1089, NS-398, or a combination of both. As shown in Figure 2, a significant suppression effect on the all treatment group from our pilot in vivo experiments in which 2 animals in each experiments. We will continue to examine with more animals for each group in the coming year.

Figure 1. Induction of VDR, and CYP24 mRNA expressions by applying COX-2 inhibitor NS-398 to LNCaP and PC-3 prostate cancer cells. LNCaP, and PC-3 cells were seeded, and then treatment with NS-398 (25 μM) for 12 hrs, and then cells were harvested and total RNA were extracted. cDNA from the two cell lines were synthesized by reverse transcription. Real-Time PCR will be performed using the Bio-Rad iQ cycler. Ct values were calculated and normalized to the level of the housekeeping gene β-microglobulin. Relative gene expression were calculated according to 2^-ΔΔCT from three independent experiments.
Figure 2. The anti-tumor effects of 1,25-Vitamin D3, NS-398, and combination effects in the LNCaP-xenograft nude mouse. Young adult male mice, at age of 8 weeks were subcutaneously injected with 2.5 x 10^6 LNCaP cells, and allow tumor to grow. Tumor volumes were calculated using the formula 0.532 x \( r_1^2 \times r_2 \) (\( r_1 < r_2 \)), and once the tumor reached to 0.5 cm^3, mice were treated with 1) control, 2) 1,25-vitamin D3 (3 μg/kg), NS-398 (1.5 mg/Kg), and combination twice per week, and tumor size were measured. The data were generated from two mice each group.

Conclusion:
As we demonstrated in our progression report, this project has moved smoothly and during this period, we generated two papers in regards to the vitamin D anti-prostate cancer action. In continue of this study we have started in vivo studies as well as established all the essential tools for further dissection the molecular mechanisms for this combination effects. We expected we will be able to obtain more promising data and then allow us to apply this concept into the clinical application.

References and Appendices:
Androgen-Receptor Coregulators Mediate the Suppressive Effect of Androgen Signals on Vitamin D Receptor Activity

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Overexpression of androgen receptors (AR) in PC-3 cell, and treatment of 5α-dihydrotestosterone in LNCaP cells lead to the suppression of VDR transactivation. Competition for shared coregulators between AR and VDR is one possible mechanism to explain the suppressive effect of androgen-AR signals on VDR activity. Among the AR coregulators we tested, ARA54, ARA70, supervillin, and gelsolin were found to enhance VDR transactivation. Further characterization of the interaction between ARA54 or ARA70 and VDR demonstrated a direct interaction between VDR and ARA70, but no association between ARA54 and VDR. The LXXLL motif of ARA70 is essential for interaction with VDR and partially responsible for its function as a coactivator of VDR. The suppression of VDR transactivation by AR signal was restored by overexpression of ARA70, but not ARA54. Together, ARA70 and ARA54 modulate VDR transactivation, and the competition for ARA70 mediates the suppressive effect of androgen-AR on VDR transactivation.

Key Words: 1α,25-Dihydroxyvitamin D₃; vitamin D₃ receptor; androgen receptor; coregulators.

Introduction

Treatment with 1α,25-dihydroxyvitamin D₃ (1,25-VD) inhibits proliferation and promotes differentiation in several types of cancers (1). The implications for clinical usefulness has triggered a number of studies regarding the use of 1,25-VD to treat prostate cancer (PCa) (2). Among several established human PCa cell lines, including LNCaP, DU145, PC-3, ALVA-3, and MDA PCa, treatment with 1,25-VD led to antiproliferation effects, but in varying degrees (3,4). For example, DU145, PC-3, ALVA-3, and MDA PCa 2a display less growth inhibition with 1,25-VD (<20% inhibition) than LNCaP and MDA PCa 2b (approx 50%). These varied degrees of 1,25-VD growth inhibition indicate cells develop resistance to 1,25-VD treatment and hence become less sensitive to 1,25-VD. The receptor for 1,25-VD (VDR), which transmits the ligand signal to the nucleus and regulates the transcription of target genes, is universally expressed in the PCa cells studied (3,4). The antiproliferation effect of 1,25-VD is suggested to be partly correlated with VDR expression level and transactivity (3). Signals affecting VDR activity are potentially involved in the development of 1,25-VD resistance. The fact that 1,25-VD-resistant cells are also androgen-independent implies that the defects in 1,25-VD signaling may be a result of the altered molecular context derived in such forms of PCa.

Androgens are important for the growth of PCa, and antiandrogens have been used therapeutically for decades. Pharmacological or surgical androgen ablation therapy is commonly used to treat PCa patients. Although tumors shrink significantly after therapy, the majority of patients develop hormone-refractory PCa. Numerous mechanisms revealed to date demonstrate that diverse signaling pathways are involved in developing resistance to androgen ablation (5). First, mutant ARs with broad ligand sensitivity have been found to utilize hormones other than androgen to transmit growth-promoting signals (5). Second, amplification of growth hormone signals, such as HER2/neu, in PCa can stimulate growth and eventually bypass AR (5). Third, overexpression of coregulators, such as SRC-1, TIF-2, and gelsolin, in androgen-independent PCa has been shown, that may magnify AR activity in the presence of trace amounts of androgen (6,7). A more recent finding also demonstrated that hydroxylflutamide, an antiandrogen, could activate the MAPK pathway, an effect that might contribute to the development of PCa resistance to androgen ablation (8). Either one of mechanisms or a combination of several mechanisms described above can result in the development of androgen-independent cell growth, and therefore play roles in 1,25-VD resistance.

Most coregulators associate with and modulate more than one steroid receptor. AR coregulators, originally identified as AR-associated proteins, also modulate many other steroid receptors. As previously reported, ARA54 enhances AR and progesterone receptor (9); ARA70 enhances peroxisome
proliferator activated receptor γ and AR (10); and gelsolin and supervillin enhance AR and glucocorticoid receptor (7,11). It is highly possible that VDR and AR share the same coregulators and the potential cross-talk between VDR and androgen-AR signals mediated by coregulators might account for vitamin D action in PCa. Therefore, evaluation of the function of androgen-AR signals and AR coregulators on VDR activity is the first step to elucidate their roles in vitamin D response. Here we characterize the effects of androgen-AR signals and AR coregulators on VDR transactivity, which may provide us information about potential mechanisms of 1,25-VD resistance in PCa cells.

Results

Androgen Signaling Suppresses VDR Transactivation

We tested how androgen-AR signals influence 1,25-VD signaling through VDR. The 1,25-VD–induced VDR activity is measured by the activation of VDR target gene, rat CYP24 promoter-controlled reporter gene, rCYP24-LUC. Overexpression of AR in PC-3 suppresses the 1,25-VD–induced VDR transactivation (Fig. 1A, lane 2 vs 5), with further suppression after addition of the androgen, 5α-dihydrotestosterone (DHT) (lane 5 vs 6). On the other hand, DHT treatment in the AR-containing cells, LNCaP, suppresses VDR activity (Fig. 1B). To examine whether the VDR amount was reduced after DHT treatment, we compared the VDR protein levels. As shown in Fig. 1C, the protein level of VDR was increased under the treatment of DHT (lane 3 vs lane 2). Therefore, the suppression of VDR activity by androgen-AR signals is not due to reduced amount of VDR under DHT treatment. The overexpression of AR in PC-3 and activation of AR in LNCaP, both suppress VDR transactivation, suggesting a possible cross-talk between AR and VDR in gene regulation.

