

Inhibition of p38 by Vitamin D Reduces Interleukin-6 Production in Normal Prostate Cells via Mitogen-Activated Protein Kinase Phosphatase 5: Implications for Prostate Cancer Prevention by Vitamin D

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Abstract

Although numerous studies have implicated vitamin D in preventing prostate cancer, the underlying mechanism(s) remains unclear. Using normal human prostatic epithelial cells, we examined the role of mitogen-activated protein kinase phosphatase 5 (MKP5) in mediating cancer preventive activities of vitamin D. Up-regulation of MKP5 mRNA by 1,25-dihydroxyvitamin-D₃ (1,25D) was dependent on the vitamin D receptor. We also identified a putative positive vitamin D response element within the MKP5 promoter that associated with the vitamin D receptor following 1,25D treatment. MKP5 dephosphorylates/inactivates the stress-activated protein kinase p38. Treatment of prostate cells with 1,25D inhibited p38 phosphorylation, and MKP5 small interfering RNA blocked this effect. Activation of p38 and downstream production of interleukin 6 (IL-6) are proinflammatory. Inflammation and IL-6 overexpression have been implicated in the initiation and progression of prostate cancer. 1,25D pretreatment inhibited both UV- and tumor necrosis factor α -stimulated IL-6 production in normal cells via p38 inhibition. Consistent with inhibition of p38, 1,25D decreased UV-stimulated IL-6 mRNA stabilization. The ability of 1,25D to up-regulate MKP5 was maintained in primary prostatic adenocarcinoma cells but was absent in metastases-derived prostate cancer cell lines. The inability of 1,25D to regulate MKP5 in the metastasis-derived cancer cells suggests there may be selective pressure to eliminate key tumor suppressor functions of vitamin D during cancer progression. These studies reveal MKP5 as a mediator of p38 inactivation and decreased IL-6 expression by 1,25D in primary prostatic cultures of normal and adenocarcinoma cells, implicating decreased prostatic inflammation as a potential mechanism for prostate cancer prevention by 1,25D. (Cancer Res 2006; 66(8): 4516-24)

Introduction

Prostate cancer (PCa) is unique among malignancies in that it generally grows very slowly, likely for decades, before symptoms arise and a diagnosis is finally made. Seemingly, the latency observed in PCa should provide a long window of opportunity for intervention by chemopreventive agents. Laboratory and epidemi-

ologic studies have shown a potential role for vitamin D in the prevention of PCa. Evidence supporting a role for vitamin D in PCa prevention began with studies that linked reduced serum levels of vitamin D metabolites to PCa incidence. Low serum levels of 1,25-dihydroxyvitamin D₃ (1,25D), the active vitamin D metabolite, are associated with increased risk of PCa in older men (1). Decreased serum levels of 25-hydroxyvitamin D₃ (25D), the circulating precursor to 1,25D, also correlate with an increased risk of PCa (1). The latter finding has become more compelling since the discovery that prostate cells are not only sensitive to circulating 1,25D but can also synthesize 1,25D from circulating 25D. Conversion of 25D to active 1,25D by vitamin D 1 α -hydroxylase (1) occurs within the normal prostate and suggests that local production of 1,25D may play a critical role in maintaining normal growth and differentiation.

Studies showing that 1,25D inhibits the growth of primary cultures of prostate cells, established PCa cell lines, and prostate xenograft tumors provide direct evidence for anticancer activity of vitamin D (1). However, mechanisms other than growth inhibition may be responsible for the prevention of PCa by vitamin D.

Using cDNA microarrays, we recently identified a new vitamin D-responsive gene, mitogen-activated protein kinase phosphatase 5 (MKP5; ref. 2). The up-regulation of MKP5, also known as dual-specificity phosphatase 10, was consistently increased by 1,25D treatment of primary cultures of prostatic epithelial cells (2). As a member of the dual-specificity MKP family of proteins that dephosphorylate mitogen-activated protein kinases, MKP5 dephosphorylates p38 and c-jun NH₂-terminal kinase, but not extracellular signal-regulated kinase (3, 4).

The potential ability of vitamin D to inhibit p38 through MKP5 is of interest to PCa prevention because p38 is activated by oxidative stress, hypoxia, and inflammation (5), all of which contribute to PCa development (6, 7). In particular, inflammation plays a causal role in the progression of many cancers including liver, bladder, and gastric cancers (8) and a similar role for inflammation in the development of PCa is now emerging (7). The strongest evidence linking inflammation to PCa is from recent findings that show (a) regular administration of nonsteroidal anti-inflammatory drugs significantly decreases PCa risk in older men by 60 to 80% (9, 10) and (b) men with chronic and/or acute inflammation of the prostate have an increased risk of developing PCa (7, 11).

Inhibitors of p38 are classically anti-inflammatory, suggesting that some of the activities attributed to vitamin D, including PCa prevention, may be a result of p38 inhibition and decreased inflammation. One of the downstream consequences of p38 protein kinase pathway activation is an increase in proinflammatory cytokine production to amplify the inflammatory response (12, 13). Interleukin 6 (IL-6) is a p38-regulated pleiotropic cytokine that has

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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been historically associated with PCa (14, 15). Elevated levels of IL-6 are found in the serum of PCa patients and primary PCa tumors overexpress IL-6 (14, 16). IL-6 is also involved in the progression of PCa to androgen-independent PCa because it can facilitate androgen receptor signaling in the absence of androgens (15, 17).

Because there is increasing evidence linking inflammation to PCa development, our studies focused on characterizing regulation of IL-6 production via p38 inhibition by 1,25D as a potentially significant cancer prevention activity. Using primary epithelial cell cultures derived from the normal peripheral zone (E-PZ; the major site of origin of prostatic adenocarcinomas; ref. 18) and from primary adenocarcinomas of the prostate (E-CA) as our model system, we characterized MKP5 induction by 1,25D and revealed MKP5 as the mediator of p38 kinase inhibition and decreased IL-6 production by 1,25D. Prostate cancer cell lines derived from metastases, however, have lost the ability to up-regulate MKP5 in response to 1,25D, suggesting selection against this anticancer activity of vitamin D.

Materials and Methods

Cell culture and reagents. Human primary prostatic epithelial and stromal cells were derived from radical prostatectomy specimens. The patients did not have prior chemical, hormonal, or radiation therapy. Histologic characterization and cell culture of the prostate cells was as previously described (19). Epithelial cells (E-PZ and E-CA) were cultured in supplemented MCDB 105 (Sigma-Aldrich, St. Louis, MO) or PFMR-4A as previously described (19). Stromal cells (F-PZ) were cultured in MCDB 105/10% fetal bovine serum (FBS). pRNSI-1 cells are immortalized E-PZ cells (20) and are cultured in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA). Human PCA cell lines LNCaP, PC-3, and DU 145 were acquired from American Type Culture Collection (Manassas, VA). LNCaP cells were cultured in MCDB 105/10% FBS and PC-3 and DU 145 in DMEM (Invitrogen)/10% FBS. Normal human keratinocytes were obtained from Cambrex (East Rutherford, NJ) and cultured according to the instructions of the supplier. Squamous cell carcinoma cell lines SCC-25 and A431 were obtained from Dr. Paul Khavari (Stanford University, Stanford, CA) and cultured in DMEM/10% FBS. All chemicals were obtained from Sigma-Aldrich unless otherwise noted. 1,25-dihydroxyvitamin D₃ (Biomol International, Plymouth Meeting, PA) was reconstituted in 100% ethanol at 10 mmol/L and stored at -20°C.

