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1α,25-dihydroxyvitamin D_3 inhibits prostate cancer cell invasion via modulation of selective proteases

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Inhibition of invasion and metastasis has become a new approach for treatment of advanced prostate cancer in which secondary hormone therapy has failed. Accumulating evidence indicates that 1α,25-dihydroxyvitamin D_3 (1,25-VD) suppresses prostate cancer progression by inhibition of tumor growth and metastasis. However, the detailed mechanisms underlying these effects remain to be determined. Here, we used the *in vitro* cell invasion assay to demonstrate that 1,25-VD inhibits the invasive ability of human prostate cancer cell lines, LNCaP, PC-3 and DU 145. Three major groups of proteases, the matrix metalloproteinases (MMPs), the plasminogen activators (PAs) and the cathepsins (CPs), that are involved in tumor invasion were then examined for changes in activity and expression after 1,25-VD treatment. We found that 1,25-VD decreased MMP-9 and CPs, but not PAs activities, while it increased the activity of their counterparts, tissue inhibitors of metalloproteinase-1 (TIMP-1) and cathepsin inhibitors. Mechanistic studies showed that 1,25-VD did not suppress MMP-9 expression at the transcriptional level, but reduced its mRNA stability. In addition, 1,25-VD increased AP-1 complexes binding to TIMP-1 promoter, which contributed to the enhancement of TIMP-1 activity, and thus resulted in inhibition of MMP activity and tumor invasion. These findings support the idea that vitamin D-based therapies might be beneficial in the management of advanced prostate cancer, especially among patients who have higher MMP-9 and CPs activities.

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 D_3; 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*, intravesical instillation of 1,25-VD inhibited the invasion in *N*-methylnitrosourea-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 MMP 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 Binderup 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% CO_2 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 either ethanol vehicle or 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 100 nM 1,25-VD for 72 h in regular medium. Cells were harvested and seeded in 24-well tissue culture plates at a density of 5×10^4 cells/well in RPMI-1640 containing 10% FBS. After incubation for 1 h at 37°C, the cells were rinsed gently with phosphate-buffered saline (PBS) and incubated with serum-free medium containing MTT (0.5 mg/ml) for another 1 h. The absorbance was recorded.

MMP-9 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 normalized with cell number. For measuring MMP-9 activity in cell-conditioned medium, we used the 'MMP-9 biotrak activity assay system' by Amersham 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 mM Tris/pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3] 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 0.1 M NaCl]. 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/pH 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 SPECTRAmax 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 proteins such as the CPs; CPIs 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′-CAAGGAGTACAACAGCTGCACTGATA-3′ and anti-sense 5′-GGTGCAGCTCTCATATTTGTTGC-3′ (34); MMP-9, sense 5′-TGGGCAAGGGCGTCGTGGTTC-3′ and anti-sense 5′-TGGTGCAGGCGGAGTAGGATT-3′ (34); TIMP-1, sense 5′-TCAACCAGACCACCTTATAC-3′ and anti-sense 5′-ATTCCTCACAGCCAACAG-3′; TIMP-2, sense 5′-GTAGTGATCAGGGCCAAAG-3′ and anti-sense 5′-TTCTCTGTGACCCAGTCCAT-3′ (35); tPA, sense 5′-ATGACACTTACGACAATG-3′ and anti-sense 5′-GGTGACTGTTCTGTTAAG-3′; uPA, sense 5′-CACGCAAGGGGAGATGAA-3′ and anti-sense 5′-ACAGCATTTTGGTGGTGACTT-3′ (36); uPAR, sense 5′-CAACGACACCTTCCACTTC-3′ and anti-sense 5′-GCACAGCCTCTTACCATATAG-3′; PAI-1, sense 5′-GCTGGTGCTGGTGAATGC-3′ and anti-sense 5′-GGCGTGGTGAACTCAGTATAG-3′; PAI-2, sense 5′-CCAGAGAACAACCAGATTG-3′ and anti-sense 5′-AGAGCGGAAGGATGAATG-3′; CP B, sense 5′-TGTGTATTCGGACTTCCTGCT-3′ and anti-sense 5′-GTGTGCCATTCTCCACTCC-3′ (37); CP H, sense 5′-CAACAATGGGAACCACACAT-3′ and anti-sense 5′-GCAAAGCTCACAGGGTTGTA-3′ (38); CP L, sense 5′-CAGTGTGGTTCTTGTTGGGCT-3′ and anti-sense 5′-CTTGAGGCCCAGAGCAGTCTA-3′ (39); Cystatin A, sense 5′-CCAAACCCGCCACTCCAGAAATC-3′ and anti-sense 5′-CAGTAGCCAGTTGAAGGAATCAGAACAC-3′; Cystatin M, sense 5′-CAGCAACAGCATCTACTAC-3′ and anti-sense 5′-ACCACAAGGACCTCAAAG-3′; β-actin, sense 5′-TGTGCCCATCTACGAGGGGTATGC-3′ and anti-sense 5′-GGTACATGGTGGTGCCGCCAGACA-3′. The quantification of each sample relative to control sample was calculated using $2^{-\Delta\Delta CT}$ method (40). The expected sizes and the absence of non-specific amplification products were confirmed by agarose gel electrophoresis and melting curve analysis.

