1α,25-Dihydroxyvitamin D (Calcitriol) Inhibits the Invasiveness of Human Prostate Cancer Cells[1]

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Abstract

1α,25-Dihydroxyvitamin D (1,25 D; also known as calcitriol), the hormonal form of vitamin D, can inhibit the proliferation and promote the differentiation of human prostate adenocarcinoma cells. However, little is known about the effects of 1,25 D on the invasive ability of prostate cancer cells. We used an *in vitro* bioassay of cell invasion (Amgel assay) to examine the effects of 1,25 D and a "noncalcemic" vitamin D analogue, 1,25-dihydroxy-16-ene-23-yne-cholecalciferol ($16-23-D_3$), on the invasiveness of three well-characterized human prostate carcinoma cell lines: DU 145, PC-3, and LNCaP. PC-3 and LNCaP cells were poorly invasive in Amgel and were hardly affected by treatment with 1,25 D or $16-23-D_3$ (<3%). Conversely, DU 145 cells were highly invasive in Amgel, and their invasion was markedly inhibited by 1,25 D and $16-23-D_3$ (maximally 66 and 59.4%, respectively). This effect was both dose-dependent (doses of 1×10^{-7}-1×10^{-13} M) and time-dependent, with maximal inhibition at 1×10^{-7} M and 72 h. Significant inhibition of invasion was observed at physiological levels of 1,25 D. Neither proliferative indices nor cell cycle kinetics were altered during the experimental exposures. Treatment with 1,25 D and $16-23-D_3$ caused a selective decrease in the secreted levels of type IV collagenases (MMP-2 and MMP-9). These findings support the hypothesis that 1,25 D reduces the risk of invasive prostate cancer and suggest a role for vitamin D compounds in the chemoprevention of invasive prostate cancer.

Introduction

In 1996, approximately 317,000 men were diagnosed with prostate cancer, and 41,100 men died of their disease (1). Other than increasing age and black race, few consistent risk factors for prostate cancer have emerged from epidemiological studies (2). A unique feature of adenocarcinoma of the prostate is the high prevalence of "incidental" or "autopsy" cases. Autopsy data indicate that approximately 30% of men over the age of 50 have histological evidence of prostate carcinoma and that the prevalence of these lesions reaches 60% in men over the age of 80 (3). These incidental tumors are histologically indistinguishable from those prostate cancers that ultimately threaten the lives of their hosts and are generally believed to represent an earlier stage in their natural history. Because incidental tumors are common among older men, a better understanding of factors that influence the development of clinical prostate cancer from its subclinical precursors is crucial to rational efforts at reducing prostate cancer mortality.

Considerable attention has focused on the role of vitamin D in this regard (4). There is now considerable data to support the hypothesis that the hormonal form of vitamin D maintains the differentiated phenotype of prostatic cells and that vitamin D insufficiency increases the risk for clinical prostate cancer (5, 6). United States mortality rates from prostate cancer are inversely related to the availability of UV light, the major source of vitamin D (7). This suggests that the progression of subclinical cancers to clinical cancer may be retarded by vitamin D.

Vitamin D is formed in the skin when sunlight activates its precursor, 7-dehydrocholesterol. Vitamin D then undergoes sequential hydroxylations in the liver and kidney to form 1,25 D,[3] the hormonal form of the vitamin. Although 1,25 D is best known for its role in calcium homeostasis, specific receptors for 1,25 D (VDRs) are present in numerous normal and malignant cells that are not involved in mineral metabolism, and 1,25 D has been shown to regulate the growth of many of these cells (8). Most human prostate cancer cell lines examined contain biologically active VDRs (9), and 1,25 D and vitamin D analogues have been shown to inhibit the proliferation and induce the differentiation of prostate cancer cells *in vitro* (10–12), in primary culture (13), and *in vivo* (14). Recent evidence indicates that nuclear VDR expression is sufficient to mediate the antiproliferative effects of 1,25 D, which occur via a genomic signaling pathway (15, 16).

Although 1,25 D is known to inhibit the proliferation of prostate cancer cells, little is known about the effect of 1,25 D on prostate cancer invasion and metastasis. Because the ability of cancerous cells to invade a basement membrane is a critical step in the evolution of tumor dissemination via hematogenous and lymphatic routes (17), we sought to investigate the effects of 1,25 D on the invasiveness of human prostate cancer cells through an artificial basement membrane-like complex ECM (Amgel). Furthermore, we sought to perform the assays in such a way that cell proliferation would not be a confounding variable.

