1α, 25-dihydroxyvitamin D₃ suppresses interleukin-8-mediated prostate cancer cell angiogenesis

Bo-Ying Bao¹,², Jorge Yao³ and Yi-Fen Lee¹,*

¹Department of Urology, ²Department of Chemical Engineering and ³Department of Pathology, University of Rochester, Rochester, NY 14642, USA

*To whom correspondence and requests for reprints should be addressed: Department of Urology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 656, Rochester, NY 14642, USA. Tel: +1 585 275 9702; Fax: +1 585 756 4133; Email: yifen_lee@urmc.rochester.edu

Angiogenesis is an essential step in initial tumor development and metastasis. Consequently, compounds that inhibit angiogenesis would be useful in treating cancer. A variety of antitumor effects mediated by 1α, 25-dihydroxyvitamin D₃ (1,25-VD) have been reported, one of which is anti-angiogenesis; however, detailed mechanisms remain unclear. We have demonstrated that 1,25-VD inhibits prostate cancer (PCa) cell-induced human umbilical vein endothelial cell migration and tube formation, two critical steps involved in the angiogenesis. An angiogenesis factor, interleukin-8 (IL-8), secreted from PCa cell was suppressed by 1,25-VD at both mRNA and protein levels. Mechanistic dissection found that 1,25-VD inhibits NF-κB signal, one of the most important IL-8 upstream regulators. The 1,25-VD-mediated NF-κB signal reduction was shown to result from the blocking of nuclear translocation of p65, a subunit of the NF-κB complex, and was followed by attenuation of the NF-κB complex binding to DNA. The role of IL-8 in PCa progression was further examined by PCa tissue microarray analyses. We found that IL-8 expression was elevated during PCa progression, which suggests that IL-8 may play a role in tumor progression mediated through its stimulation on angiogenesis. These findings indicate that 1,25-VD could prevent PCa progression by interrupting IL-8 signaling, which is required in tumor angiogenesis, and thus applying vitamin D in PCa treatment may be beneficial for controlling disease progression.

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer deaths among North American men. Although PCa is initially suppressed efficiently by medical or surgical castration, many patients treated with androgen ablation develop disease progression, and tumors eventually become hormone refractory, for which no therapy has yet demonstrated a definitive survival advantage. Therefore, the need for more options in the treatment of hormone refractory PCa is obvious.

Angiogenesis, the formation of new blood vessels from an existing vascular bed, is a crucial step in the progression of tumor growth, invasion and metastasis, and its inhibition is a putative therapeutic target (1). Angiogenesis occurs physiologically in processes including wound healing, embryogenesis and the ovulatory cycle, and in pathological states including rheumatoid arthritis, diabetic retinopathy and cancer progression (2). Initiation of angiogenesis (angiogenic switch) is controlled by local hypoxia that induces the synthesis of angiogenic factors that can activate signal pathways and transcription for endothelial cell structural reorganization. Endothelial cell reorganization is a multi-step process that includes degradation of vascular basement membrane by matrix metalloproteinases (MMPs), sprouting, elongation, migration and proliferation of endothelial cells followed by the association of endothelial cells into new tubular channels (3). These processes are tightly controlled through a balance of positive and negative regulatory factors.

Epidemiological evidences have suggested that low exposure to sunlight and vitamin D deficiency might be risk factors for PCa (4). 1α, 25-dihydroxyvitamin D₃ (1,25-VD), the active metabolite of vitamin D, has been shown to inhibit vascular endothelial growth factor (VEGF) induced endothelial cell tube formation in vitro and reduce vascularization of tumors derived from VEGF-overexpressed MCF-7 breast cancer cells xenografted into mice (5). These results indicate that 1,25-VD inhibits angiogenesis in vitro and in vivo; however, the detailed mechanisms still largely remain unknown.

Large-scale profiling of the effects of 1,25-VD on gene expression in human head and neck squamous cell carcinoma revealed that 1,25-VD downregulates interleukin-8 (IL-8), an angiogenic factor (6). The human IL-8 cDNA encodes a 99 amino acids protein, and cleavage to a 72 amino acid form is required for full activation of IL-8. IL-8 can form non-covalent dimers in solution, but dimerization is not essential for biological activity (7). IL-8, also known as CXC ligand-8 or monocyte-derived neutrophil chemotactic factor, is a member of the CXC chemokine family, and was initially identified as a regulator for the recruitment and trafficking of leukocytes, particularly neutrophils, to the sites of inflammation (8). IL-8 can induce the adhesion and migration of neutrophils through the endothelium (9), and neutralizing IL-8 inhibits neutrophil infiltration and tissue damage in several types of inflammation, suggesting a causal role of IL-8 in inflammatory reactions (10). The involvement of chronic or recurrent inflammation has been suggested in the development of PCa (11). Subsequent studies indicated that IL-8 is a common chemotactic factor involved in the regulation of pathological angiogenesis, tumor growth and metastasis (12). The receptors for IL-8, CXCR1 and CXCR2, are expressed variety of normal and tumor cells