MMP-9 mRNA stability assay

PC-3 cells were pre-treated with ethanol vehicle or 100 nM 1,25-VD for 48 h and then incubated with actinomycin D (5 μg/ml) for 2, 4, 8 and 16 h. Total 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), sense 5′-biotin-GATGGTGGGTGGATGAGTAATGCATCCAG-3′ and anti-sense 5′-CTTCCTGGATGCATTACTCATCCACCCAC-3′ (AP-1 site is underlined). For mutant-AP-1 (−105), in which the AP-1 binding site of wild-type-AP-1 (−105) was destroyed, 5′-TGAGTAA-3′ was mutated into 5′-GGACTAA-3′ (41). 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 ethanol vehicle, 100 nM TPA or 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 $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl/pH 7.5, and 0.05 mg/ml polydI–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

The results are the mean ± SD of values obtained from two or three separate experiments. ANOVA was used to analyze protease activity, real-time PCR and luciferase assay data. Data on invasion assay were analyzed by Student's *t*-test to assess the statistical significance of the difference between control 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). Densitometric data presented below bands are the fold changes compared with control sample band densities for each treatment time.

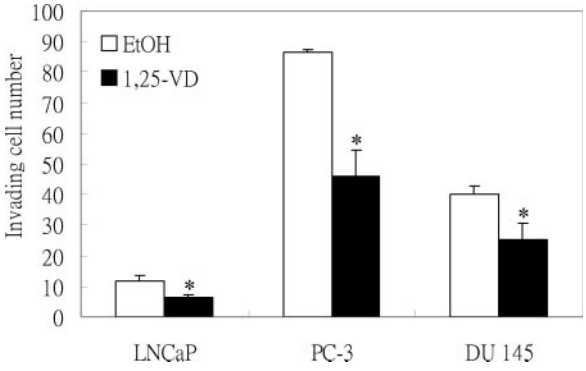

Fig. 1. The anti-invasive effects of 1,25-VD in human prostate cancer cell lines. LNCaP, PC-3 and DU 145 cells were pre-treated with ethanol vehicle or 100 nM 1,25-VD for 3 days before a 22 h invasion assay. Cells invading through Matrigel-coated membrane were stained and counted under a microscope. *Indicates significant ($P < 0.05$) differences between control and 1,25-VD-treated groups.