Some of AR Coregulators Modulate VDR Transactivation

To investigate the cross-talk between AR and VDR signaling, we hypothesize that coregulators shared by VDR and AR may mediate the cross-talk between 1,25-VD and androgen signaling pathways. We have screened several known AR coregulators for their ability to modulate VDR transactivation. The COS-1 cell line was selected for examination of coregulators’ effect on VDR due to the absence of most steroid receptors, which eliminates interference from other steroid receptors. Among several AR coregulators examined, ARA54, ARA70, gelsolin, and supervillin were found to enhance VDR (Fig. 2), whereas ARA55 and ARA24 had no effect on 1,25-VD–induced VDR transactivation (data not shown). SRC-1 and SMRT, the known VDR coactivator and corepressor, respectively, served as controls.

The relative expression levels of coregulators and VDR may affect coregulator function. By defining the minimum and maximum activity of coregulators, we can determine

Fig. 1. Modulation of VDR transactivity by androgen-AR signal. (A) PC-3 cells were plated at a density of 10⁴ cells/well in 24-well plate. Cells were co-transfected with 0.375 μg pCMV or pCMV-AR, 0.125 μg prCYP24-LUC, and 1 ng pRL-SV40 by SuperFect. After 20 h, cells were treated with EtOH or 10⁻⁷ M 1,25-VD for another 24 h before being harvested. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (B) LNCaP cells were plated at a density of 3 × 10⁴ cells/well in 24-well plates. After 24 h, cells were transfected with 0.6 μg prCYP24-LUC and 1 ng pRL-SV40 by SuperFect. After 20 h, cells were treated with EtOH, 1,25-VD or DHT, as indicated, for another 24 h. Cells were then harvested for the LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (C) The expression of AR and VDR after treatment in LNCaP cells was detected by Western blotting. LNCaP was seeded at a density of 10⁶ cells/dish in 100 mm dishes. After treating with ligands as indicated for 24 h, cell lysates were harvested for the detection of AR, VDR, and actin expression amount. The level of expression was extrapolated by densitometric analysis after correction by actin amount.
coregulator efficiency and the influence of cellular context on coregulator function. Therefore, we titrated the relative coregulator plasmid compared with that of VDR from 1 to 1 up to 8 to 1. SRC-1 was found to enhance VDR and SMRT was found to repress VDR activity starting at a ratio of 1 to 1, while most AR coregulators tested were found to enhance VDR starting at a ratio of 4 to 1 (Fig. 3). Among AR coregulators, ARA70 was shown to be the strongest enhancer of VDR activity, whereas gelsolin and ARA54 showed the weakest enhancement of VDR transactivity in COS-1 cells. Therefore, we focused on ARA70 and ARA54 to further dissect their mechanisms of actions.

**ARA70 Directly Associates with VDR But Not ARA54**

Most coregulators associate with nuclear receptors and then modulate receptor activity. We determined whether ARA54 and ARA70N associated with VDR using the mammalian two-hybrid and GST pull-down assays. Some proteins may lose their function when conjugated with Gal4 (DBD) or VP16. Therefore, we selected the one with proper function maintained after conjugation with Gal4(DBD) or VP16 for mammalian two-hybrid assay. To ensure these conjugated coregulators have proper function, we demonstrated that Gal-ARA54C can interact with VP16-AR; VP16-VDR can interact with Gal-RXRα-LBD; and the VP16-ARA70N interacts with Gal-AR, which were used as positive controls (Fig. 4A). Co-transfection of plasmids expressing Gal-ARA54C with VP16-VDR demonstrated minor interaction between VDR and ARA54C (Fig. 4A). On the other hand, Gal-VDR interacted with VP16-ARA70N in a ligand-dependent manner in the two-hybrid system (Fig. 4A). We further demonstrated the interaction by using in vitro GST pull-down assay, which showed ligand-independent interaction between ARA70 and VDR-L (Fig. 4B), whereas there was no interaction between ARA54 and VDR-L. The ligand-independent interaction between ARA70 and VDR-L in GST-pull down assay while ligand-dependent interaction between ARA70N and VDR in mammalian two-hybrid assay implied another interaction domain may be located in the C-terminus of ARA70 that is responsible for such interaction. It may also result from fusion protein characteristics, or differences in the overall assay environments.
**LXXLL Motif in ARA70 Is Important for Interaction with VDR**

LXXLL sequence motifs are important for most coregulators, including SRC-1, TIF-II, and NCoR, to associate with steroid receptors, such as estrogen receptor, RXR, and VDR. There is one LXXLL motif in ARA70 located in its N-terminus. Point-mutated ARA70N containing LXXAA instead of LXXLL was constructed and characterized for its interaction ability with VDR. By using mammalian two-hybrid assay, we demonstrated that VP16-ARA70N mutant (LXXAA) lost the interaction with Gal4(DBD)-VDR indicating the LXXLL motif in ARA70N is essential for interaction with VDR (Fig. 5A). We further tested the ability of mutant ARA70 to promote VDR transactivity. Overexpres-
Fig. 4. The interaction between VDR and ARAs was analyzed in mammalian two-hybrid and GST pull-down assays. (A) COS-1 cells were plated at a density of $3 \times 10^4$ cells/well in 24-well plates. After 24 h, the mammalian two-hybrid assay was performed by co-transfection of plasmids expressing 0.2 μg Gal-VDR, -RXRα-LBD, or -ARAs, 0.2 μg VP16, VP16-VDR, -AR or -ARAs, 0.1 μg pG5-LUC, and 1 ng pRL-SV40 as indicated. After 20 h, cells were treated with EtOH, $10^{-8} M$ DHT, or $10^{-7} M$ 1,25-VD for another 24 h, and were then harvested for the LUC assay. The LUC activity relative to lane 1 was calculated and the mean ± SD of three independent experiments is shown. (B) GST and GST-VDR-L proteins were expressed and purified from bacteria. Full-length RXR, ARA54, and ARA70 were expressed in vitro and labeled with [35S]methionine. After incubating with EtOH or $10^{-9} M$ 1,25-VD for 1 h at 4°C, GST-VDR-L was further incubated with RXR, ARA54, or ARA70 for another 2 h. The pull-down complexes were separated by 12% SDS-PAGE and analyzed by phosphorimager for the detection of radioactive signals.
sion of mutated ARA70 containing LXXAA motif in vitamin D-responsive reporter gene assay showed that coactivation of VDR was significantly reduced compared to wild-type ARA70 (Fig. 5B). However, the mutant ARA70 did not completely lose its coactivation function, which implies another interaction domain is located in the C-terminus of ARA70 that may still interact with VDR. These results indicate that the LXXLL motif in ARA70 is important for interaction with VDR and for co-activation of VDR.

**Overexpression of ARA70 Restores VDR Transactivity Suppressed by Androgen**

The fact that both VDR and AR can interact with ARA70 indicates the cross-talk between these two receptors may be mediated by competing for ARA70. We tested whether we could overexpress sufficient amounts of ARA70 for VDR transactivation even in the presence of androgen. Different amounts of plasmids encoding ARA70 protein were transfected into LNCaP where androgen treatment suppressed VDR transactivity (Fig. 1B). The androgen-suppressed VDR transactivity was dose-dependently restored by increasing the transfected amount of ARA70 plasmids (Fig. 6). However, ARA54, which promoted VDR through an indirect mechanism, could not restore VDR transactivity suppressed by androgen. Therefore, androgen treatment did not prevent ARA54 from promoting VDR transactivity, but did squelch ARA70 from interacting with VDR, hence decreasing VDR transactivity.