Abbreviations: 1,25-VD, 1α, 25-dihydroxyvitamin D₃; BPH, benign hyperplasia; CM, conditioned media; ELISA, enzyme-linked immunosorbent assay; HG, high-grade adenocarcinoma; HUVEC, human umbilical vein endothelial cells; IHC, immunohistochemistry; IL-8, interleukin-8; MK, midkine; MMP, matrix metalloproteinase; N, normal; PCa, prostate cancer; PCR, polymerase chain reaction; PIN, prostatic intraepithelial neoplasia; TGF-β, transforming growth factor-β; TMA, tissue microarray; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.
(13,14). Serum levels of IL-8 are elevated in PCa patients, with levels increasing as PCa progresses, suggesting that IL-8 could be a potential independent predictor for PCa (15). Therefore, the suppression of IL-8 production might be able to delay PCa progression.

In this present study, we investigated how 1,25-VD inhibits the PCa cells secreting angiogenic factors, with the focus on IL-8 signals to suppress angiogenesis. Our study suggested that 1,25-VD exerts anti-angiogenic effects by modulating IL-8 signals to control tumor growth and could potentially be used in the management of advanced PCa.

Materials and methods

Cells, plasmids and materials

1,25-VD was the generous gift of Dr Lise Binderup from Leo Pharmaceutical Products (Ballerup, Denmark), and the IL-8 promoter construct was kindly provided by Dr Antonella Casola from University of Texas Medical Branch. NF-kB reporter construct, p65, and dominant negative inhibitor of NF-kB (mIkB) expression plasmids were kindly provided by Dr Edward Schwarz from University of Rochester. The anti-p65 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); recombinant human IL-8 and anti-IL-8 antibody (for neutralizing) were purchased from R&D systems (Minneapolis, MN). Anti-IL-8 antibody for immunohistochemical (IHC) studies was purchased from Biosource (Camarillo, CA). Tumor necrosis factor-α (TNF-α) was purchased from Calbiochem (San Diego, CA). Human umbilical vein endothelial cell (HUVEC) were obtained from Dr Patricia Simpson-Hadaris from University of Rochester, and HUVEC culture media (Medium 200) was obtained from Cascade Biologies (Portland, OR). LNCaP, PC-3, DU145 and RWPE-1 cells were obtained from the American Type Culture Collection (Manassas, VA), and HPr-1 cells were a generous gift of Dr Yong-Chuan Wong from the University of Hong Kong. Cell culture medium (RPMI-1640 for LNCaP, PC-3 and DU145 cells; keratinocyte serum-free medium for RWPE-1 and HPr-1 cells) was obtained from Gibco BRL (Carlsbad, CA).

Prostate tissue microarray (TMA)

Over a 2-year period (2002–2003), many prostatic adenocarcinoma cases were reviewed at the University of Rochester Medical Center, Strong Memorial Hospital, and 80 cases were selected for microarray. Areas for sampling were designated as normal (N), benign hyperplastic (BPH), prostatic intraepithelial neoplasia (PIN), low-grade adenocarcinoma (LG) and high-grade adenocarcinoma (HG). Tumors were classified as follows: Gleason pattern 1, 2 and 3 were labeled LG, and Gleason pattern 4 and 5 were labeled HG. A total of 50 N, 82 BPH, 35 PIN, 104 LG and 82 HG areas were chosen for sampling, averaging 4–6 cores per sample (16). The TMA was constructed using a manual tissue arrayer (Beecher instruments; Sun Prairie, WI). Core samples were retrieved from the selected region in each donor paraffin block and transferred to a receiver paraffin block. Tissue cores were 0.6 mm in diameter and 1.0–3.0 mm in length, depending on the tissue thickness in the donor block. The array blocks were then incubated 10 min at 37°C to improve adhesion between cores and paraffin of the recipient block. A section from each block was cut, stained and reviewed to ensure quality control. Sections for IHC were placed on charged glass slides. Two array blocks were constructed, one containing N, BPH and PIN cores, the other containing LG and HG adenocarcinoma cores. Each core was examined under a light microscope and separately scored. Cores that had <50% of original tissue present were disregarded. Scoring included percentage of staining and staining intensity (0, 1+, 2+, 3+), and results were recorded as positive (≥5%, 2+ or 3+) or negative (<5% staining or staining intensity <2+). Data obtained were analyzed using Fisher’s exact test (17).