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

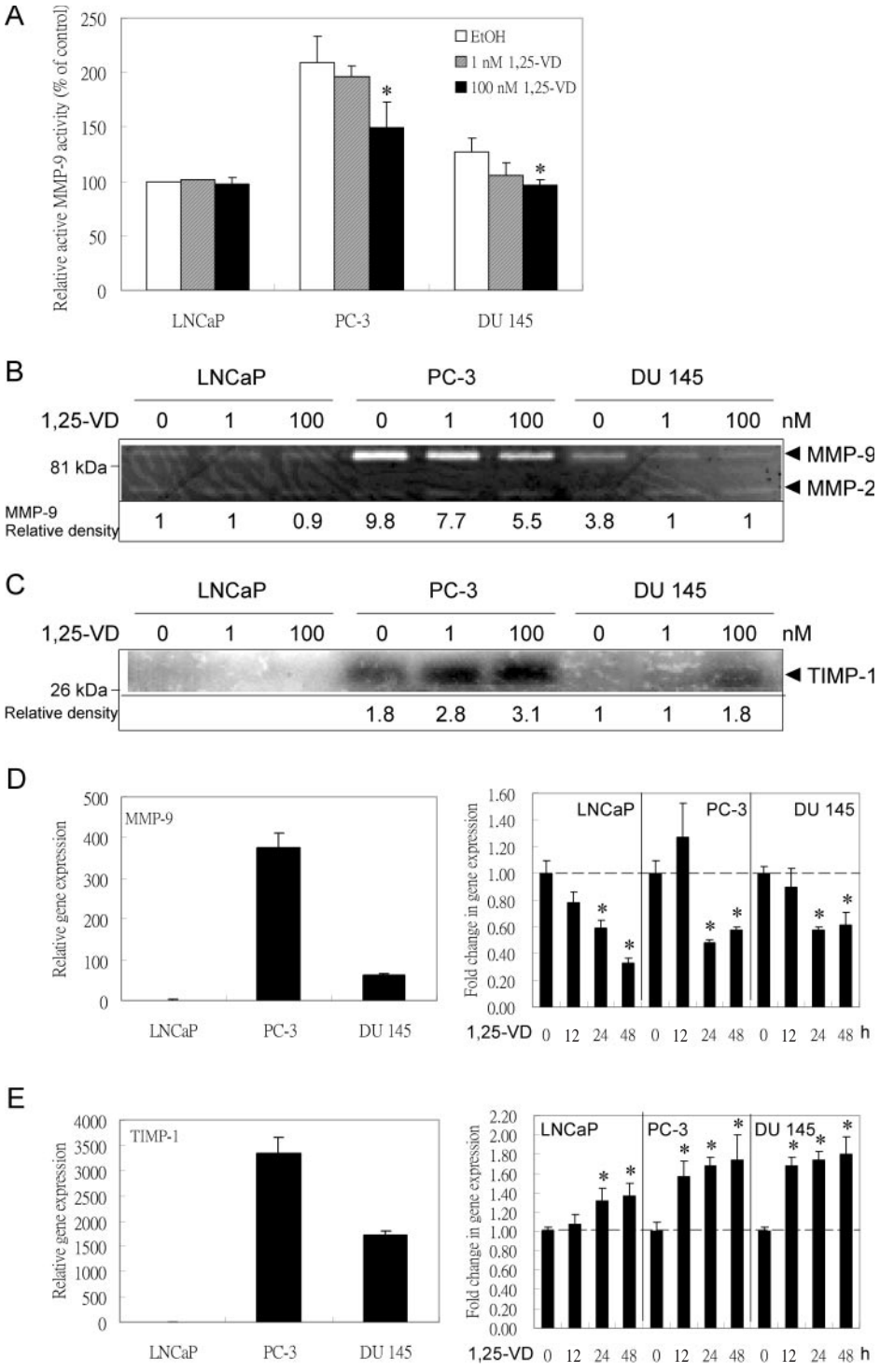

Fig. 2. The effect of 1,25-VD on gelatinolytic matrix metalloproteinase system. Down-regulation of MMP-9 activity (**A** and **B**), and up-regulation of TIMP-1 activity (**C**), by 1,25-VD. LNCaP, PC-3 and DU 145 cells were treated with ethanol vehicle or the indicated concentrations of 1,25-VD for 48 h, and then the secreted MMPs and TIMPs were analyzed separately by MMP-9 activity (A), gelatin zymographic (B), and reverse zymographic (C) assays. The MMP-9 activity from untreated control LNCaP cells were set as 100% (A). The activity was extrapolated by densitometric analysis and values represent the fold changes relative to untreated control LNCaP for MMP-9 (B), and to DU 145 cells for TIMP-1 (C). (**D**) The mRNA expression of endogenous MMP-9 (left panel) and 1,25-VD effects on its expression (right panel) in prostate cancer cell lines. (**E**) The mRNA expression of endogenous TIMP-1 (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. *Indicates significance ($P < 0.05$).