RNA isolation and quantitative real-time reverse transcription-PCR. RNA was isolated from cells by Trizol (Invitrogen) followed by chloroform extraction. The aqueous phase was then precipitated in 100% isopropanol and the pellet washed in 75% ethanol before resuspension in water. RNA concentration and quality were determined by absorbance ratio at 260/280 nm using a UV spectrophotometer. Total RNA (2 µg) was reverse transcribed using Thermoscript RT (Invitrogen). Resulting cDNA was used for quantitative PCR amplification with gene-specific primers and the DyNAMO SYBR Green kit (Finnzymes, Espoo, Finland) in the Opticon 2 thermocycler (MJ Research, South San Francisco, CA). PCR conditions for all primer sets were optimized and have similar amplification efficiency under the following thermocycler conditions: 95°C 5 minutes, 34 × (95°C 30 seconds, 58°C 30 seconds, 72°C 60 seconds), 72°C 5 minutes. Relative mRNA levels were calculated from the point where each curve crossed the threshold line (Ct) using the following equation: $\text{Rel. value} = 2^{-[\text{Ct}(\text{control}) - \text{Ct}(\text{test})]_{\text{test gene}} / 2^{-[\text{Ct}(\text{control}) - \text{Ct}(\text{test})]_{\text{housekeeping gene}}}$ (21). Reactions were done in triplicate and the values normalized to the expression of the housekeeping gene TATA-box binding protein (TBP; ref. 22). Primer sets were TBP, 5'-tgctgagaagagtgtgctggag-3' and 5'-tctgaataggctgtggggtc-3'; total MKP5, 5'-atcttgccctctctgttct-3' and 5'-attgctgttgcctttgac-3'; MKP5 isoform 1 specific, 5'-tgaatgtgagtcctatage-3' and 5'-gttagcagggcaggtgtag-3'; MKP5 isoform 2 specific, 5'-tggatgcagctgagattctg-3' and 5'-ggttctgctt-gtctgtctca-3'; MKP5 isoform 3 specific, 5'-attatgaagtgacttagt-3' and

5'-ggttctgctgttctgtctca-3'; and CYP24, 5'-ggcaacagttctgggtgaat-3' and 5'-tatttgcgacaatccaaca-3'.

Cell lysate preparation and immunoblot. Cells were lysed in ice-cold 1× Cell Lysis Buffer (Cell Signaling, Beverly, MA) containing 1 µmol/L phenylmethylsulfonyl fluoride and 100 nmol/L okadaic acid. Cells were disrupted by sonication and insoluble cell debris removed by centrifugation at 15,000 × g, 4°C. Protein concentrations of the cell lysates were quantified using the Bio-Rad Protein Dye (Bio-Rad, Hercules, CA). Cell lysates were used fresh or stored at -70°C. Cell lysates (10-30 µg) were mixed with LDS NuPAGE Sample Buffer and separated by electrophoresis through 10% NuPAGE Bis-Tris Gels (Invitrogen) and transferred onto polyvinylidene difluoride membrane. Fresh cell lysates were used for analysis of phosphorylated proteins. Membranes were probed with the following primary antibodies: anti-phospho-p38 rabbit polyclonal and anti-p38 rabbit polyclonal from Cell Signaling, anti-vitamin D receptor (VDR) monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti-actin (Santa Cruz Biotechnology). Following primary antibody incubation overnight at 4°C, the blots were incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (Cell Signaling) and developed with HyGlo enhanced chemiluminescence reagent (Denville Scientific, Metuchen, NJ).

Small interfering RNA transfection. Cells at 75% confluency were transfected with 10 nmol/L of negative control (Ambion, Austin, TX), VDR-specific small interfering RNA (siRNA; Santa Cruz Biotechnology), or MKP5-specific siRNA (Ambion) using siPORT NeoFX (Ambion). Cells were used for experiments after transfection as indicated in results and figure legends.

Chromatin immunoprecipitation. Chromatin immunoprecipitation assays were carried out using Upstate Biologics (Waltham, MA) protocol and reagents. Briefly, cells (1-100-mm dish per treatment) were cross-linked with 1% formaldehyde, harvested, and sonicated before immunoprecipitation. One microgram each of anti-VDR (H-81) and anti-VDR (N-20) rabbit polyclonal antibodies (Santa Cruz Biotechnology) was used for overnight 4°C immunoprecipitation. Protein A-agarose beads were then used to pull down immune complexes. Beads were washed, then reverse cross-linked with NaCl at 65°C. The DNA was extracted with spin columns. PCR (30 cycles) was done on 10% of the recovered DNA using primers flanking the vitamin D response element (VDRE) in the MKP5 promoter; VDRE-MKP5: 5'-ccagagccgagtgcaaatag-3' and 5'-gcaatttctctcagttcc-3'. Primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (5'-cggctac-tagcggttttacg-3' and 5'-aagaatgcggctgactgt-3') were used as a negative control. PCR products were electrophoresed through 1.5% agarose gels containing 1 µg/mL of ethidium bromide and visualized by UV.

UV irradiation. Prostate cell cultures were exposed to 1,000 J/m² (~45 seconds) of UVB irradiation using calibrated UVB bulbs with a Kodaceal filter. The lid of the cell culture dish and phenol red-containing media were removed during UV irradiation.

In vitro p38 kinase assay. All reagents, antibodies, and protocol for this assay were supplied by Cell Signaling Technologies. Fresh cell lysate was prepared as described above under *Cell lysate preparation and immunoblot*. Cell lysates containing 250 µg of protein were incubated overnight with immobilized phospho-p38 monoclonal antibody to immunoprecipitate activated p38. Bead-immune complexes were washed and resuspended in 1× kinase buffer containing 200 µmol/L ATP and recombinant activating transcription factor-2 (ATF-2) fusion protein as the substrate and incubated at 30°C for 30 minutes to allow phosphorylation of ATF-2. Reactions were terminated by addition of LDS sample buffer. Samples were then heated at 95°C for 5 minutes and kinase activity was determined by immunoblot analysis with phospho-ATF-2 antibody. Input protein (20 µg) was also immunoblotted and probed with anti-p38.