Preparation of conditioned media (CM) and measurement of IL-8 by enzyme-linked immunosorbent assay (ELISA)

We seeded 1 × 10⁵ cells/well in 24-well plates and allowed them to attach overnight, and then treated them with either vehicle or indicated concentrations of 1,25-VD for 24 h. The culture medium was then removed, and the cell layers were washed and incubated with serum-free medium. The CM were collected 24 h after incubation and normalized with cell number for subsequent experiments. IL-8 amounts were assayed by ELISA kit according to the manufacturer’s suggested procedures (R&D Systems, D8000C).

In vitro tube formation assay

1 × 10⁴ HUVEC were plated on a growth factor-reduced Matrigel (BD Biosciences; Bedford, MA) coated 96-well plate in serum-free medium, medium containing IL-8 or PCa CM. Following 6 h of incubation, the plate was examined for tube formation under a microscope and photographed. For each treatment, three images were captured and the length of tubes formed was quantified using ImageJ (NIH Image; http://rsb.info.nih.gov/ij/).

Cell migration assay

Cell migration assay was performed as described previously (18). Briefly, 5 × 10⁴ HUVEC were seeded to Matrigel-coated inserts (BD Labware; Bedford, MA) in serum-free medium, medium with IL-8 or PCa CM. After 4 h of incubation, the cells remaining on the top of the Matrigel were removed by a cotton swab and the residual cell amounts were measured by MTT assay.

Gelatin substrate gel zymography

HUVEC were incubated with serum-free medium containing either PBS or IL-8 for 24 h, and then CM were collected and analyzed by gelatin zymography as described previously (18). Briefly, CM were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), under non-reducing conditions, in gels copolymerized with 0.1% gelatin. Following electrophoresis, gels were washed 30 min twice in wash buffer [50 mM Tris (pH 7.4) and 2.5% Triton X-100], then rinsed in incubation buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM CaCl₂ and 0.02% NaN₃] and incubated at 37°C for 18 h (long development) or 48 h (long development). Enzyme activities were visualized by staining with Coomassie blue.

DNA pull-down assay

Oligonucleotides corresponding to the NF-κB site of the IL-8 promoter were synthesized according to published sequences (19). Sequences of the oligonucleotides were as follows: sense 5'-biotin-TCGTGGAAATTCTCTCTGAA-3' (–84 to –67) and anti-sense 5'-TCAGAGGAATTCACACGA-3' (NF-κB binding site is underlined). Nuclear extracts from LNCaP cells were prepared according to the protocol of Andrews et al. (20). For DNA pull-down assays, 50 μg nuclear extracts were incubated with probe for 30 min at room temperature. To capture the complexes, streptavidin-agarose was added, incubated for 1 h at 4°C and then washed three times, eluted from the beads by the addition of 2X laemmil buffer and heating to 95°C for 5 min. Proteins were then separated by 10% SDS–PAGE and analyzed by immunoblot for p65.

Statistical and densitometric analyses

The results are the mean ± standard deviation of values obtained from two or three separate experiments. Student’s t-test was used in IL-8-neutralizing experiments. ANOVA was used to assess the statistical significance of the differences between control, IL-8-treated and 1,25-VD-treated groups. A statistically significant difference was considered to be present at P < 0.05. Autoradiograms/bands were scanned, and the mean density of each band was analyzed by the Quantity One program (Bio-Rad; Hercules, CA). Densitometric data presented below bands are the fold changes compared with control sample band densities.

Real-time polymerase chain reaction (PCR) analysis, cell proliferation (MTT) assay, transient transfection, luciferase assay and western blot analysis

The assays above were performed as described previously (21). IL-8 sense primer 5'-TCCCTAAGGGCAAAACTTCCTCAG-3' and anti-sense primer 5'-GTCCACTCTCAATCTACCTCAG-3', and primers for MMP-2, MMP-9 and β-actin were described previously (18).