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

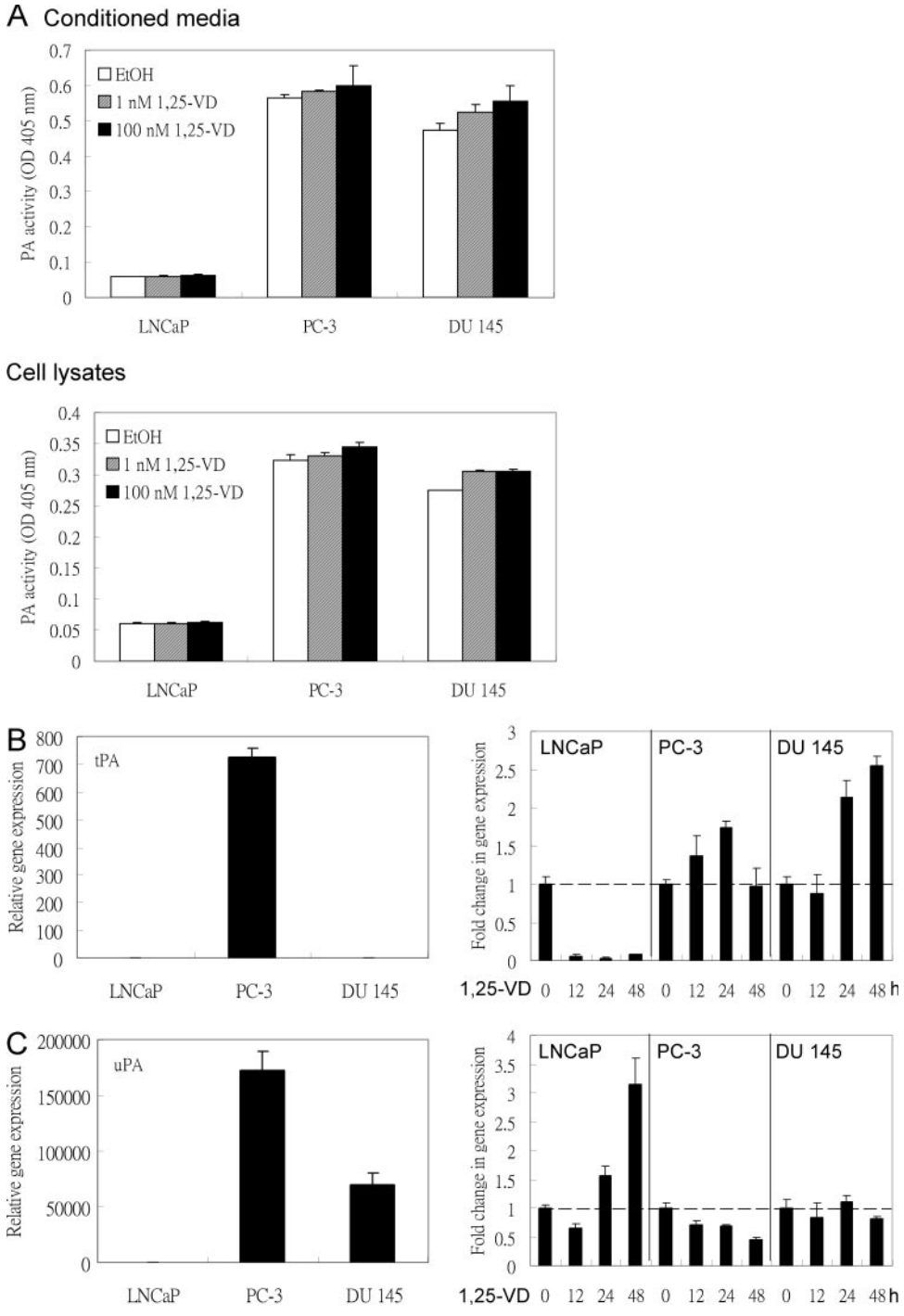

Fig. 3. The effect of 1,25-VD on plasminogen activator system. (**A**) The effect of 1,25-VD on PA activity. LNCaP, PC-3 and DU 145 cells were treated with ethanol vehicle or the indicated concentrations of 1,25-VD for 48 h, and then conditioned media and cell lysates were analyzed by PA activity assay. (**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.

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

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.