IL-6 ELISA. Prostate cells (10⁷) were plated in 24-well culture plates. After 24 hours, fresh media containing various agents were added (as described in Results and figure legends). Conditioned media were collected following treatment and used at a 2:1 dilution to determine amount of secreted IL-6 with the Human IL-6 ELISA Kit II (BD Biosciences, San Diego, CA). Results were calculated from a standard curve and are expressed as pg/mL IL-6/10⁶ cells or pg/10⁵ cells.

IL-6 promoter activity. A pGL3 luciferase construct containing a 651-bp fragment of the IL-6 promoter and pRL-null-renilla (Promega, Madison, WI) were transiently transfected into E-PZ cells using NeoFX reagent. pGL3-IL-6 was a generous gift from Dr. Oliver Eikelberg at the University of Giessen (Giessen, Germany). Eight hours after transfection, cells were treated with vehicle or 50 nmol/L 1,25D. Cells were UV irradiated 14 hours after vehicle or 1,25D treatment. Luciferase activity was measured 24 hours after UV using the Dual-Luciferase Assay Kit (Promega). The ratio of luciferase to renilla-luciferase was determined to correct for transfection efficiency.

Results

1,25D increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells. We previously showed that MKP5 mRNA was increased 3- to 10-fold after 6 hours of treatment with 50 nmol/L of 1,25D in three independent primary cultures of normal human prostatic epithelial cells (E-PZ; ref. 2). The optimal concentration of 1,25D required to up-regulate MKP5 mRNA was determined by quantitative reverse transcription-PCR (RT-PCR). A dose-response curve showed that 1 nmol/L of 1,25D was sufficient to increase MKP5 mRNA in E-PZ cells by 6 hours, but 50 nmol/L of 1,25D was required to maintain MKP5 mRNA up-regulation at 24 hours (Fig. 1A). Higher concentrations of 1,25D are needed at the time points >12 hours because high density E-PZ cells rapidly metabolize and inactivate 1,25D (23). In all subsequent experiments, 50 nmol/L of 1,25D was used to treat subconfluent cultures of E-PZ cells. Upon treatment with 50 nmol/L of 1,25D, increased MKP5 expression was observed as early as 3 hours and maintained for 24 hours in E-PZ cells (Fig. 1B).

The *MKP5* gene is located on chromosome 1 and is transcribed into three distinct mRNA splice variants that putatively encode two different proteins (24). MKP5 splice variant 1 encodes the full-length 52-kDa protein whereas variants 2 and 3 both encode a truncated 16-kDa protein that only contains the dual-specificity phosphatase domain (Fig. 1D). Because the biological significance of the MKP5 splice variants has not yet been characterized, we examined the ability of 1,25D to regulate the mRNA expression of the three MKP5 splice variants by quantitative RT-PCR. All of the

MKP5 mRNA splice variants were induced following 6 hours of treatment with 50 nmol/L of 1,25D, but splice variant 1 seemed to be most abundant and achieved the highest level of mRNA induction in the E-PZ cells (Fig. 1C). Primers within exons 3 to 5 of MKP5, the conserved region present in all MKP5 mRNAs, were used for all subsequent experiments.

Up-regulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR on 1,25D treatment. The rapid induction of MKP5 mRNA by 1,25D suggested that MKP5 is a direct target of 1,25D. Direct targets of 1,25D contain one or more VDREs in the promoter region, which mediate transcriptional regulation by VDR binding. Knockdown of VDR levels by VDR siRNA in E-PZ cells abolished the induction of MKP5 by 1,25D (Fig. 2A), showing that induction of MKP5 by 1,25D is VDR dependent. The mRNA expression of vitamin D 24-hydroxylase (CYP24), a well-characterized target of 1,25D, was similarly suppressed in cells transfected with VDR siRNA (Fig. 2A) whereas the expression of *TBP*, a housekeeping gene, was not affected by VDR siRNA transfection. On sequence analysis, a putative VDRE was identified -1,320 bp upstream of the 5' untranslated region in the MKP5 promoter (Fig. 2B). The putative MKP5-VDRE was highly similar to the characterized VDREs present in the promoters of CYP24 and parathyroid hormone-related protein. Chromatin immunoprecipitation assay showed an increased interaction between VDR and the putative MKP5-VDRE that exhibited time-dependent changes on stimulation with 50 nmol/L of 1,25D in E-PZ cells (Fig. 2C). The cyclic nature of VDR interaction with the MKP5 promoter observed in the chromatin immunoprecipitation experiments is consistent with previously described interactions of nuclear hormone receptors with DNA (25). The siRNA and chromatin immunoprecipitation data together provide strong support that MKP5 is directly regulated by 1,25D at the transcription level.

MKP5-mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines. Phosphorylation of p38 is required for activation of p38 kinase activity. MKP5 dephosphorylates p38, thus reducing p38 kinase activity. Vitamin D has been shown to

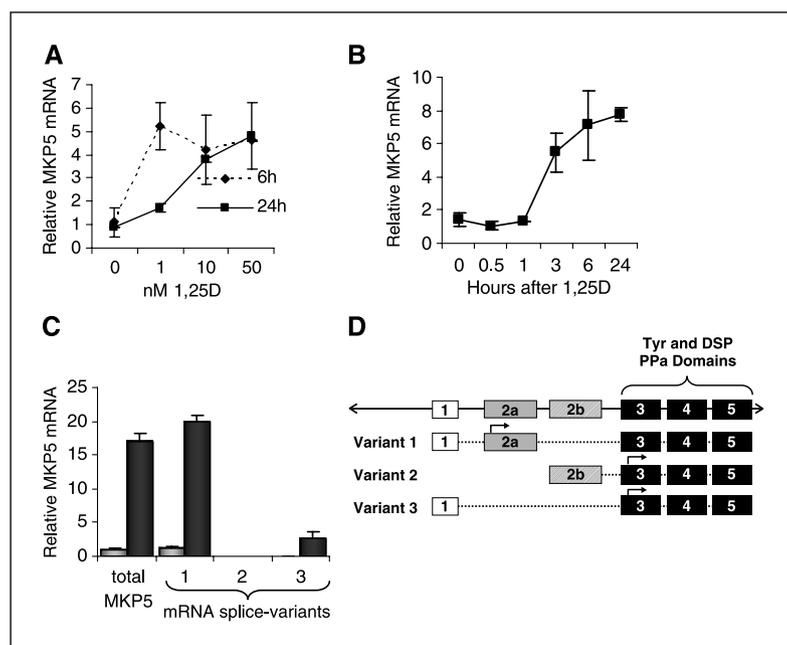


Figure 1. 1,25D increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells (E-PZ). Quantitative RT-PCR measurement of MKP5 mRNA levels in E-PZ cells, 6 hours (dashed line) and 24 hours (solid line) after treatment with 1, 10, and 50 nmol/L 1,25D (A) and 0.5, 1, 3, 6 and 24 hours following 50 nmol/L 1,25D treatment (B). C, quantitative RT-PCR analysis of MKP5 mRNA splice variants in E-PZ cells after 6-hour vehicle (light columns) or 50 nmol/L 1,25D (dark columns) treatment (mRNA levels for isoform 2 treated with vehicle and 1,25D are 0.003 ± 0.0007 and 0.014 ± 0.0004 , respectively, but are not visible on this graph). D, diagram of MKP5 mRNA splice variants. Quantitative RT-PCR results are shown relative to untreated control and normalized to expression of housekeeping gene *TBP*. Each experiment was run in triplicate and graphs are representative of two or more separate experiments with different patient-derived E-PZ cells. Bars, SD.