[3] The abbreviations used are: 1,25 D, 1α,25-dihydroxyvitamin D; ECM, extracellular matrix; $16-23-D_3$, 1,25-dihydroxy-16-ene-23-yne-cholecalciferol; TGF, transforming growth factor.

Materials and Methods

Cell Culture. Human prostate adenocarcinoma DU 145, PC-3, and LNCaP cell lines were obtained from American Type Culture Collection (Rockville, MD) and were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 10,000 units/ml pencillin G, 100 μg of streptomycin sulfate (Life Technologies, Inc., Grand Island, NY), and 200 mM L-glutamine (Life Technologies). All cell lines were tested and found to be free of *Mycoplasma* contamination. Stock solutions of 1,25 D and of the vitamin D analogue 16-23-D$_3$ (Ro23-7553) were resuspended in 1% absolute ethanol. 16-23-D$_3$ was provided by Dr. Milan Uskokovic (Hoffman-La Roche, Nutley, NJ).

Cell Growth and Proliferation Studies. Tumor cells (1×10^5 cells/well) from each cell line were seeded onto 6-well plates, allowed to attach for 3 h, and treated with 1×10^{-7} M 1,25 D, an identical concentration of 16-23-D$_3$, or 1% ethanol vehicle as control, in 2 ml of medium. After 0, 1, 2, and 3 days of incubation, cells were trypsinized, harvested, washed in PBS, and counted in a Coulter counter. In parallel, cells were labeled with [^{3}H]thymidine (1 μCi/ml) in complete medium for the indicated time periods, and the radioactivity was measured by liquid scintillation counting. Cell viability at each end point was monitored by trypan blue exclusion. At the end of each time course, the cells were harvested from each well and counted with a Coulter counter.

Flow Cytometry Analysis. Cells from the control and treated groups were subjected to cytofluorometric analysis using a Cycle TEST Plus DNA reagent kit (Becton Dickinson). Briefly, cells (2×10^6 cells/ml) were washed, harvested in citrate buffer, and incubated with a trypsin-RNase mixture, followed by staining with 0.5% propidium iodide. Cell cycle parameters were obtained by a FACScan flow cytometer and analyzed using Mod FIT software. Approximately 20,000 ungated events were collected and presented as histograms.

***In Vitro* Human Invasion (Amgel) Bioassay System.** The characteristics of Amgel have been described previously. Briefly, Amgel is a processed yet physiological human ECM derived from a nontumor tissue source, acellular amnions (18). The major components of Amgel include collagens I and IV, laminin, entactin, tenascin, and heparin sulfate proteoglycan. Neither the growth factors epidermal growth factor, platelet-derived growth factor, TGFβ1, TGFβ2, and fibroblast growth factor nor the M_r 72,000/M_r 92,000 metalloproteases (type IV collagenases/gelatinases) are detectable in this biomatrix. Amgel maintains a stable gel-like matrix architecture both at 4 and 37°C. Amgel-based coculture systems thus mimic a "defined" human cell-matrix environment and allow dissection of tumor cell adhesion, migration, and matrix-degradation steps of the invasion cascade (19–21).

The invasion assay protocol used Amgel-coated inert filters (8-μm pore size; Poretics Corp., Livermore, CA) as an extracellular membrane barrier sandwiched between custom-made 6-mm diameter Lucite chambers. Details of this 72-h assay, including the coating of Amgel onto filters and the incubation and recovery of [^{3}H]thymidine-labeled cells, have been described by Siegal *et al.* (18). Briefly, stock Amgel (4 mg of protein/ml) was prepared in our laboratory, diluted with 1 × PBS and 1 M HEPES (1:1.05, v/v), and coated onto the filters. The Amgel-coated filters were then rehydrated for 1 h in serum-free medium using constant agitation. Cells growing in logarithmic phase were labeled with 1 μCi/ml [^{3}H]thymidine (60 Ci/mmol) for 24 h and washed thoroughly to remove
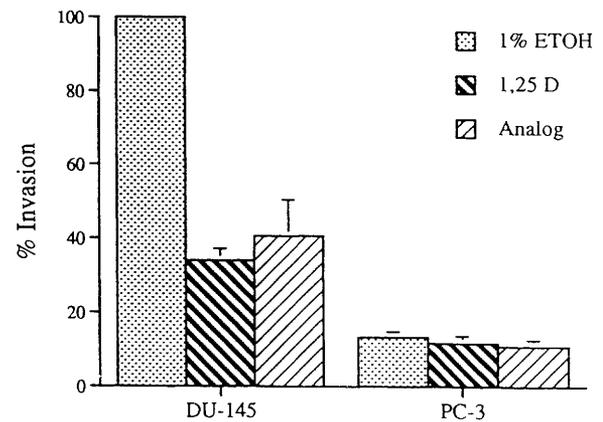