Results

1,25-VD suppresses the IL-8 expression in human prostate epithelial cells

To define the role of IL-8 in PCa progression, we checked the amounts of IL-8 secretion and mRNA expression in several normal and malignant human prostate epithelial cell lines. As shown in Figure 1, the secretion and mRNA expression of IL-8 were higher in PC-3 and DU145 PCa cells, two androgen-independent and more aggressive human PCa cell lines, than in LNCaP, an androgen-dependent PCa cell line, or in immortalized normal human prostate epithelial cell lines, HPr-1 and RWPE-1. This correlation of IL-8 expression with PCa aggressiveness suggested that IL-8 might be involved in PCa progression.

The inhibition effects of 1,25-VD on PCa cells secreting IL-8 were then examined. As shown in Figure 2, 1,25-VD can suppress IL-8 amounts secreted from PCa cells to different...
extents, that is, in LNCaP cells, 1,25-VD dose-dependently suppressed IL-8 secretion; in PC-3 cells, a low dose (1 nM) of 1,25-VD was sufficient to suppress IL-8 secretion; however, a much higher dose (100 nM) of 1,25-VD was required to suppress IL-8 in DU145 cells and was possibly due to high endogenous 24-hydroxylase activity (22). Interestingly, we found that 1,25-VD can suppress immortalized normal prostate epithelial cells secreting IL-8, which suggested that 1,25-VD could suppress prostate inflammation, a critical step in tumor initiation.

1,25-VD and IL-8-neutralizing antibody block PCa-stimulated HUVEC tube formation

We applied the HUVEC tube formation, an in vitro angiogenesis assay, to examine the effects of IL-8 and 1,25-VD on PCa-stimulated angiogenesis. HUVEC were seeded on Matrigel-coated plates in serum-free medium without and with treatment of IL-8 (1 and 10 ng/ml) for 6 h, and then tube formation was examined. As shown in Figure 3A, IL-8 dose-dependently stimulated HUVEC network formation, as well as the CM from LNCaP, PC-3 and DU145 human PCa cells (Figure 3B), suggesting that PCa cells secrete some angiogenic factors, such as IL-8, to stimulate HUVEC tube formation.

The 1,25-VD effects on the HUVEC tube formation were then examined. We found that 1,25-VD has no direct effect on HUVEC tube formation (data not shown), but it can partially abolish PCa-stimulated HUVEC tube formation. IL-8-neutralizing antibody can inhibit PC-3 and DU145 cells, but not LNCaP cells-induced tube formation. Due to low amounts of IL-8 secretion from LNCaP cells, therefore, 1,25-VD might inhibit the LNCaP-stimulated HUVEC tube formation by
Fig. 3. 1,25-VD and IL-8-neutralizing antibody block PCa CM-stimulated HUVEC tube formation. (A) $1 \times 10^4$ HUVEC were plated on growth factor-reduced Matrigel-coated 96-well plates with serum-free medium alone, or medium containing indicated concentrations of IL-8. (B) $1 \times 10^5$ HUVEC were plated on growth factor-reduced Matrigel-coated 96-well plates with serum-free medium. Then, vehicle, 100 nM 1,25-VD-treated or 200 ng/ml IL-8-neutralized PCa CM were added and incubated for another 6 h. The plates were examined for tube formation under microscope and photographed. The length of tubes was quantified using image analysis. Asterisk indicates significant difference ($P < 0.05$).
regulating other angiogenic factors. Together, our data found that IL-8 and other angiogenic factors secreted from PCa cells could stimulate endothelial cell tube formation, and suppression of IL-8 secretion by 1,25-VD and its neutralizing antibody could suppress the HUVEC tube formation and possibly delay PCa progression.

1,25-VD inhibits PCa-stimulated HUVEC migration

Endothelial cell proliferation and migration, two important steps in the process of angiogenesis, were examined. HUVEC were treated with serum-free medium, vehicle- or 1,25-VD-treated PCa cell CM for 4 days, and HUVEC proliferation was determined by MTT assay. The CM from three PCa cell lines can stimulate HUVEC proliferation, compared with serum-free medium. However, 1,25-VD-treated PCa cell CM had no significant effect on HUVEC proliferation compared with vehicle-treated CM (data not shown).

The stimulatory effects of PCa CM on HUVEC migration were then measured. The CM from three PCa cell lines stimulated HUVEC migration from 1.6- to 2.5-fold (Figure 4A, crossed versus open bar), and 1,25-VD-treated CM showed less stimulatory effects on HUVEC migration (Figure 4A, crossed versus striped and black bars). IL-8 enhanced HUVEC migration in a dose-dependent manner (Figure 4B), and IL-8-neutralizing antibody, not IgG control, can partially reverse the CM-stimulated HUVEC migration (Figure 4C).