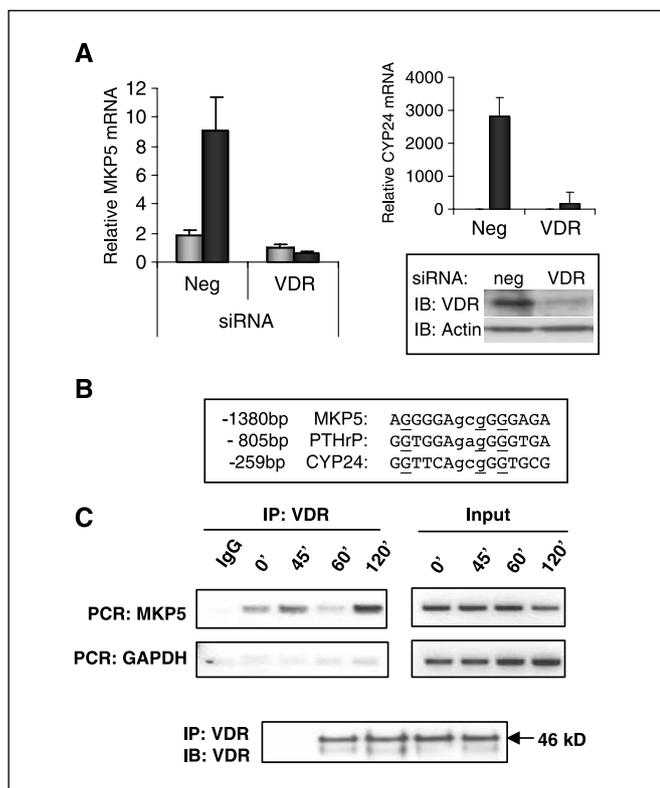


Figure 2. Up-regulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR on 1,25D treatment. **A**, quantitative RT-PCR analysis of MKP5 mRNA 6 hours after treatment with vehicle (light columns) or 50 nmol/L 1,25D (dark columns) in E-PZ cells that were transfected for 24 hours with negative control siRNA or VDR siRNA. CYP24 gene expression was included as positive control for VDR knockdown (untreated mRNA level of CYP24 is equal to one and is not visible on this graph) and immunoblot of nuclear lysate (10 μ g) verified VDR knockdown by the siRNA. **B**, diagram of putative VDRE located -1,380 bp of 5'UTR in MKP5 promoter aligned with validated VDREs in parathyroid hormone-related protein and CYP24. **C**, chromatin immunoprecipitation analysis and PCR of putative VDRE in MKP5 promoter and GAPDH promoter following 50 nmol/L 1,25D treatment using rabbit polyclonal VDR antibody for pulldown. Immunoblot of protein precipitate probed with mouse monoclonal VDR antibody shows specific pulldown of VDR. Results are representative of four or more separate experiments with different patient-derived E-PZ cells. Quantitative RT-PCR results are shown relative to untreated control and normalized to expression of housekeeping gene *TBP*. Each experiment was run in triplicate and graphs were representative of two or more separate experiments. Bars, SD.

inhibit osmotic stress-stimulated p38 phosphorylation in keratinocytes (26). We observed a similar inhibition of p38 phosphorylation by 1,25D in E-PZ cells (Fig. 3A). E-PZ cells were pretreated with 1,25D for 14 hours to allow for sufficient up-regulation of MKP5 protein. MKP5 protein levels could not be directly monitored in this study due to lack of an appropriate antibody. After pretreatment with 1,25D, osmotic stress-stimulated phosphorylated p38 levels were appreciably decreased compared with levels in cells not treated with 1,25D. Transfection with MKP5-specific siRNA attenuated induction of MKP5 mRNA by 1,25D compared with negative control siRNA (Fig. 3B) and abolished the suppression of p38 phosphorylation by 1,25D (Fig. 3A), implicating MKP5 as the mediator of p38 inactivation by 1,25D.

We examined the effect of 1,25D on MKP5 in various other prostate-derived cells in comparison with the primary cultures of E-PZ cells. The results showed that, like E-PZ cells, pRNS-1-1 cells also induce MKP5 (Fig. 3C). In contrast, prostate stromal cells (F-PZ) and established PCa cell lines (PC-3, LNCaP, and DU 145)

did not up-regulate MKP5 mRNA following 1,25D treatment (Fig. 3C). pRNS-1-1 cells were generated by SV40 transformation and immortalization of E-PZ cells and are not growth inhibited by 1,25D, although they retain VDR and other responses to 1,25D (1). Prostatic stromal cells too express VDR and show certain responses to 1,25D despite lack of induction of MKP-5 in these cells by 1,25D (1). The PCa cell lines PC-3, LNCaP, and DU 145 all express VDR and respond to 1,25D in other ways (1). Immunoblot analysis showed that in DU 145, PC-3, and LNCaP cells, 1,25D pretreatment did not alter NaCl-induced p38 phosphorylation (Fig. 3D). These data suggest that MKP5 may specifically mediate 1,25D activity in normal prostate cells and that this activity is lost in advanced PCa.

We suspect that MKP5 up-regulation by 1,25D is not unique to prostatic epithelium. Because inactivation of p38 by 1,25D was reported in keratinocytes, we examined the expression of MKP5 in these cells. Similarly to prostatic epithelial cells, normal human keratinocytes showed up-regulation of MKP5 mRNA on treatment with 1,25D, suggesting that MKP5 may mediate p38 inactivation in keratinocytes as well (Supplementary Fig. S1A). Similarly to the PCa cell lines, up-regulation of MKP5 by 1,25D was attenuated in the human squamous cell carcinoma cell lines A431 and SCC-25 (Supplementary Fig. S1B).

MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D. Published studies have shown that 1,25D inhibits UV-induced IL-6 production in keratinocytes; however, no mechanism has been proposed (27). IL-6 overexpression has been strongly associated with PCa progression and, therefore, inhibition of IL-6 may play an important role in PCa prevention. Because IL-6 induction is downstream of p38 activation and often dependent on p38 activation (13, 28), we tested the role of MKP5 in regulating IL-6 expression in E-PZ cells. An *in vitro* p38 kinase activity assay, using ATF-2 as the substrate, showed that 1,25D pretreatment decreased basal and UV-stimulated p38 activity in E-PZ cells (Fig. 4A). The 1,000 J/m² dose of UVB irradiation used in these experiments did not induce apoptosis or necrosis in E-PZ cells (data not shown). Consistent with p38 inactivation, secreted levels of IL-6 protein following UV treatment were suppressed by 1,25D pretreatment (Fig. 4B). SB202190, a specific p38 inhibitor, similarly decreased UV-stimulated IL-6 production (Fig. 4B). A decrease in IL-6 mRNA expression was observed in 1,25D pretreated samples and this decrease was blocked by transient transfection with MKP5 siRNA (Fig. 4C). The primary mechanism for increased IL-6 production following p38 activation is through IL-6 mRNA stabilization rather than increased mRNA transcription (29). To determine if this is also true for 1,25D regulation of IL-6 mRNA, we examined the effect of 1,25D on IL-6 promoter activity in the absence or presence of UV irradiation. We found that neither UV irradiation nor 1,25D treatment significantly altered IL-6 promoter activity as determined by luciferase assay in E-PZ cells (Fig. 4D1), suggesting that 1,25D is not altering mRNA transcription. When 1 μ mol/L of actinomycin D was used to inhibit new mRNA transcription, we observed that UV irradiation caused IL-6 mRNA stabilization and 1,25D pretreatment decreased the UV-induced IL-6 mRNA stabilization (Fig. 4D2). Under basal conditions, IL-6 mRNA half-life was <45 minutes in E-PZ cells. In UV-irradiated cells, IL-6 mRNA half-life increased to >90 minutes whereas UV irradiation did not significantly alter IL-6 mRNA half-life in 1,25D pretreated cells (Fig. 4D2).

1,25D inhibits tumor necrosis factor α -stimulated p38 activation and IL-6 production. The ability of 1,25D to inhibit

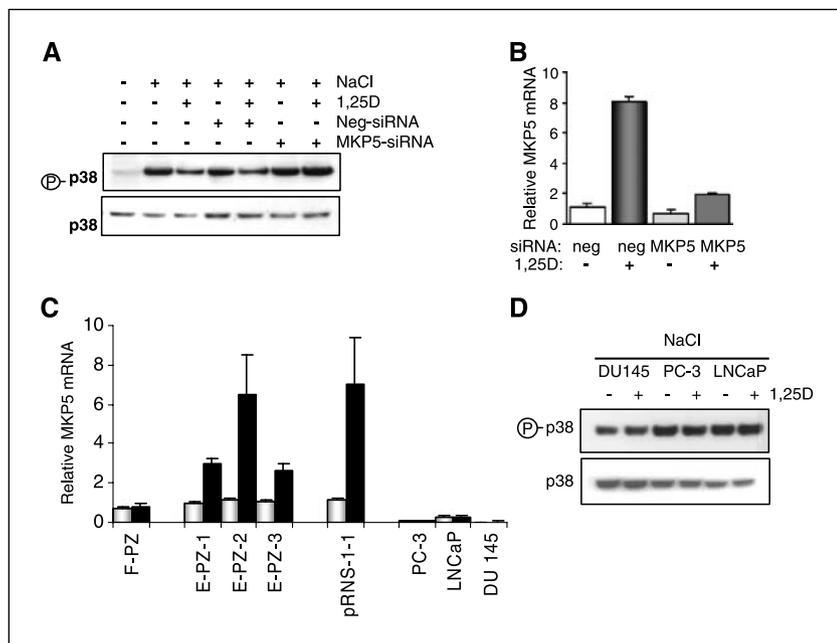


Figure 3. MKP5-mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines. **A**, immunoblot analysis of p38 phosphorylation in 20 μ g of E-PZ cell lysate 20 minutes after treatment with 0.5 mol/L NaCl. Before NaCl treatment, E-PZ cells were transfected with negative control or MKP5 siRNA for 4 hours, then treated with vehicle or 50 nmol/L 1,25D for 14 hours. **B**, quantitative RT-PCR analysis of MKP5 mRNA expression in E-PZ cells transfected with negative control siRNA (*open columns*) or MKP5 siRNA (*striped columns*) 4 hours before vehicle (*light columns*) or 1,25D (*shaded columns*) treatment for 12 hours. Representative of at least three separate experiments with different patient-derived E-PZ cells. Results are displayed relative to negative siRNA control and normalized to expression of the housekeeping gene *TBP*. **C**, quantitative RT-PCR analysis of MKP5 mRNA 6 hours after treatment with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*) in primary cultures of prostate stroma (F-PZ) in three different cultures of normal primary prostatic epithelial cells (derived from normal peripheral zone designated E-PZ) and in prostate cell lines pRNS-1-1, LNCaP, PC-3, and DU 145. Quantitative RT-PCR results are displayed relative to control and normalized to expression of the housekeeping gene *TBP*. **D**, immunoblot analysis of p38 phosphorylation 20 minutes after treatment with 0.5 mol/L NaCl in DU 145, PC-3, and LNCaP cells pretreated for 14 hours with vehicle or 50 nmol/L 1,25D.

p38 phosphorylation was further investigated using a more physiologic stress, tumor necrosis factor α (TNF- α). TNF- α is a proinflammatory cytokine released by inflammatory cells that can trigger cell proliferation, necrosis, apoptosis, and induction of other cytokines (12). Interestingly, elevated serum levels of TNF- α are associated with aggressive pathology and decreased survival of PCa

patients (16). In E-PZ cells, TNF- α does not induce apoptosis but does significantly slow cell growth (30). TNF- α binds cell-surface receptors which signal through multiple pathways, including p38 kinase, to increase production of IL-6 and other cytokines (12). Immunoblot analysis showed that TNF- α -stimulated p38 phosphorylation was attenuated by 1,25D pretreatment, similar to the