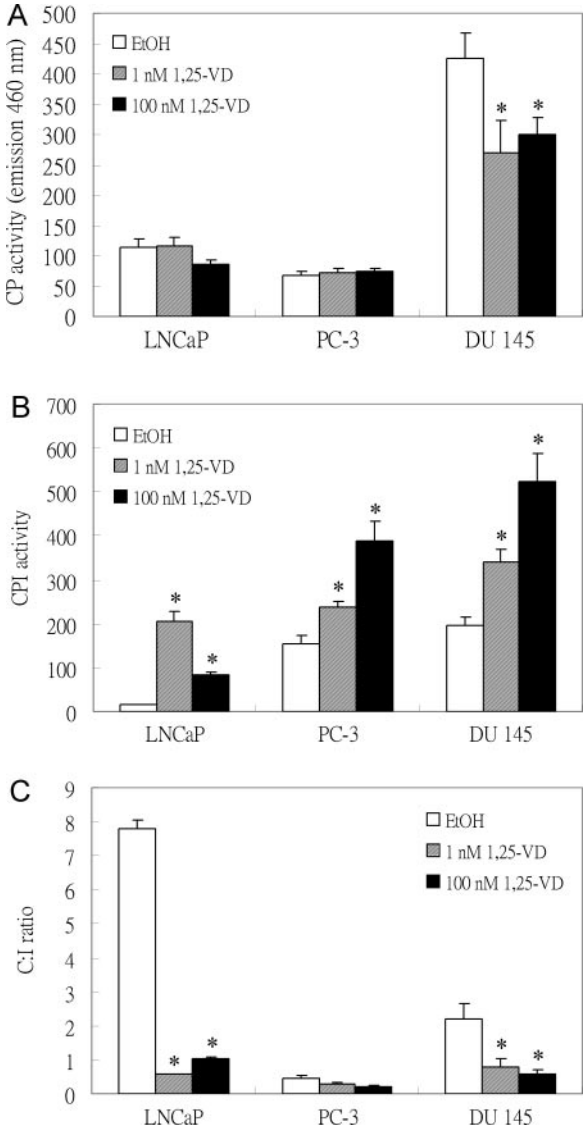

Fig. 4. The effect of 1,25-VD on cathepsin activity. (**A**) Regulation of CP activity by 1,25-VD in prostate cancer cells. (**B**) Up-regulation of CPI activity by 1,25-VD in prostate cancer cells. LNCaP, PC-3 and DU 145 cells were treated with the indicated concentrations of 1,25-VD for 48 h, and then cell lysates were analyzed for CP and CPI enzyme activity. (**C**) Ratio of CP to CPI activity (C:I) in prostate cancer cell lines in response to 1,25-VD. *Indicates significance ($P < 0.05$).