**Discussion**

The finding that androgen-AR signal can attenuate VDR transactivation led us to investigate whether AR coregulators can interact with VDR and enhance VDR transactivation. The interaction between AR coregulators and VDR suggests the cross-talk between AR and VDR is mediated by shared coregulators. The interaction between ARA70 and VDR implies that AR and VDR could be linked together by ARA70, or that they may compete for binding to ARA70. Most coregulators utilize the LXXLL sequence motifs to associate with steroid receptors, while AR coregulators also utilize a unique motif, FXXLF (12, 13). Interestingly, these two motifs co-exist in ARA70, implying that AR and another steroid receptor may associate with ARA70 without competing with each other. Further investigation showed that VDR associates with ARA70 through the LXXLL motif, but not the FXXLF motif (data not shown). Our result showing that VDR transactivity suppressed by androgen was restored by overexpression of ARA70 indicates that the competition for association with ARA70 between VDR and AR mediates this cross-talk. Another AR coregulator, ARA54, also enhances VDR transactivity, but no direct interaction between these two proteins was observed in either the mammalian two-hybrid or GST pull-down assay. A recent study has shown that ARA54 may act as a Ub-ligase (E3) and exert proteasome function (14). Therefore, the degradation of VDR partners may be regulated by ARA54, an effect that indirectly modulates VDR transactivity. This mechanism may exist in spite of no direct interaction with VDR. Because androgen signals did not squelch ARA54 from promoting VDR transactivity, the overexpression of ARA54 could not restore VDR transactivity in the presence of androgen.

This observation also suggests that the context of AR and AR coregulators in cells can contribute to how androgen-AR affects the vitamin D-VDR signaling. Among all the co-
activators we tested, only some show the enhancing effect. In fact, ARA55 did not promote VDR transactivation (data not shown). The order of promoting capacity is SRC-1 > ARA70 > supervillin > gelsolin > ARA54 (Fig. 3). Some studies report the overexpression of coregulators, including SRC-1 and gelsolin, in hormone-refractory PCa. This implies enhanced VDR transactivity, hence the reponse to vitamin D may occur in hormone-refractory PCa. More studies are needed to characterize the expression levels of other coregulators in various stages of PCa. Nonetheless, one report demonstrated that 31% of hormone-refractory tumors contained AR gene amplification (15). Increased expression of AR during PCa progression may result in suppressed VDR activity and diminished antiproliferation effects of vitamin D in high-grade PCa patients. The composition of AR and coregulators in cells provides an environment where VDR activity is affected. Therefore, when evaluating the antiproliferation effect of vitamin D in prostate cancer, the impact from overexpression of AR and coregulators should both be considered.

Several studies demonstrate the antiproliferation effect of vitamin D in androgen-responsive PCa LNCaP cells is dependent on functional AR. First, the expression of AR is induced by 1,25-VD and this induction correlates with growth inhibition effect of 1,25-VD (16). Second, anti-androgen treatment attenuates the growth-inhibitory effect of 1,25-VD in LNCaP cells (16). Third, AR RNAi depleted AR expression results in less growth inhibitory effect of 1,25-VD (17). However, antiandrogen did not affect the anti-proliferation effect of 1,25-VD in androgen-refractory PCa cell lines, MDA PCa, and LNCaP-104R1 (4,18). Overexpression of AR in ALVA 31 did not restore the response to 1,25-VD (18). Those results indicate that 1,25-VD no longer depends on AR signaling for growth inhibition in these cell lines. The dilemma that 1,25-VD inhibits PCa growth through functional AR while functional AR suppress VDR transactivity complicates the outcome from the cross-talk between VDR and AR. Therefore, whether the functional AR affected VDR transactivity can be translated into the antiproliferation effect of 1,25-VD may depend on how cells rely on androgen-AR signal for growth.

In addition to PCa cells, VDR is widely expressed and functions in many tissues, such as parathyroid gland, bone, skin, and intestine (19). The fact that AR coregulators modulate VDR suggests that AR can suppress VDR activity by competing for the same coregulators in common target tissues of VDR and AR, such as the hair follicle and bone. Androgenetic alopecia occurring in men is androgen-dependent, and androgen-regulated factors secreted from dermal papilla cells are believed to inhibit the growth of components of the hair follicle (20). Because alopecia also occurs in VDR knockout mice, the androgenetic alopecia might result from the suppression of VDR activity by androgen signals in the hair follicle (21). In addition, the bone volume
in AR knockout mice (ARKO) is reduced, and osteopenia may result from increasing numbers of osteoclasts (22). The formation of osteoclasts is induced by active VDR (23), which implies a potential mechanism for the stimulation of osteoclasts in ARKO mice. The physiological significance of AR signaling on VDR target genes and the roles of coregulators in mediating this cross-talk should be further investigated.

In summary, the fact that overexpression of AR in PC-3 cells and treatment of LNCaP cells with DHT both suppress endogenous VDR transactivation, supports the existence of cross-talk between VDR and AR in gene regulation. To investigate the underlying mechanism, we demonstrated that several AR coregulators, including ARA54, ARA70, gelsolin, and supervillin, all promote VDR. Furthermore, ARA70, but not ARA54, associates with VDR and overexpression of ARA70 can restore the androgen–AR signal suppressed VDR transactivity. Therefore, the cross-talk between VDR and AR is through sharing same coregulators. The altered composition of AR and AR coregulators, which frequently occurs during PCA progression, may contribute to 1,25-VD resistance in PCa. This cross-talk could possibly exist in other tissues and further studies are required to elucidate the physiological significance resulting from the interplay between androgen signal, androgen coregulators, and 1,25-VD signals.

Materials and Methods

Plasmids

pCYP24-LUC was constructed by inserting the fragment containing −950 bp−−55 bp region of pCYP24 promoter released from rat 25-hydroxyvitamin D3 24-hydroxylase gene promoter CAT construct (−2.2 kb−+188 bp), a kind gift from Dr. Yoshihiko Ohyama (Hiroshima University, Japan), by Mscl and Sacl into Sacl and Smal sites of pGL3-TK (a gift from Dr. Eungseok Kim, University of Rochester, Rochester, NY). The cDNA encoding full-length VDR was amplified by PCR and inserted into the pCMX-VP16 vector and pCMX-Gal4.N vector to generate the VP16-VDR and Gal4-VDR expression plasmids. pCMV-Gal4-ARA54C (amino acids 361–474), pCMV-Gal4-retinoid x receptor α (RXRα)–ligand binding domain (LBD), and pG4AB1-hAR(DE) (amino acids 624–918) were used for expression of Gal4-DNA binding domain (DBD)–conjugated proteins. The plasmids pCMX-VP16-ARA70N (amino acids 1–401) and pCMX-VP16-AR were used to express VP16-conjugated ARA70N and AR full-length proteins. The cDNA encoding the LBD of VDR (VDR-L) was amplified by PCR and inserted into the pGEX-KG vector to generate the GST-VDR-L expression plasmid. The plasmid expressing VP16-conjugated mutant ARA70N (LXXAA) for mammalian two-hybrid, pCMX-VP16-ARA70N(LX XAA), was described previously (24). pSG5-ARA70(LX XAA) was constructed by replacing a fragment from EcoRI and BglII digested pSG5-ARA70 with a fragment released from EcoRI and BglII digested pCMX-VP16-mtARA70.