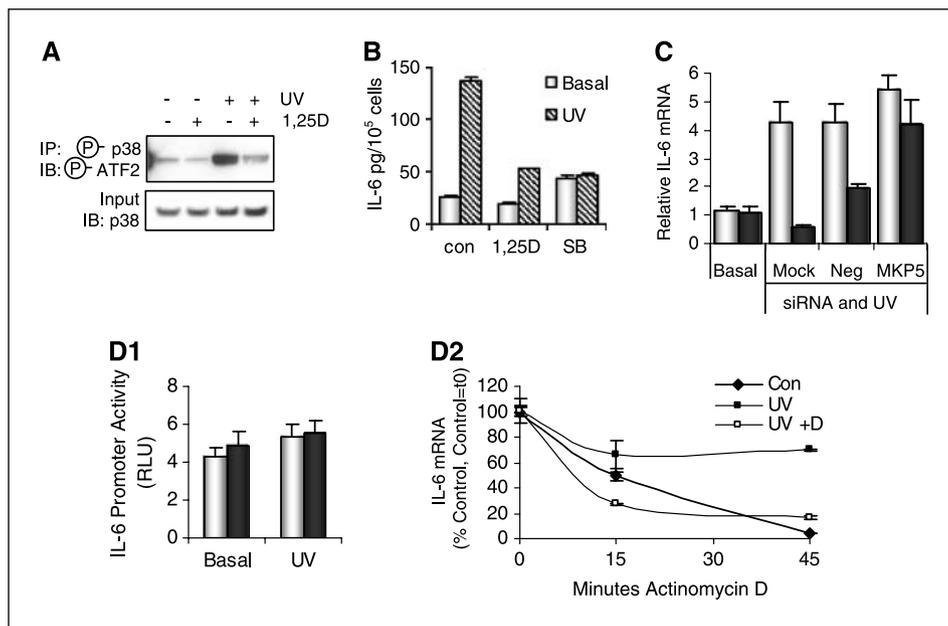


Figure 4. MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D. **A**, *in vitro* p38 kinase activity assay, using ATF-2 as a substrate, in E-PZ cells 20 minutes after 1,000 J/m² UVB irradiation in E-PZ cells pretreated 14 hours with vehicle or 50 nmol/L 1,25D. **B**, ELISA measurement of secreted IL-6 in cell culture media 24 hours after UVB irradiation (*hatched columns*) in E-PZ cells pretreated with vehicle, 50 nmol/L 1,25D for 14 hours, or 1 μ mol/L SB202190 for 1 hour. **C**, quantitative RT-PCR measurement of IL-6 mRNA in E-PZ cells 24 hours after UV irradiation. Cells were either transiently transfected with negative siRNA or MKP5 siRNA for 4 hours, then pretreated for 14 hours with either vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*) before UV. **D1**, luciferase activity of pGL3-IL-6 24 hours after UV irradiation in E-PZ cells pretreated for 14 hours with vehicle (*light columns*) or 50 nmol/L 1,25D (*dark columns*). **D2**, quantitative RT-PCR analysis of IL-6 mRNA stability in E-PZ cells following UV irradiation. Cells were treated with 1 μ mol/L actinomycin D for 0, 15, and 45 minutes. Actinomycin was dosed 30 minutes after UV and cells were pretreated 14 hours before UV with vehicle (■) or 50 nmol/L 1,25D (□). IL-6 mRNA levels under basal conditions after 15 to 45 minutes of treatment with 1 μ mol/L actinomycin D were shown as control (◆).

effect of 1,25D on UV-stimulated p38 phosphorylation in E-PZ cells (Fig. 5A). Changes in IL-6 mRNA and secreted protein levels were followed over a time course after TNF- α stimulation and showed that 1,25D pretreatment attenuated the initial production of IL-6 and completely inhibited the accumulation of IL-6 in the media (Fig. 5B and C).

1,25D up-regulates MKP5 mRNA and inhibits TNF- α -stimulated IL-6 production in matched pairs of normal and tumor cells from individual PCa patients. Because we observed a difference in regulation of MKP5 by 1,25D between E-PZ cells and PCa cell lines, we analyzed the effect of 1,25D on MKP5 and IL-6 in matched pairs of normal (E-PZ) and tumor (E-CA) cells from two PCa patients with localized Gleason grade 4/3 cancers (individuals A and B). Quantitative RT-PCR analysis showed that MKP5 mRNA was up-regulated by 1,25D in E-CA as well as E-PZ cells from both individuals (Fig. 6A). In addition, IL-6 mRNA and protein levels, following TNF- α stimulation, were decreased by 1,25D pretreatment in all of the matched pairs of normal and cancer cultures (Fig. 6B and C). Although patient-to-patient variability was evident

in the extent of TNF- α -stimulated IL-6 production, cancer cells from primary adenocarcinomas retain the ability to up-regulate MKP5 in response to 1,25D, in contrast to the established metastases-derived cell lines (DU 145, LNCaP, and PC-3).

Discussion

The purpose of these studies was to explore the potential significance of MKP5 in mediating PCa prevention by vitamin D. We previously identified MKP5 as a target of 1,25D in normal human prostatic epithelial cells by microarray analysis (2). The results of our studies suggest that the ability of vitamin D to inhibit p38 signaling, via MKP5 up-regulation, may be a significant antitumor activity of vitamin D (Fig. 7).

MKP5 is likely a direct target of 1,25D, regulated by a positive VDRE in the promoter region of the gene. Treatment of E-PZ cells with 1,25D produced a time- and dose-dependent increase in MKP5 mRNA that was dependent on VDR expression. Furthermore, VDR was found to associate with the VDRE in the MKP5 promoter on 1,25D treatment. These data support direct transcriptional activation of the *MKP5* gene by 1,25D. Consistent with increased levels of MKP5, 1,25D inhibited p38 phosphorylation. MKP5 siRNA blocked p38 inactivation by 1,25D, which further showed that MKP5 mediates p38 inactivation by 1,25D.

The ability of 1,25D to inactivate p38 led us to examine the regulation of IL-6, which is downstream of p38 activation (28), by 1,25D. Suppression of UV-stimulated IL-6 production by 1,25D was previously shown in keratinocytes (27) and we suspected that similar activity could be mediated by MKP5 in normal prostatic epithelial cells. Using MKP5 siRNA, we showed that 1,25D inhibited UV-stimulated p38 activity and IL-6 production in a MKP5-dependent manner in E-PZ cells. UV irradiation did not increase IL-6 promoter activity but did increase mRNA half-life, indicating posttranscriptional regulation of IL-6 mRNA expression. 1,25D pretreatment was able to attenuate the UV-stimulated increase in IL-6 mRNA half-life. Induction of IL-6 by a more physiologically relevant stress, TNF- α , was similarly inhibited by 1,25D.

IL-6 has been shown to be negatively regulated by androgens and the androgen receptor in murine bone marrow-derived fibroblasts (31). It is unlikely that androgen receptor contributes to IL-6 regulation observed in E-PZ cells because E-PZ cells are typical of prostatic basal epithelial cells and do not express androgen receptor (32). There is evidence of cross-talk and regulation of androgen receptor by vitamin D (1). In cells where androgen receptor and VDR are both expressed, such as the luminal cells of the prostate, it is possible that cooperation of the two pathways could facilitate a further decrease in IL-6 expression.