1,25-VD inhibits PCa-stimulated MMP-9 expression in HUVEC

The effects of 1,25-VD on the expression of MMPs, downstream targets of IL-8 (23), were examined. Real-time PCR analysis demonstrated that treatment of IL-8 in HUVEC induced MMP-9, but not MMP-2 mRNA expression (Figure 5A). Gelatin zymography assay further confirmed that IL-8 stimulated MMP-9, but only slightly enhanced MMP-2 activity in HUVEC (Figure 5B). PCa CM induced the mRNA level of MMP-9, not MMP-2, and 1,25-VD-treated PCa CM showed less MMP-9 induction (Figure 5C). Similar to 1,25-VD treatment, IL-8 antibody reduced the PC-3 and DU145 cells CM-stimulated MMP-9, but not MMP-2, mRNA expression (Figure 5D). Taken together, these data demonstrated that angiogenic factors, including IL-8, secreted from PCa cells can induce MMP-9 expression, which might be able to stimulate HUVEC migration, and this IL-8-mediated MMP-9 induction can be inhibited by 1,25-VD and IL-8-neutralizing antibody.

1,25-VD suppresses TNF-α-induced IL-8 expression in human PCa cell lines

In order to study the underlying mechanism by which 1,25-VD regulates IL-8 expression in PCa cells, TNF-α was used to stimulate IL-8 signals. LNCaP, PC-3 and DU145 cells were treated with or without 1,25-VD for 1 h and then stimulated with TNF-α for another 24 h. We found that TNF-α significantly induced IL-8 secretion (Figure 6A) and mRNA expression (Figure 6B), and that both can be suppressed by 1,25-VD treatment, except in PC-3, where a much lower TNF-α-induced IL-8 secretion and no significant 1,25-VD inhibitory effects were seen. To examine if 1,25-VD regulates IL-8 expression at the transcriptional level, IL-8 promoter reporter (IL-8-Luc) assays were performed. We found that the basal level of IL-8-Luc activity is higher in PC-3 cells than in DU145 and LNCaP cells (Figure 6C), and 1,25-VD can partially inhibit TNF-α-induced IL-8-Luc activity in LNCaP cells, while there is no significant TNF-α induction nor 1,25-VD inhibition effect in PC-3 and DU145 cells (Figure 6D).

1,25-VD inhibits TNF-α-induced p65 translocation in LNCaP cells

So far, no vitamin D response element has been identified in the IL-8 promoter, so we suspect that 1,25-VD might regulate IL-8 expression through cross-talk with other regulatory factors. The NF-κB pathway, one of the most important pathways regulating IL-8 expression, was examined by NF-κB DNA-binding luciferase (NF-κB-Luc) reporter assays. As shown in Figure 7A, the basal level of NF-κB-Luc is higher in PC-3 and DU145 than in LNCaP cells, which corresponds with the IL-8-Luc promoter activity. Next, we applied p65 and miκB to modulate the NF-κB activities to examine if alteration of NF-κB could affect IL-8-Luc activity. As shown in Figure 7B, p65 stimulates NF-κB-Luc, as well as IL-8-Luc activities, and miκB suppresses both NF-κB-Luc and IL-8-Luc activities. The induction of NF-κB by p65 was lower in

Fig. 4. 1,25-VD and IL-8-neutralizing antibody block PCa CM-stimulated HUVEC migration. 5 × 10⁴ HUVEC were seeded onto Matrigel-coated chamber with serum-free medium. Then, the lower chambers were incubated with (A) PCa CM from samples pre-treated with vehicle, or 1,25-VD (1nM or 100 nM), 100 nM), or (B) incubated with serum-free medium containing various concentrations of IL-8 or (C) incubated with PCa CM pre-treated with IgG, or anti-IL-8 antibody. After 4 h of incubation at 37°C, the cells remaining on the top of the Matrigel were removed by cotton swabs and the residual cells were determined by MTT assays. Values represent the fold increase of migrated cells relative to serum-free medium treated control. Asterisk indicates significant difference (P < 0.05).
PC-3 and DU145 cells, which might be due to constitutively active NF-κB in these two cell lines so that exogenous transfection of p65 would not induce NF-κB activity further (Figure 7A).

We then examined how 1,25-VD affects NF-κB activity. As shown in Figure 7C, a 56-fold NF-κB-Luc activation by TNF-α treatment was detected in LNCaP cells, and 1,25-VD partially suppressed the TNF-α-induced NF-κB-Luc activity. Due to the high basal level of active NF-κB, there is no obvious TNF-α induction of NF-κB activity in PC-3 and DU145 cells, so we mainly focused on the regulation of IL-8 by 1,25-VD in LNCaP cells in the following experiments.