Fig. 1. Invasiveness of DU 145 and PC-3 cells treated with 1×10^{-7} M 1,25 D or 16-23-D$_3$. Control invasion of DU 145 cells was set at 100%. *Columns,* means of three experiments performed in quadruplicate; *bars,* SE.

unincorporated radioactivity, and their specific gravity was determined.

Labeled cells (7×10^4) that were resuspended in 0.4 ml of RPMI with 0.1% BSA were seeded onto rehydrated Amgel filters (70 μg of protein), and the lower chambers were filled with 2.5 ml of medium containing 5% dialyzed fetal bovine serum. Cells were incubated at 37°C for 6 h to foster cell attachment. The following day, the medium in the chambers was replaced with medium containing various concentrations of 1,25 D or 16-23-D$_3$ (1×10^{-7}–1×10^{-13} M). The medium in the top chambers was replaced at 24- and 48-h time intervals with fresh medium containing the test compounds, and incubation was continued for a total of 72 h. The cells traversing through the Amgel-coated filters were collected by treatment of lower chambers and the underside of the coated filters with 0.25% trypsin, followed by 1× PBS washes after trypsinizing. The cells from the lower chamber (*i.e.*, cells treated with initial medium, a trypsin wash, and PBS washes) were collected by vacuum filtration onto a 0.45-μm Millipore filter to resolve cell-bound and free radioactivity. The radioactivity of filter-bound cells was measured by liquid (β) scintillation counting. Percentage of invasion was determined by formula as follows: postcounting/precounting × 100. This method of quantitation provided highly reproducible results and proved advantageous over other techniques of cell counting.

Gelatin Zymography. Tumor cells were grown to confluence, washed twice with 1 × PBS, and then cultured in serum-free medium for 48 h in the presence or absence of 1×10^{-7} M 1,25 D or 16-23-D$_3$. Conditioned media were concentrated by a 0–60% ammonium sulfate precipitation, dialyzed, and dissolved in SDS-PAGE buffer without a reducing agent. Samples (40 μg of protein), without heating, were resolved on 10% separation/4% stacking acrylamide gels copolymerized with gelatin (0.1%). HT-1080 human fibrosarcoma cells were used as a positive control. Gels were run at a constant current and voltage (40 mA and 150 V) for 3 h at 4°C. SDS was removed by rinsing the gels in 2.5% Triton X-100, followed by an overnight incubation in 50 mM CaCl$_2$, 2 μM ZnCl$_2$, and 1% Triton X-100 at room temperature. Finally, gels were stained with 0.1% amido black, destained, and analyzed by densitometry. The molecular weight markers were always reduced and included on the same gels for the estimation of size/position.
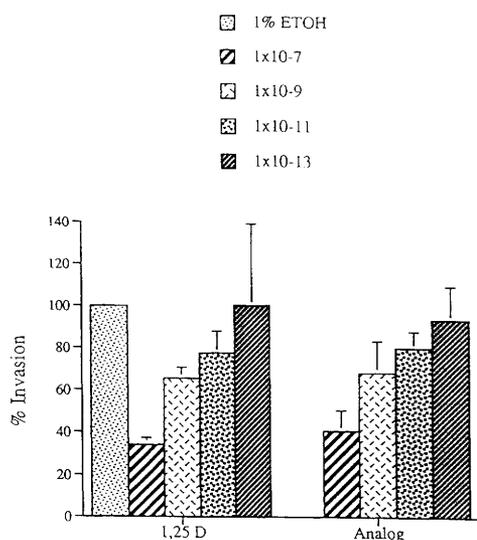
Fig. 2. Invasiveness of DU 145 cells that were exposed to different concentrations of 1,25 D and 16-23-D$_3$. As the concentration of the two compounds decreased, the invasion rates increased, reaching the control level at 1×10^{-13} M. *Columns*, means of five experiments performed in quadruplicate; *bars*, SE.