Transfection Studies

LNCaP and PC-3 cells were maintained in RPMI-1640 containing 10% FBS. COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS. Cells were seeded 24 h before transfection, then transfected according to the “SuperFect Transfection” instructions (Qiagen, Valencia, CA). After 2–3 h incubation, culture medium was replaced with medium supplemented with 10% charcoal–dextran treated FBS (CD-FBS). After 20 h, cells were treated with EtOH, 1,25-VD, or DHT, as indicated, then further incubated at 37°C for 16–24 h before harvesting. Cell lysates were prepared and used for the luciferase (LUC) assay according to the manufacturer’s instructions (Promega, Madison, WI). LUC activity was normalized with internal control Renilla-LUC activity. The results were obtained from at least three independent experiments and presented as means ± SD.

Mammalian Two-Hybrid Assay

COS-1 cells were plated at a density of 3 × 10^6 cells/well in 24-well plates. After 24 h, the mammalian two-hybrid assay was performed by co-transfection of plasmids expressing 0.2 μg Gal4(DBD)-conjugated receptors or ARAs, 0.2 μg VP16 alone, VP16 conjugated receptors, or ARAs, 0.1 μg pG5-LUC, and 1 ng pRL-SV40. After 20 h, cells were treated with EtOH, 10^-8 M DHT, or 10^-7 M 1,25-VD for another 24 h, and were then harvested for the LUC assay. The LUC activity represents interaction between receptors and coregulators tested.

Glutathione S-Transferase (GST) Pull-Down Assay

GST-VDR-L fusion protein, and GST control protein were purified (25). The GST fusion proteins were pulled down by glutathione (GSH) beads at 4°C for 1 h and washed three times with washing buffer. The purified GST fusion proteins and beads were incubated in 100 μL binding buffer with EtOH or μM 1,25-VD for 1 h, at 4°C. In vitro-translated 35S-methionine-labeled RXR, ARA54, and ARA70 full-length proteins were produced using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Five microliters of RXR, ARA54, or ARA70 was then added. After incubating for 2 h at 4°C, GSH beads were washed with washing buffer three times. Protein complexes were separated by 12% SDS-PAGE and visualized by Phosphor Imager (Molecular Dynamics Amersham Bioscience, Piscataway, NJ).

Preparation of Cellular Protein and Western Blots

LNCaP cells were seeded in 100-mm dishes and treated with ligands. After 24 h, cells were collected and suspended in lysis buffer, and centrifuged. Aliquots corresponding to
100 µg protein of each sample were loaded onto a 8% SDS-PAGE for Western blotting. After blotting, the membrane was blocked with 5% nonfat milk and probed with antibody against AR (NH27), VDR (Santa Cruz Biotechnology, Santa Cruz, CA), and actin (Santa Cruz Biotechnology) at a dilution of 1:2000 in PBS containing 0.1% Tween 20, for 1 h. After washing, the membrane was incubated with the alkaline phosphatase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology) for 45 min, washed again, and the immunoreactive bands were visualized for alkaline phosphatase activity with the 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium phosphatase substrate (Bio-Rad Laboratories, Hercules, CA).

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References

1α,25-dihydroxyvitamin D₃ inhibits prostate cancer cell invasion via modulation of selective proteases

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Introduction

Prostate cancer is the second leading cause of cancer deaths among North American men. The initial treatment of advanced stage prostate cancer is suppression of testicular androgen production by medical or surgical castration, but nearly all patients develop disease progression. Hormone refractory prostate cancer (HRPC) remains a challenge in the management of prostate cancer patients. Since no therapy has yet demonstrated a definitive survival advantage, the need for more options in the treatment of HRPC is obvious.

Inhibition of invasion and metastasis might be a good approach for treatment of HRPC in which hormone therapy has failed. Cancer cell metastasis is a step-wise process that includes detachment of cells from the primary tumor, local proteolysis of the basement membrane, intravasation, survival in the circulation, arrest in a distant organ, extravasation and invasion into the surrounding tissue and growth (1). Metastasis necessarily involves penetration of the extracellular matrix (ECM) and basement membrane, and is thought to require the action of proteases.

There are three major groups of proteases, the matrix metalloproteinases (MMPs), the plasminogen activators (PAs), and the cathepsins (CPs), known to be involved in tumor invasion. The MMPs are a family of >20 zinc-dependent proteases that are capable of degrading the components of the ECM (2,3). Among the MMPs, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are key enzymes for degrading type IV collagen, a major component of the basement membrane (4,5). Most MMPs are secreted as inactive pro-enzymes and their proteolytic activities are regulated by other proteases or inhibited by specific inhibitors, tissue inhibitors of metalloproteinase (TIMPs). This implies that the balance between MMP and TIMP levels is a critical determinant of the net proteolytic activity. The increased activities of MMP-2 and MMP-9 have been associated with increasing tumor metastases in various human cancers, suggesting an important functional role for these proteases in the metastatic process (6).

The serine proteases urokinase PA (uPA) and tissue PA (tPA) can convert plasminogen to plasmin, which is capable of promoting tumor growth and angiogenesis, degrading the ECM and basement membrane, and activating pro-MMPs (7). PA activity is negatively regulated by plasminogen activator inhibitors (PAIs), PAI-1 and PAI-2. PAIs function by direct binding to uPA and tPA, and subsequently form inactive complexes (8). Over-expression of uPA and its cell surface receptor (uPAR), along with high PA activity are correlated positively with both the invasive activity of cancer cell lines as well as poor patient prognosis (9,10).

Increased CPs activity and expression, and changes in localization have been observed in many different cancers (11–15). CPs can also degrade components of the ECM, suggesting that these proteases are involved in cancer cell invasion and metastasis (16–18). CPs activities are down-regulated by endogenous inhibitors, such as cystatins. Loss of expression and activity of certain members of the cystatin superfamily have been shown to correlate with the metastatic ability of some cancer cells (19–21).

Epidemiological evidence suggests that low exposure to sunlight and vitamin D deficiency might be risk factors for prostate cancer mortality (22,23). Much research has focused on 1,25-VD, the active metabolite of vitamin D, and its ability

Abbreviations: 1,25-VD, 1α,25-dihydroxyvitamin D₃; CP, cathepsin; CPI, cathepsin inhibitor; ECM, extracellular matrix; HRPC, hormone refractory prostate cancer; MMP, matrix metalloproteinase; PA, plasminogen activator; PAI, plasminogen activator inhibitor; TIMP, tissue inhibitors of metalloproteinase; tPA, tissue PA; TPA, 12-O-tetradecanoylphorbol-13-acetate; uPA, urokinase PA.