To determine whether 1,25-VD can prevent the NF-κB complex from binding to its corresponding DNA sequence, DNA pull-down assays were performed. Biotin-labeled oligonucleotides, corresponding to the NF-κB binding site in the IL-8 promoter, were used to pull down the NF-κB complex from TNF-α-stimulated LNCaP nuclear extracts, with or without treatment with 1,25-VD. As shown in Figure 7D, TNF-α treatment enhanced the amounts of p65/NF-κB DNA-binding complex, and 1,25-VD decreased the TNF-α-stimulated p65/NF-κB DNA-binding complex. Moreover, p65 nuclear translocation, an essential step for NF-κB activation, induced by TNF-α (Figure 7E, lane 2 versus 1), was retarded by 1,25-VD treatment (Figure 7E, lanes 3 and 4 versus 2). Therefore, we concluded that 1,25-VD could reduce the nuclear translocation of p65 and prevent DNA binding, which consequently suppressed NF-κB-mediated IL-8 transcriptional activity.

Correlation of IL-8 expression with PCa progression
To further investigate the role of IL-8 in PCa progression in vivo, we examined IL-8 expression in a large number of prostate carcinoma cases using a TMA. Five different types of prostate tissue including N, BPH, PIN, LG and HG were collected. As demonstrated in Figure 8, a cytoplasmic staining...
was observed in all positive cores, similar to the positive control using endometrial tissue (data not shown). Negative IL-8 staining was shown in one N and one BPH sample (Figure 8A and B) and positive IL-8 cytosolic staining in LG (Figure 8C) and HG (Figure 8D) samples. After reviewing and scoring, we summarized the results as shown in Figure 8E. The positive IL-8 staining was 40% (46 out of 114) in benign tissue cores (normal and BPH), 75% (18 out of 24) in PIN cores and 72% (81 out of 112) in carcinoma cores (LG and HG). Significant increases of IL-8 expressions were found between benign and carcinoma tissues ($P < 0.01$), as well as between benign and PIN tissues ($P < 0.01$). Additionally, there was a significant IL-8 staining increase between LG and HG carcinomas ($P < 0.05$). These results demonstrated that IL-8 expressions correlated with PCA aggressiveness, indicating that IL-8 might serve as a prognostic factor for human PCA.

**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 inhibition can be achieved by inducing cell cycle arrest, differentiation or apoptosis (21,24). Second, it reduces tumor metastasis, which involves modulation of proteases (18,25). Third, 1,25-VD has been shown to inhibit angiogenesis of cancer cells (5). In this study, we focused on how 1,25-VD suppresses PCA-stimulated angiogenesis.

Our results have provided several pieces of evidence that suggest that IL-8, one of the most important angiogenic factors secreted by PCA cells, stimulates angiogenesis. First, a positive correlation of IL-8 expression with aggressiveness of PCA was demonstrated in the cell lines and human prostate tissues (Figures 1 and 8). Second, CM from PC-3 and DU145 cells induced more HUVEC tube formation and migration than LNCaP cells (Figures 3B and 4C), which also correlated with IL-8 expression and the aggressiveness of PCA cells. Third, 1,25-VD and IL-8-neutralizing antibody treatment had better inhibitory effects on HUVEC tube formation and migration in PC-3 and DU145 than in LNCaP cells (Figures 3B and 4C), suggesting that 1,25-VD could suppress PCA-induced angiogenesis via inhibition of IL-8, especially in PCA cells with high IL-8 expression.