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

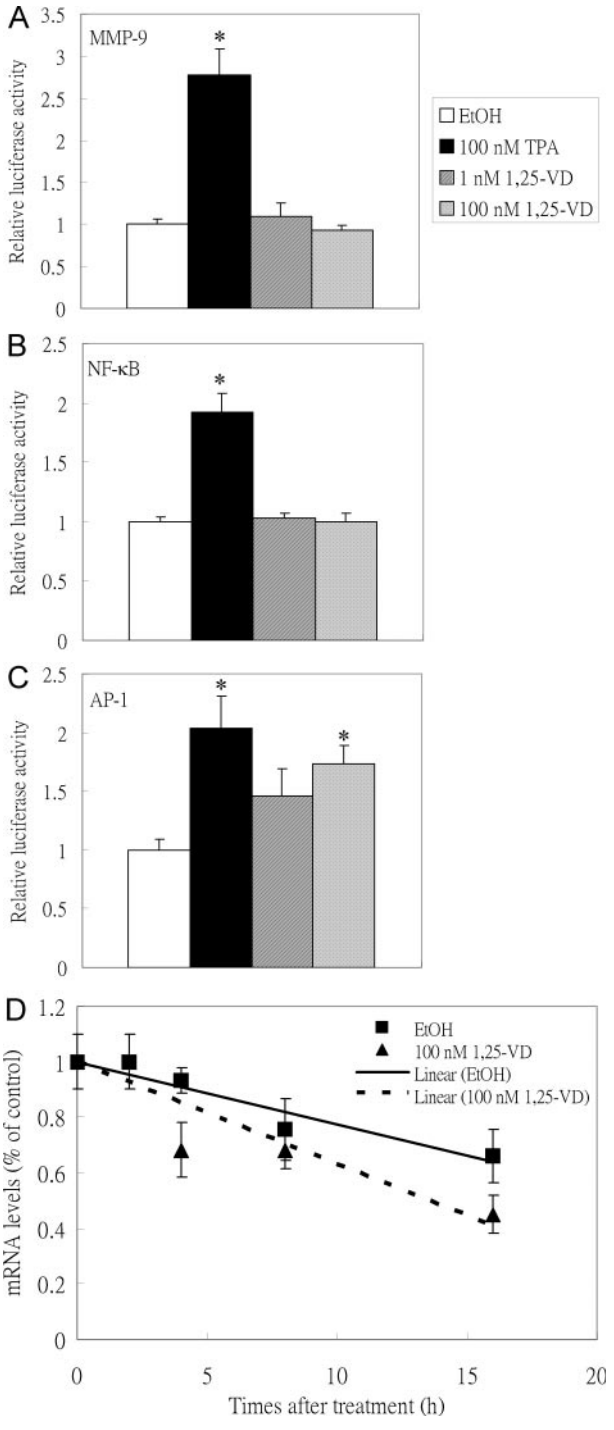

Fig. 5. 1,25-VD has no direct effect on matrix metalloproteinase-9 promoter. Effects of 1,25-VD on the MMP-9 promoter containing luciferase reporter gene activity (**A**) on NF-κB response element containing luciferase reporter gene activities (**B**), and on AP-1 site containing luciferase reporter gene activity (**C**). PC-3 cells were transiently transfected with 0.8 μg/well of MMP-9, NF-κB or AP-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. (**D**) Effects of 1,25-VD on MMP-9 mRNA stability in PC-3 cells. PC-3 cells were pre-treated with ethanol vehical or 100 nM 1,25-VD for 48 h and then incubated with actinomycin D (5 μg/ml) for 2, 4, 8 and 16 h. Total mRNA was prepared and analyzed by real-time PCR. The MMP-9 mRNA levels before actinomycin D treatment were set as 100%. Data are expressed as the mean ± SD of triplicate samples.