IL-6, as well as other interleukins and/or their receptors, are overexpressed in PCa tissue and/or serum of PCa patients (15, 33). Elevated serum levels of IL-6 and TNF- α are associated with aggressive pathology and decreased survival of PCa patients (16). Increased IL-6 staining is observed in malignant prostate tissue compared with adjacent normal tissue and IL-6 also contributes to the development of hormone-refractory cancer by androgen-independent activation of the androgen receptor (17). *In vitro*, the PCa cell lines DU 145, PC-3, and LNCaP all express the IL-6 receptor and are responsive to exogenous IL-6; however, DU 145 and PC-3 also greatly overexpress endogenous IL-6 whereas the LNCaP cell line does not express any IL-6 (15). The mechanism(s) leading to constitutive overexpression of IL-6 in PCa cells and

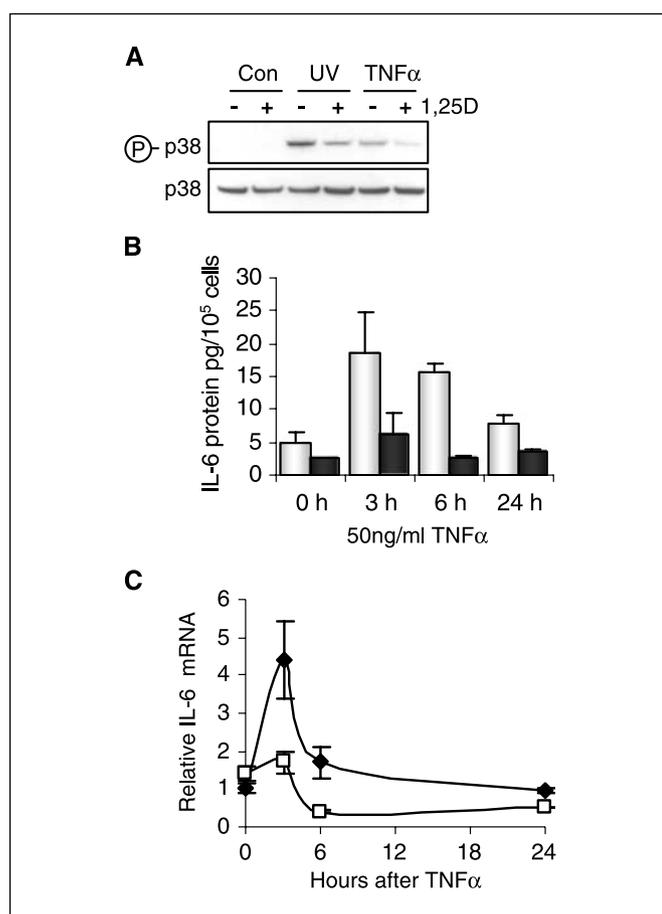


Figure 5. 1,25D inhibits TNF- α -stimulated p38 phosphorylation and IL-6 production. **A**, immunoblot analysis of p38 phosphorylation 20 minutes after treatment with 1,000 J/m² UVB or 50 ng/mL TNF- α in E-PZ cells pretreated 14 hours with vehicle or 50 nmol/L 1,25D. **B**, ELISA measurement of secreted IL-6 in cell culture media 3, 6, and 24 hours after 50 ng/mL TNF- α in E-PZ cells pretreated 14 hours with vehicle (light columns) or 50 nmol/L 1,25D (dark columns). **C**, quantitative RT-PCR analysis of IL-6 mRNA 3, 6, and 24 hours after 50 ng/mL TNF- α in E-PZ cells pretreated for 24 hours with vehicle (\blacklozenge) or 50 nmol/L 1,25D (\square). IL-6 gene expression relative to untreated control and normalized to expression of the housekeeping gene *TBP*. Bars, SD of triplicate samples.

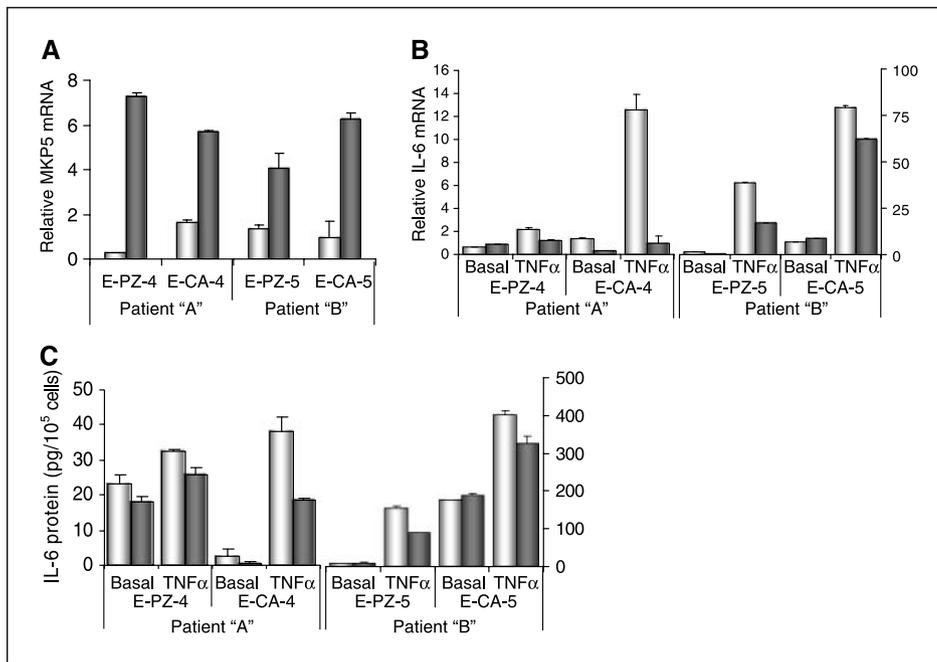


Figure 6. 1,25D up-regulates MKP5 mRNA and inhibits TNF- α -stimulated IL-6 production in matched pairs of normal and tumor cells from individual PCa patients. **A**, quantitative RT-PCR measurement of MKP5 mRNA levels in patient A E-PZ and E-CA cells and patient B E-PZ and E-CA cells 15 hours after treatment with vehicle (open columns) or 50 nmol/L 1,25D (shaded columns). **B**, quantitative RT-PCR analysis of IL-6 mRNA 3 hours after 50 ng/mL TNF- α in cells pretreated 15 hours with vehicle (open columns) or 50 nmol/L 1,25D (shaded columns). MKP5 and IL-6 gene expressions are shown relative to untreated control and normalized to expression of the housekeeping gene TBP. Bars, SD of triplicate samples. **C**, ELISA measurement of secreted IL-6 in cell culture media 3 hours after 50 ng/mL TNF- α in E-PZ and E-CA cells pretreated for 14 hours with vehicle (light columns) or 50 nmol/L 1,25D (dark columns).

tissues has not been fully characterized but seems to be transcriptional rather than posttranscriptional (34).

Although proinflammatory factors are overexpressed in PCa, it is unclear whether increased levels of these factors are required for development of the cancer originally or are a consequence of the cancer. On one hand, inflammation may trigger the initial expression of these factors in normal prostate tissue and when PCa arises, the PCa cells maintain these features for a survival advantage. On the other hand, because there is significant inflammatory infiltrate in PCa lesions, the possibility of the inflammatory genes becoming expressed later in PCa development cannot be excluded. If the former situation occurs, then our results suggest that the ability of 1,25D to suppress the synthesis of IL-6, and perhaps other inflammatory factors, may be a key component in blocking carcinogenic events associated with inflammation.