We noted that neutralizing IL-8 only partially inhibits PCA-stimulated endothelial cell tube formation, migration and MMP-9 expression, compared with 1,25-VD treatment (Figures 3B, 4C and 5D), suggesting that in addition to
IL-8, some other angiogenic factors could be regulated by 1,25-VD. Gene microarray analysis allows global, unbiased evaluation of a broad number of genes and can make the process of studying gene regulation more efficient. By using this technique, large-scale profiling of 1,25-VD effects on gene expression has been conducted in human head and neck squamous cell carcinoma (6), revealing that 1,25-VD upregulates some anti-angiogenic factors, such as bone morphogenetic protein 2A (BMP-2A) and transforming growth factor-β (TGF-β), as well as downregulates some angiogenic factors, such as endothelin-1 (ET-1), chorionic gonadotropin beta subunit, retinoic acid inducible factor midkine (MK), VEGF-related protein, Cytb61 and IL-8. The effects of 1,25-VD on these genes’ regulation were also tested by RT–PCR in human PCa cell lines (data not shown). TGF-β regulates cell proliferation, migration, extracellular matrix production and differentiation in a wide variety of cell types (26). TGF-β also inhibits endothelial cell proliferation and induces extracellular matrix deposition, thus controlling the resolution phase of angiogenesis (27). However, stimulatory effects of TGF-β on angiogenesis have also been reported in vivo (28). Therefore, the cellular responses mediated by TGF-β in endothelial cell function are complex, being either stimulatory or inhibitory, depending on the differentiation status of cells and cues from the surrounding environment (29). BMPs, other members of the TGF-β superfamily, have been reported to suppress vascular smooth muscle cell proliferation and increase the expression of smooth muscle differentiation markers (30). ET-1 is a potent mitogen for both endothelial cells and vascular smooth muscle cells, and it may indirectly enhance endothelial cell proliferation through stimulation of VEGF production by other cell types (31–33). Elevated ET-1 plasma

**Fig. 7.** Regulation of NF-κB activity by 1,25-VD in human PCa cell lines. (A) Endogenous NF-κB activity in human PCa cell lines. Cells were transiently transfected with 0.8 μg NF-κB reporter constructs for 24 h. Reporter activities were determined by Luc assay. The fold changes in the NF-κB activity are presented relative to LNCaP cells set at 1. (B) Effects of p65 and mIkB on IL-8 promoter activity. Cells were transiently transfected with 0.4 μg NF-κB or IL-8 reporter constructs, and 0.6 μg control vector, p65 or mIkB expression plasmids as indicated for 24 h. Reporter activities were determined by Luc assay. The fold changes in the reporter activity are presented relative to control vector-transfected cells set as 1. (C) 1,25-VD moderately suppressed TNF-α-induced NF-κB activity. Cells were transiently transfected with 0.8 μg NF-κB reporter constructs, and treated with vehicle or various concentrations of 1,25-VD for 1 h and then treated with TNF-α (200 U/ml) for 24 h. Reporter activities were determined by Luc assay. The fold changes in the NF-κB activity are presented relative to untreated cells set as 1. Asterisk indicates significant difference (P < 0.05). (D) 1,25-VD suppresses NF-κB DNA binding capacity. Nuclear extracts were prepared from LNCaP cells treated with vehicle or various concentrations of 1,25-VD for 1 h and then treated with TNF-α (200 U/ml) for another 1 h. Fifty micrograms of nuclear extracts were incubated with NF-κB binding DNA probes as described in Materials and methods. After DNA pull-down assay, proteins in the resulting DNA-protein complexes were separated by 10% SDS–PAGE and analyzed by immunoblot for the p65. (E) TNF-α-induced p65 translocation is suppressed by 1,25-VD. Cells were treated with vehicle or various concentrations of 1,25-VD for 1 h and then treated with TNF-α (200 U/ml) for another 1 h. Cell lysates were fractionated into nuclear and cytoplasmic fractions, and then analyzed by western blotting with p65 antibody.
levels have been detected in patients with various tumors including PCa, and levels are increased in metastatic and hormone refractory stages (16). The expression of MK was initially considered to be restricted in embryonic development and the adult brain (34). However, recently, MK has also been shown to be expressed in a range of primary human tumors, and the expression of MK in invasive bladder carcinomas correlates with poor patient survival (35). Cyr61 is a secreted, extracellular matrix-associated, angiogenic regulator, and it can stimulate endothelial cell proliferation and migration \textit{in vitro} and induces angiogenesis \textit{in vivo} (36,37). Cyr61-null mice suffer embryonic death due to loss of vascular integrity in the embryo (38). Furthermore, overexpression of Cyr61 promotes tumor growth in vascularization and is associated with human breast cancer (39). Therefore, the anti-angiogenic effects of 1,25-VD might be not only via the suppression of IL-8 but also via the regulation of other angiogenic factors, which might need further investigations.

The role of chronic or recurrent inflammation in the development of PCa has been suggested (11). IL-8 is a potent chemotactic factor for neutrophils and is well associated with the initiation of an inflammatory response. Ferrer et al. (40) found that PCa specimens stained positively for IL-8, whereas BPH and normal tissue exhibited little staining. Serum levels of IL-8 were also elevated by $>2$-fold in patients with stages A–C PCa in comparison with healthy individuals, and patients with stage D PCa had 4-fold elevation of their IL-8 serum level (15). As we have shown in Figure 8, TMA results also strongly supported that IL-8 expressions were elevated during the PCa progression and that the suppression of IL-8 production might be beneficial for the control of various types of inflammatory reactions or even cancer development.