Fig. 3. Growth curve of DU 145 cells in the presence of 1×10^{-7} M 1,25 D, 1×10^{-7} M 16-23-D$_3$, or ethanol vehicle. Compared to the control, even at this high concentration, neither 1,25 D nor 16-23-D$_3$ had any effect on the growth rate of DU 145 cells over the 3-day time course. *Data points*, means of two experiments performed in duplicate.

Results

Three well-characterized human prostate adenocarcinoma cell lines were exposed to 1×10^7 M 1,25 D or 1×10^7 M 16-23-D$_3$, and their invasive ability was determined by use of a 72-h *in vitro* invasion assay. As seen in Fig. 1, 1,25 D and 16-23-D3 decreased the invasion rate of DU 145 cells (mean $\pm$ SE) to 34.0 $\pm$ 3.20% and 40.6 $\pm$ 9.84%, respectively. In comparison with DU 145 cells, PC-3 cells had an invasion rate through Amgel of only 13.3 $\pm$ 1.57%. In the presence of an equal concentration of 1,25 D and 16-23-D3, this invasive ability was reduced minimally, to 11.6 $\pm$ 2.04% and 10.8 $\pm$ 1.76%, respectively. Additionally, no significant changes were noted in the presence of the test compounds with LNCaP tumor cells (data not shown).

Because 1,25 D and its synthetic analogue selectively inhibited the invasive ability of human prostate cancer cells, we next sought to determine whether this response was dose dependent. In Fig. 2, we demonstrate that, indeed, this response was sensitive to dosage manipulation with either compound, with the response being completely abated at 1×10^{-13} M. (For reference, 10^{-7} M 1,25 D is a pharmacological dose; 10^{-10} M is a physiological dose; and 10^{-13} M is a subphysiological dose.) The anti-invasive effect was also time dependent, with maximal inhibition at 72 h (data not shown).

Because previous studies have demonstrated an antiproliferative effect of 1,25 D and various analogues, growth curves and flow cytometric analysis were performed to determine whether the anti-invasive effects we observed could be accounted for either in altered proliferative rates or modulation of the cell cycle. As seen in Fig. 3, DU 145 cell counts as determined over 72 h show nearly identical growth kinetics in the presence or absence of drug. The results of automated cell counting were superimposable on the results obtained by scintillation counting of cell-bound radioactivity. Similarly, PC-3 and LNCaP exhibited nearly identical results (data not shown).

When cells were incubated for greater than 24 h in either 1,25 D or 16-23-D$_3$ and subjected to fluorescence-activated cell sorting analysis, the thre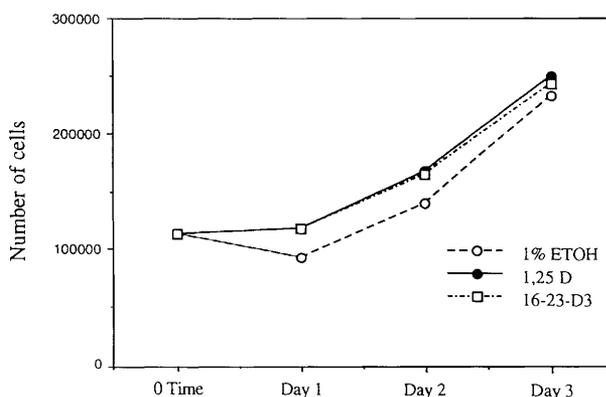e histograms were again nearly superimposable. The DU 145 cells were determined to be diploid with a G$_0$-G$_1$ corresponding to 56.3 $\pm$ 1.6%, a G$_2$-M of 17.1% $\pm$ 0.5%, and a total percent S-phase fraction (representing the actively dividing cells) of 26.7 $\pm$ 1.9% (Fig. 4). The coefficient of variation was very narrow, at 3.58 $\pm$ 0.07%. Therefore, we conclude that 1,25 D and 16-23-D3 did not alter the growth rates or the cell cycle parameters in DU-145, PC-3, and LNCaP cell lines under short-term (48 h) experimental conditions. Thus, neither cell cycle nor growth rate effects can account for the dramatic results obtained in the Amgel invasion assays.