Another important finding from our study was that 1,25D up-regulated MKP5 in primary cultures derived from normal prostatic

epithelium or primary adenocarcinomas of the prostate and in SV40 Tag-immortalized prostatic epithelial pRNS-1-1 cells, but not in the prostatic stromal cells or in PCa cell lines. pRNS-1-1 cells are not sensitive to growth inhibition by 1,25D yet retain functional VDR (1). Stromal cells (F-PZ) derived from normal prostatic tissue and PCa cell lines did not induce MKP5 when treated with 1,25D although these cells express VDR and are growth-inhibited by 1,25D (1). Furthermore, the squamous cell carcinoma cell lines A431 and SCC-25 displayed attenuated MKP5 up-regulation following 1,25D treatment compared with normal keratinocytes although they too retain VDR and growth inhibition by 1,25D (35, 36). We observed up-regulation of MKP5 by 1,25D in the matched pairs of E-PZ and E-CA cells from individual patients. These E-CA cells were derived from localized PCa whereas PC-3, DU 145, and LNCaP were all derived from PCa metastases. These data suggest that localized PCa may still be responsive to the anti-inflammatory properties of vitamin D. Together these observations show that (a) induction of MKP5 by

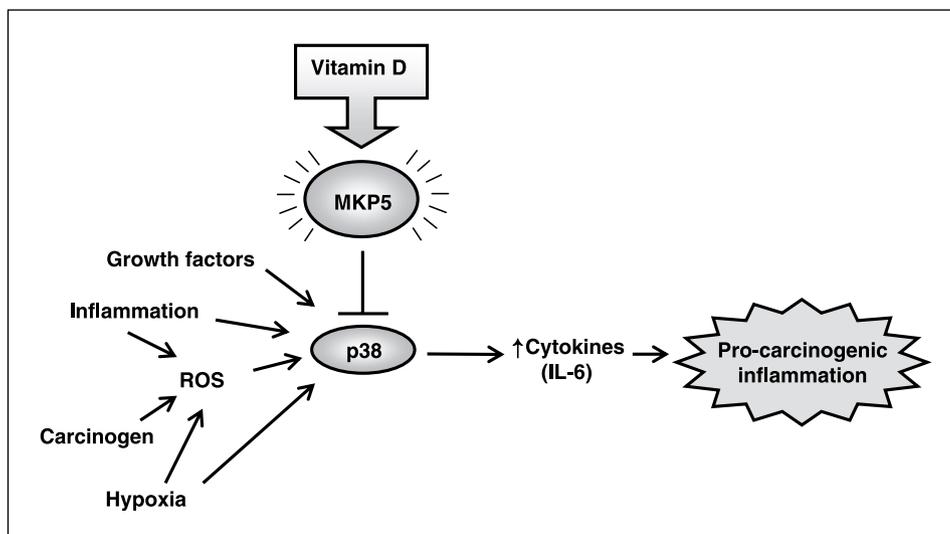


Figure 7. Proposed mechanism for anti-inflammatory activity and PCA prevention by vitamin D.

1,25D is specific to normal and localized malignant prostatic epithelial cells and does not occur in normal prostatic stromal cells; (b) induction of MKP5 is independent of growth inhibition by 1,25D; and (c) lack of 1,25D-induced MKP5 in PCa cell lines is not a result of immortalization per se because immortalized pRNS-1-1 cells up-regulate MKP5 in response to 1,25D.

The quantitative RT-PCR data show that the basal levels of MKP5 are lower in the PCa cell lines compared with normal prostatic epithelial cells. Because the established metastasis-derived PCa cell lines have low levels of MKP5 and are unable to induce MKP5 in response to 1,25D, it is tempting to speculate that loss of MKP5 may occur during PCa progression as the result of selective pressure to eliminate tumor suppressor activity of MKP5 and/or 1,25D. A number of MKP family members have been suggested to be tumor suppressors. In PCa MKP1 has been shown to be down-regulated (37). Candidate MKP tumor suppressors in other malignancies include MKP7, which is frequently deleted in lymphoblastic leukemia (38), MKP3, hypermethylated or deleted in pancreatic cancer (39, 40), and MKP2, which is deleted in breast carcinoma (41).

The link between inhibition of p38 by 1,25D via MKP5 and PCa prevention becomes more apparent when the many different ways the p38 pathway may become activated are considered. In addition to inflammatory cytokines, osmotic stress, UV irradiation (which we used in these studies), reactive oxygen species and hypoxia also activate p38 (42). Reactive oxygen species can amplify p38 activation because they are generated during hypoxia and as a by-product of inflammation (refs. 6, 8; Fig. 7). Hypoxia has been implicated in PCa metastasis and progression to androgen independence (6).

In addition to a potential role in PCa prevention, p38 inhibition by 1,25D via MKP5 may be farther reaching and mitigate activities of vitamin D in other tissues. Recent cDNA microarray analyses have shown MKP5 up-regulation by vitamin D in skin (43), colon (44), and ovarian cells (45). Furthermore, the overall immunomodulatory activity of vitamin D on the VDR-expressing cells of the innate and adaptive immune system (46) is highly similar to the

immunomodulatory role of MKP5 that was shown by Zhang et al. (47) using MKP5 knockout mice. The mechanism by which vitamin D reduced IL-6 mRNA stability, through MKP5-mediated p38 inactivation, may also be responsible for down-regulation of other mRNAs. Activation of the p38 pathway causes a robust and rapid increase in inflammatory response proteins by mRNA stabilization and increased translation through AU-rich elements in the 3' untranslated region (UTR; ref. 48). Posttranscriptional regulation of inflammatory genes is the basis for the anti-inflammatory activity of p38 inhibitors (48). Decreased mRNA stabilization by vitamin D has been shown to mediate down-regulation of granulocyte macrophage colony-stimulating factor (49) and this mechanism may decrease the stability of other AU-rich 3'-UTR-containing proinflammatory mRNAs by vitamin D.

It is becoming apparent that inflammation, both chronic and acute, contributes to PCa development. The epidemiologic evidence combined with the molecular pathogenesis of PCa supports this hypothesis (7). If inflammation is a significant risk factor for PCa, then PCa prevention will best be achieved with agents, such as vitamin D, which inhibit inflammation and/or decrease the cellular stress response that accompanies inflammation. Our study shows that MKP5 is a mediator of anti-inflammatory effects of 1,25D and suggests that vitamin D may play a significant role in PCa prevention by facilitating p38 inhibition and reduced IL-6 production in prostatic epithelial cells.

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Inhibition of p38 by Vitamin D Reduces Interleukin-6 Production in Normal Prostate Cells via Mitogen-Activated Protein Kinase Phosphatase 5: Implications for Prostate Cancer Prevention by Vitamin D

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