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to induce either apoptosis or differentiation in many cancer cells. However, little is known about how 1,25-VD regulates cancer cell invasion and metastasis. In vitro, 1,25-VD has been demonstrated to inhibit the invasion of a number of cultured cancer cells through Matrigel or Amgel, including breast, prostate and lung cancer cells (24–26). In vivo, intravas-tilational injection of 1,25-VD inhibited the invasion in N-methyl-nitrosourea-induced bladder cancer in rats (27). In a Phase II study, weekly high-dose vitamin D and docetaxel resulted in significant reduction of prostate-specific antigen, increased patients’ quality of life, and delayed the time of disease progression in men with metastatic androgen-independent prostate carcinoma (28,29). Regarding mechanisms, 1,25-VD has been shown to inhibit certain proteases, such as some components of the PA and MPM systems, which are important determinants of tumor invasion. Decreased activity of uPA and tPA and increased activity of PAI in response to 1,25-VD have been described in MDA-MB-231 human breast cancer cells (30). In addition, a 1,25-VD-responsive region was identified between nucleotides −2350 and −1870 of the uPA promoter. Decreased activity of MMP-2 and MMP-9 in breast and prostate cancer cells after 1,25-VD treatment have also been demonstrated (25,30).

In this study, we focus on how 1,25-VD modulates the activities of proteases and their inhibitors to inhibit prostate cancer invasion. We systematically examined the activity and gene expression levels of three major groups of proteases, the MMPs, the PAs and the CPs, after 1,25-VD treatment. We found that the activity of MMP-9 and CPs, but not PAs, decreased and that the activities of their counterparts, TIMP-1 and cathepsin inhibitors (CPIs), increased after 1,25-VD treatment. In addition, we have provided a mechanism of how 1,25-VD up-regulates TIMP-1 and down-regulates MMP-9 activity to influence cancer cell invasion. Our results support the idea that vitamin D-based therapeutics are beneficial and may lead to the design of better combination therapies in the management of advanced prostate cancer.

**Materials and methods**

**Cells, plasmids and materials**

1,25-VD was the gift from Dr Lise Binderep of Leo Pharmaceutical Products, 12-O-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma and MMP-9 promoter construct was kindly provided by Dr Yasuyuki Sasaguri from University of Occupational and Environmental Health, Japan. AP-1 and NF-κB reporter constructs were kindly provided by Dr Andrew M.-L.Chan from Mount Sinai School of Medicine, NY. TIMP-1 promoter constructs were kindly provided by Dr Ian M.Clark from University of East Anglia, UK. The LNCaP, PC-3 and DU 145 cells were obtained from the American Type Culture Collection. Cell culture media (RPMI-1640) was obtained from Gibco BRL.

**Cell culture, transfection and luciferase assays**

LNCaP, PC-3 and DU 145 cells were maintained in RPMI-1640 containing penicillin (100 IU/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum (FBS) at 5% CO2 and 37°C. Transfections were performed by using SuperFect according to the manufacturer’s suggested procedures (Qiagen). After transfection, cells were treated for 24 h with charcoal-stripped FBS medium containing ethanol vehicle or the ligands. Cell lysates were prepared, and the luciferase activity was normalized for transfection efficiency using pRL-CMV as an internal control. Luciferase assays were performed using the dual-luciferase reporter system (Promega, Madison, WI).

**Invasion assay**

LNCaP, PC-3 and DU 145 cells were incubated with ethanol vehicle or 100 nM 1,25-VD for 72 h in regular medium. Cells were harvested and counted, and 5 × 10^4 cells/chamber were used for each invasion assay. Cells were added to Matrigel coated inserts (Becton Dickinson Labware, Bedford, MA) in serum-free media containing ethanol vehicle or 100 nM 1,25-VD. The lower chambers contained medium with 10% FBS and ethanol vehicle or 100 nM 1,25-VD. The chambers were incubated for 22 h at 37°C. The cells that had invaded to the lower surface of the membranes were fixed and stained with 1% Toluidine blue, and total invading cell number in five random fields was counted under a light microscope.

**Cell attachment assay**

LNCaP, PC-3 and DU 145 cells were incubated with ethanol vehicle or 1,25-VD under serum-free conditions for 48 h, and then conditioned media was collected and normalized with cell number. For measuring MMP-9 activity in cell-conditioned medium, we used the ‘MMP-9 biotrik activity assay system’ by Amershams Pharmacia (RPN 2634) according to the manufacturer’s instructions.

**Gelatin substrate gel zymography**

LNCaP, PC-3 and DU 145 cells were incubated with ethanol vehicle or 1,25-VD under serum-free conditions for 48 h, and then conditioned media was collected and normalized with cell number. To analyze the MMP-2, MMP-9 and TIMPs activities in cell-conditioned medium, regular gelatin zymography and reverse gelatin zymography were used. Briefly, samples were subjected to 12% SDS–PAGE, under non-reducing conditions, in gels co-polymerized with 0.1% gelatin for gelatin zymography or 0.1% gelatin plus 40 ng/ml MMP-2 and MMP-9 (Chemicon International) for reverse gelatin zymography. Following electrophoresis, gels were washed twice for 30 min in wash buffer (50 M Tris/PH 7.4 and 2.5% Triton X-100), then rinsed in incubation buffer (50 M Tris/PH 7.4, 150 mM NaCl, 10 mM CaCl2 and 0.02% NaN₃) and incubated at 37°C for 24 h. Enzyme activities were visualized by staining with Coomassie blue.

**Plasminogen activator activity assay**

LNCaP, PC-3 and DU 145 cells were incubated with ethanol vehicle or 1,25-VD under serum-free conditions for 48 h, and then conditioned media was collected and analyzed by the PA activity assay. PA activity was measured using the chromogenic substrate S-2251 (H-D-Val-Leu-Lys-p-nitroanilide) (31). In brief, plasminogen is converted into plasmin by plasminogen activator, and the generated plasmin hydrolyzes S-2251 to release p-nitroaniline. The released p-nitroaniline is measured by absorbance at 405 nm. The assay solution was prepared by mixing 20 μl of 1 mg/ml purified bovine plasminogen and 80 μl of 1 mM S-2251 in dilution buffer [0.05 M Tris–HCl (pH 7.4) and 1 mM MgCl2]. To determine the total PA activity in each sample, an equal volume of the assay solution was added to 100 μl of the sample. Following incubation at 37°C for 1 h, the absorbance at 405 nm was measured with a microplate photometer, and normalized to the protein concentration.

**CP and CPI activity assays**

LNCaP, PC-3 and DU 145 cells were incubated with ethanol vehicle or 1,25-VD under serum-free conditions for 48 h, and then cell lysates were collected and analyzed by the CP and CPI activity assay. Specific catalytic activity of total proteases was determined fluorometrically by hydrolysis of 500 μM synthetic substrate Z-phe-arg-NMec (32). Proteases activity in cell lysates was measured using Z-phe-arg-NMec as substrate in buffer consisting of 250 mM sodium acetate/H 5.4, 40 mM acetic acid, 2.5 mM EDTA and 1 μM DTT. Total CP activity was abolished with 1.53 mM cysteine proteinase inhibitor E-64, therefore activities of CPs were differentiated by inactivation with E-64. Fluorescence was measured in a SPECTRMax GEMINI spectrofluorometer at an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

Total CPI activity was measured by incubating cell lysates with the cysteine proteinase papain as follows. Samples were boiled for 5 min to denature heat-sensitive proteases such as the CPs. CPs are heat stable (33). The denatured proteins were removed by centrifugation at 14 000 × r.p.m. for 10 min at 4°C. Aliquots of the sample were incubated with 10 μl of 10 mM papain and remaining papain activity was measured essentially as described for CP activity assay using Z-phe-arg-NMec as substrate. Total papain activity was determined in assays containing aliquots of PBS.
Real-time PCR analysis