IL-8 can be regulated both at the transcriptional and post-transcriptional levels. In the 3' flanking region, IL-8 gene contains the repetitive ATTTA motif, which is responsible for destabilization of various cytokine mRNAs (41). Also, within the IL-8 5'-promoter region, there are potential binding sites for many transcription factors, such as AP-1, AP-2, AP-3, HSE, HNF-1, IRF-1, glucocorticoid receptor (GR), NF-κB, NF-IL-6 and octamer factor that can regulate IL-8 expression (42). The sequence located at $-94$ to $-70$ of the IL-8 promoter is essential for responding to various stimuli, mainly through NF-κB (43). For example, the inhibition of inflammatory cytokine expression by glucocorticoid is GR-dependent via either direct interaction with the p65 subunit of NF-κB or upregulation of NF-κB inhibitor IκBα (44,45). 1,25-VD has been shown to reduce levels of p50 and its precursor p105 and then decrease PMA-induced NF-κB binding to the

![Fig. 8. Elevation of IL-8 expression during PCa progression in TMA analyses. (A) A typical example of negative staining of IL-8 in normal prostate tissue sample. (B) A typical example of negative staining of IL-8 in BPH tissue sample. (C) An intense cytoplasmic staining of IL-8 IHC in LG carcinoma sample. (D) An intense cytoplasmic staining of IL-8 IHC in HG carcinoma sample. (E) Correlation of IL-8 expression with PCa progression. Data obtained were analyzed using Fisher's exact test. ×400 magnification.](image-url)

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<td>47.14%</td>
</tr>
<tr>
<td>Benign</td>
<td>46</td>
<td>68</td>
<td>114</td>
<td>40.35%</td>
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<tr>
<td>PIN</td>
<td>18</td>
<td>6</td>
<td>24</td>
<td>75.00%</td>
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<tr>
<td>LG</td>
<td>43</td>
<td>24</td>
<td>67</td>
<td>64.18%</td>
</tr>
<tr>
<td>HG</td>
<td>38</td>
<td>7</td>
<td>45</td>
<td>84.44%</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>81</td>
<td>31</td>
<td>112</td>
<td>72.32%</td>
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<table>
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<th>Benign vs Carcinoma</th>
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<tr>
<td></td>
<td>LG vs HG</td>
<td>0.011</td>
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</table>

The data obtained were analyzed using Fisher's exact test.
IL-6 promoter in human lymphocytes (46). 1,25-VD can also partially inhibit NF-κB activity in MRC-5 normal human fibroblasts by targeting DNA binding of NF-κB (47). In our studies, we demonstrated that 1,25-VD downregulates TNF-α-induced IL-8 promoter activity via reduction of p65 nuclear translocation in LNCaP human PCa cell line (Figure 7E). 1,25-VD can significantly reduce IL-8 secretion, but only moderately suppress IL-8 promoter and NF-κB activities, indicating that 1,25-VD might downregulate IL-8 by mechanisms other than the inhibition of NF-κB DNA binding. Therefore, it might be possible that 1,25-VD can also affect the stability of IL-8 mRNA through the ATTGA motif in the 3′-flanking region or other post-transcriptional regulations.

The principal clinical problem of cancer is metastasis. Angiogenesis is a critical step in tumor progression, and its inhibition has obviously become a therapy target. Our study demonstrates that 1,25-VD significantly inhibits human PCa cell-­induced endothelial cell migration and tube formation. This inhibition was associated with the suppression of IL-8 secretion from cancer cells. The inhibition of the angiogenic activity of cancer cells by 1,25-VD supports the clinical use of 1,25-VD, either alone or in combination with other chemotherapeutic drugs in the management of advanced PCa.

Acknowledgements

We are grateful to Dr Lise Binderup from Leo Pharmaceutical Products for providing the 1,25-VD; Dr Antonella Casola from University of Texas Medical Branch for IL-8 promoter construct; and Dr Edward Schwarz from University of Rochester for p65, miR8 and NF-κB reporter constructs. We also thank Fred Wu and Karen Wolf for help in manuscript preparation. This work was supported by the Department of Defense grant PC040630.

Conflict of Interest Statement: None declared.

References


Received September 20, 2005; revised March 22, 2006; accepted March 31, 2006