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 neo-angiogenesis 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 oncogenes, including FGF-2, EGF, HGF, TNF-α and Ras, mainly through binding to AP-1 and NF-κB binding sites (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

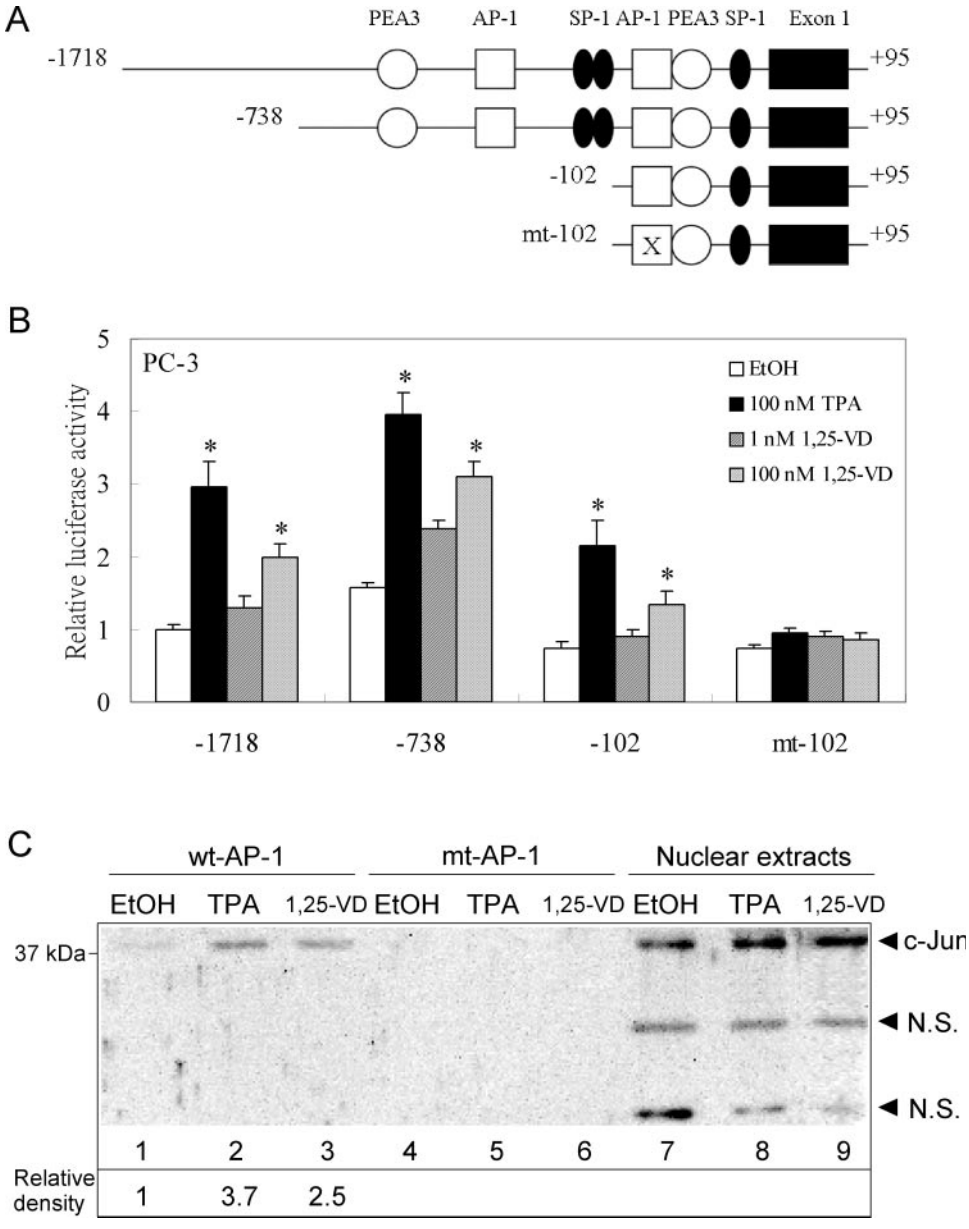

Fig. 6. Regulation of tissue inhibitors of metalloproteinase-1 promoter activity by 1,25-VD. (**A**) Schematic structure of TIMP-1 promoter constructs used for testing luciferase activity. (**B**) Effects of 1,25-VD on the activities of TIMP-1 promoter constructs. PC-3 cells were transiently transfected 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.

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

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 C:I 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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