LNCaP, PC-3 and DU 145 cells were cultured and treated with either ethanol vehicle or 100 nM 1,25-VD for 12, 24 and 48 h, then total RNA was extracted using Trizol (Invitrogen). We carried out reverse transcription with the Super Script II kit (Invitrogen) and PCR amplifications with SYBR Green PCR Master Mix on an iCycler IQ multi-color real-time PCR detection system (Bio-Rad). The PCR was performed as follows: initial denaturation at 95°C for 10 min, and 45 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 30 s. Primer sequences were MMP-2, sense 5’-CAAGGATACACGCTCAGTATA-3’ and anti-sense 5’-GGTG-CAGCTTCTATTTGGTG-3’ (34); MMP-9, sense 5’-TGGCAGGCGTGAGTTCC-3’ and anti-sense 5’-TGGTGACGAGTTGACGTAG-3’; TIMP-2, sense 5’-GACTGATGACCGGCAAGCGAGC-3’ and anti-sense 5’-ATCTTCAAAGAACCAGACAC-3’; TIMP-1, sense 5’-CTAACGAGACACCAACTTAC-3’ and anti-sense 5’-TTTCACTAGCCGACACCACT-3’ (35); tPA, sense 5’-ATGACATCTTACGAGATTTCC-3’ and anti-sense 5’-GTTGAAGGCGTCGTAACACT-3’; uPAR, sense 5’-CAAGAACCACCTCCACTTCTC-3’ and anti-sense 5’-GGAACTTTTCTGG-3’; TIMP-1, sense 5’-CTGTTGTTCTTGTTGGGCT-3’ and anti-sense 5’-TGTTCTGTTAAG-3’; PAI-1, sense 5’-CAAACTGGAGAACAACCAGTTG-3’ and anti-sense 5’-AGAGCGGAAGGATGAATG-3’; PAI-2, sense 5’-CCAGAGAACAACCAGTTG-3’ and anti-sense 5’-AGAGCGGAAGGATGAATG-3’; MMP-9 mRNA stability assay

MMP-9 mRNA was prepared and analyzed by real-time PCR described above.

DNA pull-down assay

Oligonucleotides corresponding to the AP-1 site were synthesized according to published sequences (41). Sequences of the oligonucleotides were as follows: wild-type-AP-1 (−105) strand, sense 5’-biotin-GATGGTGTTGGGATGATCGT-3’ and anti-sense 5’-TCTTCTGAGCATCTCCTC-3’. Double-stranded probes were made by annealing a 50 μM mixture of complementary oligonucleotides in TNE (10 mM Tris–HCl, 50 mM NaCl and 1 mM EDTA), heating to 95°C for 5 min and then slowly cooling to room temperature. Nuclear extracts were prepared from PC-3 cells that were serum-starved for 24 h and stimulated with 1,25-VD for 3 h (42). For pull-down assays, 30 μg of nuclear extracts were incubated in a 25 μl reaction mixture consisting of 10 μM probe and 1× binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl/pH 7.5, and 0.05 mg/ml poly(dI-dC)). After incubation for 30 min at room temperature, the reaction volume was increased to 0.5 ml with modified binding buffer, which does not contain poly(dI-dC). To capture the complexes, streptavidin-agarose was added, and incubated for 1 h at 4°C. The complexes were washed three times with modified binding buffer, and eluted from the beads by the addition of 2× Laemmli buffer and heating to 95°C for 5 min. Proteins were then separated by 10% SDS–PAGE and analyzed for the c-Jun (SC-44, Santa Cruz) by immunoblot analysis.

Statistical and densitometric analysis

Results

1,25-VD inhibits human prostate cancer cell invasion in vitro

We first explored the vitamin D effect on the prostate cancer cell invasion ability by in vitro matrigel invasion assay as described previously (25,30). We treated cells with 1,25-VD for 3 days and followed with a 22 h invasion period. The invasion potentials of three prostate cancer cell lines were determined by counting the invading cells in the lower membrane. As shown in Figure 1, 1,25-VD inhibited LNCaP, PC-3 and DU 145 cells invasion by 43, 47 and 38%, respectively. According to others’ and our previous study, 3 days of 1,25-VD treatment can inhibit LNCaP, but not PC-3 and DU 145 cell proliferation (43–45). In addition to cell proliferation, we also examined 1,25-VD effects on cell attachment, and we found that 1,25-VD can decrease PC-3 attachment by 5%, but there was no effect on LNCaP or DU 145 cell attachment (data not shown). Therefore, these data suggest that neither decreased cell proliferation nor cell attachment contributes to 1,25-VD anti-invasive effects in prostate cancer cells.

1,25-VD regulates matrix metalloproteinase activities

The mechanisms underlying the anti-invasive effects of 1,25-VD on prostate cancer cells were then examined. We first tested whether 1,25-VD inhibits cell invasion via modulation of MMP activities. MMP-9 activity assay for determining active-MMP-9 activity, gelatin zymography for determining pro-MMP-2 and pro-MMP-9 activities, and reverse gelatin zymography for determining TIMP-1 activities were applied. As shown in Figure 2, treatment of PC-3 and DU 145 cells, but not LNCaP cells, with 1,25-VD decreased active- and pro-MMP-9 activity (Figure 2A and B), associated with a concomitant increase in secreted TIMP-1 activity (Figure 2C). We then tested whether the regulation of MMP-9 and TIMP-1 activities by 1,25-VD occurred directly at the transcriptional level. The mRNA levels of MMP-9 and TIMP-1 were measured by quantitative real-time PCR. As shown in Figure 2D, the endogenous MMP-9 transcripts expressed highest in PC-3, then DU 145, least in LNCaP, which corresponds to the enzyme activity we observed in Figure 2A and B. The MMP-9 transcripts were suppressed by 1,25-VD in all three prostate cancer cell lines we tested in a 1,25-VD treated time-dependent manner. Similar to MMP-9, its counterpart TIMP-1 has a similar
endogenous expression level among three prostate cancer cell lines. In contrast, when treated with 1,25-VD, TIMP-1 transcripts were induced in a time-dependent manner (Figure 2E), which correlates with the enzyme activities. However, the mRNA level of MMP-2 was slightly increased and there was no consistent change on TIMP-2 after 1,25-VD treatment (Supplementary Figure 1). In summary, we concluded that 1,25-VD may inhibit human prostate cancer cell invasion.
through modulation of selective MMP activities, including those of MMP-9 and TIMP-1.

1,25-VD has selective effects on PA and CP systems
Serine and lysosomal cysteine proteases have been implicated in cancer cell invasion and metastasis, not only in degradation of ECM, but also through activation of many other protease zymogens, including pro-MMP-9. We have shown that 1,25-VD inhibits prostate cancer cell invasion by modulating MMP-9 and TIMP-1 activities. Therefore, we examined the ability of 1,25-VD to regulate PA and CP activities. As shown in Figure 3A, PA activities have no significant change in prostate cancer cell conditioned medium or cell lysates after 1,25-VD treatment. The mRNA expression of molecules involved in the

![Image of Figure 3](image.png)

**Fig. 3.** The effect of 1,25-VD on plasminogen activator system. (A) The effect of 1,25-VD on PA activity. LNCaP, PC-3 and DU 145 cells were treated with ethanol vehicle or the indicated concentrations of 1,25-VD for 48 h, and then conditioned media and cell lysates were analyzed by PA activity assay. (B) The mRNA expression of endogenous tPA (left panel) and 1,25-VD effects on its expression (right panel) in prostate cancer cell lines. (C) The mRNAs expression of endogenous uPA (left panel) and 1,25-VD effects on its expression (right panel) in prostate cancer cell lines. LNCaP, PC-3 and DU 145 cells were cultured and treated with either ethanol vehicle or 100 nM 1,25-VD for 12, 24 and 48 h. Total mRNA was prepared and analyzed by real-time PCR. Data are expressed as the mean ± SD of triplicate samples. Values represent the fold changes in gene expression relative to LNCaP cells or untreated control.
PA system, including tPA, uPA, uPAR, PAI-1 and PAI-2 were measured by quantitative real-time PCR. Similar to MMP-9, PC-3 cells express the highest tPA and uPA mRNA; however, 1,25-VD had little or no consistent effect on the expression of PA-related genes among the cell lines tested (Figure 3B and C, and Supplementary Figure 2).