Last, in an attempt to understand the mechanism whereby 1,25 D and its congener modulate the invasive phenotype of DU 145 cells, we examined the secreted type IV collagenase levels by zymography in the presence or absence of the two compounds. In Fig. 5, the gelatinolytic activity of MMP-2 and MMP-9 were seen to be markedly decreased in the presence of both drugs, supporting the hypothesis that calcitriol and its synthetic analogue inhibit MMP activity, which, in turn, reduces proteolytic digestion of the ECM, resulting in decreased invasive ability.

Discussion

The principal clinical problem in prostate cancer is metastatic disease (22). Because invasion of the basement membrane is a critical step in the metastatic cascade, agents that modify the invasive phenotype have obvious potential as anticancer drugs. The present study demonstrates that 1,25 D and the synthetic analogue 16-23-D$_3$ significantly inhibit the invasive phenotype of human prostate cancer cells. This inhibition of invasion was independent of any effect on cell proliferation and was associated with a decrease in the production of proteolytic enzymes.

The ability of a cell to metastasize *in vivo* is highly complex and multifactorial. It has been shown in multiple systems, however, that, compared to poorly metastatic clones, highly metastatic cell populations often demonstrate increased invasive ability. Invasive cell behavior has been represented as a three-step model. Step 1 involves tumor cell attachment to basement membrane via cell surface receptors, such as the laminin receptor. Step 2 involves the proteolytic degradation of the matrix by metalloproteinases or other enzymes, and step 3 involves the active migration of cells through the disintegrated ECM to distant sites (23, 24).
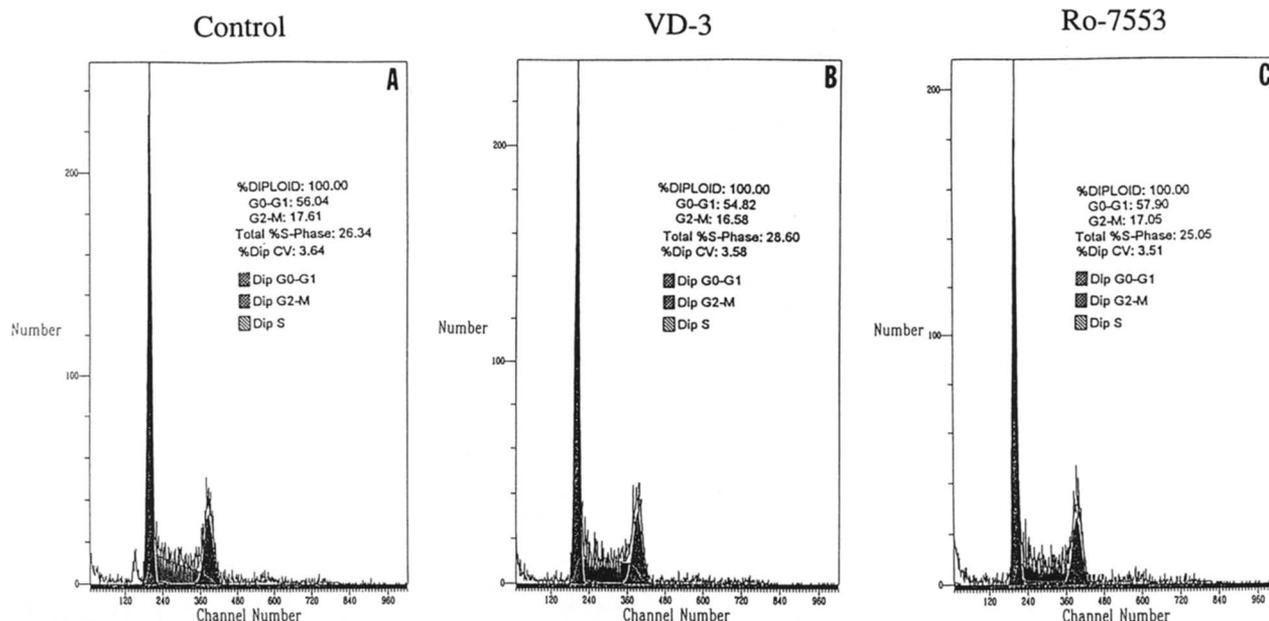
Control