Next, we examined 1,25-VD effects on CP activities. LNCaP, PC-3 and DU 145 cells were treated with increasing concentrations of 1,25-VD for 48 h, and then cell lysates were collected for determination of CP and CPI activities. CP L + B activities were measured using Z-phe-arg-NMec as substrate, which is mainly hydrolyzed by CP L and to a small extent by CP B (46). As shown in Figure 4A, 1,25-VD inhibited CP activity in DU 145 cells, but had less effect on LNCaP and PC-3 cells. Total heat-stable CPI was measured, as shown in Figure 4B. 1,25-VD significantly induced CPI activity in all three cell lines we tested. The ratio of CP to CPI activity (C:I ratio), which represents invasion potential, was calculated and shown to decrease in all cell lines (Figure 4C). The mRNA expression level of potential genes involved in regulation of CP activities, such as CP B, CP H, CP L, cystatin A and cystatin M were measured. However, there was no significant or consistent change of all those CP-related genes we tested upon 1,25-VD treatment among the cell lines (Supplementary Figure 3). Taken together, we concluded that 1,25-VD might decrease C:I ratio and then consequently inhibit prostate cancer cell invasion, yet the 1,25-VD targets and detailed mechanisms need to be further examined.

The suppression of MMP-9 activity by 1,25-VD was not regulated at the transcriptional level

We have shown in Figure 2 that 1,25-VD inhibited both secreted MMP-9 activity and MMP-9 transcripts in PC-3 and DU 145 cells, so the regulation of MMP-9 by 1,25-VD was then examined using a 1.9 kb MMP-9 promoter luciferase reporter gene assay in PC-3 cells. As shown in Figure 5A, luciferase activity was induced ~2.7-fold when cells were treated with ethanol vehicle or 100 nM TPA, however, there was no change when cells were treated with 1,25-VD. AP-1 and NF-κB have been shown to activate the MMP-9 promoter, therefore we tested whether 1,25-VD modulated MMP-9 activity indirectly through down-regulation of AP-1 or NF-κB by testing with AP-1 and NF-κB responsive DNA element containing luciferase constructs. As shown in Figure 5B and C, 1,25-VD had no effect on NF-κB-response element driven luciferase activity, and slightly enhanced AP-1-response element driven luciferase activity, suggesting that the suppression of MMP-9 mRNA expression might not be regulated at the transcriptional level.

To test whether 1,25-VD could affect the post-transcriptional events of MMP-9 mRNA, we performed actinomycin D experiments. PC-3 cells were treated with ethanol or 100 nM 1,25-VD for 48 h before transcription was blocked by actinomycin D. We found that 1,25-VD increased the decay of MMP-9 mRNA (Figure 5D). In conclusion, these data suggested that 1,25-VD inhibited MMP-9 activity and mRNA expression might result from the decrease of MMP-9 mRNA stability.

Transcriptional up-regulation of TIMP-1 by 1,25-VD

We have shown in Figure 2 that 1,25-VD induced TIMP-1 mRNA expression and activity, thus the regulation was examined further. As illustrated in Figure 6A, four TIMP-1 promoter constructs that contain three different lengths of promoter, −1718, −738, −102 and one AP-1 mutated (mt −102) luciferase reporter were tested in PC-3 cells. As shown in Figure 6B, TPA, serving as a positive control, induced luciferase activity to ~3-fold, and 1,25-VD activated the TIMP-1 promoter activity in a dose-dependent manner in all lengths of TIMP-1 promoter constructs we tested. Similar results were observed in DU 145 cells (data not shown). However, mutation of AP-1 (mt −102) results in a diminished response to both TPA and 1,25-VD. Therefore, AP-1 might be involved in 1,25-VD-mediated TIMP-1 activation. To further test our hypothesis, AP-1 responsive DNA binding capacity in PC-3 cells was examined, after 1,25-VD treatment, by DNA pull-down assay. Biotin-labeled oligonucleotides corresponding to the AP-1 site in the TIMP-1 promoter were used to pull down the AP-1 complex from TPA or 1,25-VD treated...
PC-3 nuclear extracts. As shown in Figure 6C, increased amounts of c-Jun proteins, one component of the AP-1 complex, were pulled-down by wild-type-AP-1 DNA when cells were treated with TPA or 1,25-VD (lanes 2 and 3 versus 1); however, no c-Jun protein was pulled-down by mt-AP-1 DNA (lanes 4–6). These results indicated that 1,25-VD activated TIMP-1 mRNA expression and its activity through the up-regulation of AP-1 complexes, and the enhancement of TIMP-1 results in, at least partly, inhibition of MMP activity and invasiveness of cancer cells.

Discussion

There are several steps in tumor progression that could be regulated by 1,25-VD. First, 1,25-VD is a potent growth inhibitor for cells of epithelial origin or distal metastasis, and this is achieved by inducing cell cycle arrest, differentiation or apoptosis (47). Second, 1,25-VD reduces tumor metastasis, and this is thought to involve the regulation of proteases (25,30). Third, 1,25-VD has been shown to inhibit neoangiogenesis of cancer cells (48). In this study, we found 1,25-VD decreased cell invasion of three human prostate cancer cell lines, LNCaP, PC-3 and DU 145, to a similar degree by modulating the activity of selective proteases and their corresponding gene expression.

Type IV collagen is a major structural protein in the basement membrane and ECM. A number of studies have linked elevated MMP-2 and MMP-9 levels with an increased tumor metastatic potential. In human prostate cancer cells and mononuclear phagocytes, 1,25-VD has been reported to reduce MMP-9 activity (25,49), which is similar to our results (Figure 2A and B). In our data, we found that 1,25-VD inhibits MMP-9 transcript expression in all three cell lines (Figure 2D), which led us to further dissect the molecular mechanisms underlying this suppression. It is known that the human MMP-9 promoter contains regulatory elements for AP-1 (533, 79), NF-κB (600), SP-1 (558) and polyoma enhancer A3 (PEA3) (540) (50). The expression of MMP-9 is regulated by various growth factors, cytokines and oncoproteins (51–54). 1,25-VD has been reported to inhibit NF-κB activity in human lymphocytes and fibroblasts by either decreasing NF-κB DNA binding capacity or decreasing the expression of its precursor protein (55,56). Thus, we hypothesized that 1,25-VD might decrease NF-κB activity and consequently decrease transcription of MMP-9. However, we failed to show that 1,25-VD decreased the transcriptional activity of 1.9 kb of the MMP-9 promoter (Figure 5A) or NF-κB transcriptional activity (Figure 5B), whereas AP-1 activity was increased (Figure 5C). Therefore, cell-specific factors, other than NF-κB, or some post-transcriptional modifications might be involved in 1,25-VD mediated suppression of MMP-9 gene transcription in human prostate cancer cells, and such factors have yet to be determined.

Involvement of 1,25-VD in the regulation of the PA system has been reported in human keratinocytes, rat osteogenic sarcoma cells, U-937 mononuclear phagocytes and human breast cancer cells (30,57–59). Down-regulation of uPA by 1,25-VD was found at the transcriptional level in HT-1080 human keratinocytes. The uPA promoter contains SP-1, c-ets-1, cAMP responsive elements and two AP-1 sites (60). Promoter activity analysis of the uPA suggested that the 1,25-VD responsive
regulatory region is located between nucleotides -2350 and -1870 (30), yet no known vitamin D inhibitory sequences were found within that region. The changes of PA systems in response to 1,25-VD seem to be cell-type specific. In keratinocytes and breast cancer cells, 1,25-VD down-regulates PAs and up-regulates PAI-1, whereas in sarcoma cells and phagocytes PA activities are enhanced and PAIs are suppressed (30,57–59). Hoosein et al. (61) reported that the presence of uPAR in PC-3 and DU 145 cells was correlated with high invasive ability, whereas LNCaP cells, which lack uPAR, have poor invasive ability. We also found that endogenous mRNA expressions of uPA and uPAR are much higher in PC-3 and DU 145 compared to LNCaP cells (Figure 3C and Supplementary Figure 2), however, 1,25-VD treatment slightly induced total PA activities. These data indicate that PA activities might be important for cancer cell invasion, but that 1,25-VD has no effect on the PA system in achieving its anti-invasive effects in human prostate cancer cell lines.

Increased expression and activity of CPs are seen in osteoclastomas, melanomas, gliomas, breast, colorectal, gastric, lung and prostate carcinomas (12–15), suggesting that these proteases might be involved in the development, invasion and

Fig. 6. Regulation of tissue inhibitors of metalloproteinase-1 promoter activity by 1,25-VD. (A) Schematic structure of TIMP-1 promoter constructs used for testing luciferase activity. (B) Effects of 1,25-VD on the activities of TIMP-1 promoter constructs. PC-3 cells were transiently transfected with 0.8 µg/well of different lengths of TIMP-1 reporter constructs, and treated with ethanol vehicle, 100 nM TPA, 1 nM or 100 nM 1,25-VD, as indicated, for 24 h. Reporter gene expression was measured via the luciferase assay. The fold induction of luciferase activity is presented relative to the transactivation observed upon vehicle treatment. *Indicates significant (P < 0.05) difference between control and TPA- or 1,25-VD-treated groups. (C) 1,25-VD increases AP-1 DNA binding on the TIMP-1 promoter. Nuclear extracts were prepared from PC-3 cells that were serum-starved for 24 h and stimulated with ethanol vehicle, 100 nM TPA or 1,25-VD for 3 h. 30 µg of nuclear extract was incubated with either wild-type- or mt-AP-1 probes as described in Materials and methods. After DNA pull-down assay was performed, proteins in the resulting DNA–protein complexes were separated by 10% SDS–PAGE and analyzed for the c-Jun by immunoblot analysis. The nuclear extracts (lanes 7–9) represent 50% of protein used in the pull-down assay. The level of DNA binding was extrapolated by densitometric analysis and values represent the fold changes relative to untreated control PC-3 cells. N.S., non-specific.
metastasis of cancer cells. However, up-regulation of CP B and increased apoptosis after 1,25-VD treatment was found in the MCF-7 human breast cancer cell line (62). Similarly, 1,25-VD induced CP D gene expression and differentiation in the HL-60 human myeloid leukemia cell line (63). These findings suggested that CP might have other biological functions besides promoting cancer cell invasion. Nevertheless, the effects of 1,25-VD on CPs and CPIs in prostate cancer development and metastases have not been established. CP H and cystatin M have been shown to be down-regulated and up-regulated by 1,25-VD in squamous carcinoma cells, respectively (64). Cystatin A expression and promoter activity also can be up-regulated by 1,25-VD in human keratinocytes (65). From our data, 1,25-VD effects on CP activity were not consistent among the three cell lines we used. CP activity was down-regulated in LNCaP and DU 145 cells, but up-regulated in PC-3 cells (Figure 4A), yet 1,25-VD enhanced CPI activities in all the cell lines (Figure 4B). However, the mRNA expression of potential targets, CP B, CP H, CP L, cystatin A and cystatin M, showed no significant change upon 1,25-VD treatment. Therefore, the net CP protease activities, calculated by the C1 ratio, were decreased by 1,25-VD treatment, which might contribute to the anti-invasion action of 1,25-VD, but potential targets and detailed mechanisms need to be further investigated.

Among the three major groups of proteases and their inhibitors we tested, TIMP-1 is the most promising target for the anti-invasive effects of 1,25-VD in human prostate cancer cells. Experiments have shown that recombinant TIMP-1 (rTIMP-1) inhibits the invasion of tumor cells through amniotic membranes (66). Administering rTIMP-1 to mice injected with metastatic B16 melanoma cells also inhibits the formation of lung metastases (66). TIMPs are able to inhibit the active forms of all of the MMPs. These data all suggest that the invasive and metastatic ability of cancer cells can be altered by changing the MMP:TIMP ratio. A concomitant increase in the secretion of TIMP-1 and, to a slightly lower extent, TIMP-2 by 1,25-VD was observed in MDA-MB-231 human breast cancer cells (30). As we have shown in Figure 2C and E, TIMP-1 activity and expression were increased by 1,25-VD treatment. The 1.7 kb TIMP-1 promoter contains at least 10 consensus binding sites for SP-1, 6 for AP-1, 6 for PEA3, 12 for AP-2 and 5 CCAAT boxes. (41). Point mutations confirmed that the AP-1 site at –92/–86 is essential for basal expression and for TPA to induce this gene. Several lines of evidence indicate that 1,25-VD can increase the gene transcriptional activity via modulation of AP-1 abundance or DNA binding activity (67,68). Here, we provide strong evidence showing that 1,25-VD activates the TIMP-1 promoter through an AP-1 site, and the AP-1 site with a point-mutation in the TIMP-1 promoter diminishes the 1,25-VD response (Figures 5C and 6B). DNA pull-down assays demonstrated that 1,25-VD induced the active AP-1 complexes, which then bound to the TIMP-1 promoter to induce TIMP-1 expression.

Metastases are responsible for most cancer mortalities, and any indication of metastatic cells would therefore justify aggressive therapy. Invasion of the basement membrane is a critical step in the metastatic cascade, therefore agents that inhibit invasiveness have obvious potential as anticancer drugs. Our study demonstrates that 1,25-VD significantly inhibits human prostate cancer cell invasion. This inhibition of invasion is associated with a decrease in MMP-9 protease activity and an increase in the production of protease inhibitors, such as TIMP-1 or CPIs. The ability of 1,25-VD to inhibit cancer cell invasion supports clinical uses of 1,25-VD in the treatment of advanced stage prostate cancer, and may lead to more effective vitamin D-based therapeutics designed to control the metastatic potential of many tumors.

Supplementary material
Supplementary material can be found at: http://www.carcin.oxfordjournals.org/

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References
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