VD-3

Ro-7553

Fig. 4. Effect of 1,25 D and 16-23-D_3 on cell cycle kinetics of human prostate DU 145 adenocarcinoma cells. Cells were incubated with or without 1×10^{-7} M 1,25 D or 1×10^{-7} M 16-23-D_3 for 48 h and subjected to fluorescence-activated cell sorting analysis. G_0-G_1 (2C DNA) and G_2-M (4C DNA) cell cycles are shown, as is the S-phase population representing the actively replicating cells. No difference is apparent among the three groups.
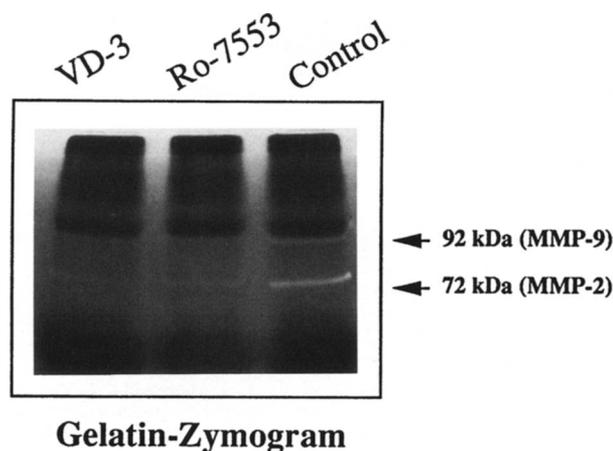

Gelatin-Zymogram

Fig. 5. Effect of 1,25 D and 16-23-D_3 on type IV collagenase (MMP-2/MMP-9) expression and secretion. DU 145 tumor cells were cultured in serum-free medium for 48 h with or without 1×10^{-7} M 1,25 D or 1×10^{-7} M 16-23-D_3. Concentrated medium was analyzed by gelatin-embedded SDS-PAGE zymography. *Clear bands*, gelatinolytic activity of MMP-2 (M_r 72,000 collagenase) and MMP-9 (M_r 92,000 collagenase).

Matrix metalloproteinases, especially M_r 72,000 (MMP-2) and M_r 92,000 (MMP-9) type IV collagenases, are candidate proteases that are thought to play a central role in tumor invasion and metastasis by acting at step 2 of invasion (see above; Ref. 25). MMPs are initially produced as proenzymes, and only after activation are they secreted into the extracellular milieu. MMP-2 is known to localize to invasive cell membrane surfaces secondary to its direct binding to members of the integrin cell adhesion protein family (26). We used 0.1% gelatin-based zymography to measure the levels of M_r 72,000 and

M_r 92,000 gelatinases in serum-free conditioned medium, having previously demonstrated a close relationship between MMP-2 activity and the invasive index of tumor cells as seen in the Amgel assay. Recently, Stearns and Stearns (27, 28) reported that the activated type IV collagenase, MMP-2a, was expressed in prostate cancer and that increased expression was associated with neoplastic progression. In a series of studies using SDS-PAGE, Western blotting, zymography, quantitative ELISAs, and quantitative computer-assisted imaging analysis of immunohistochemically stained sections, increasing levels of MMP-2a were identified as the Gleason grade of the prostate cancer increased, indicating an inverse relationship between differentiated state and enzyme level.

A number of studies have used intact human basement amnion or Matrigel-based reconstituted basement membrane in *in vitro* invasion assays systems to study invasive ability of prostate and other transformed human cells (29, 30). Hoosein *et al.* (31) reported that the presence of cell surface urokinase receptors on PC-3 and DU 145 cells correlated with high invasive ability through short-term exposure to Matrigel, whereas LNCaP cells, which lack urokinase receptors, had poor invasive ability. However, Matrigel has several limitations with regard to its use in human systems. First, Matrigel is prepared from malignant mouse (EHS) tumor and contains numerous growth and differentiation factors as well as proteases. The presence of these factors makes the interpretation of observed cell-matrix interactions more complex and the subject of controversy (32–34). Second, Matrigel contains little or no types I and IV collagen, the essential footprint for epithelial cell-ECM interactions. Unlike Matrigel, Amgel represents a predominately growth factor- and collagenase-free human ECM environment. Thus, we believe that the use of Amgel represents a methodological advance over Matrigel-based assay systems for the study of human cancer cell invasion.

In addition to 1,25 D, other steroids (*e.g.*, estradiol-17β

and progesterone) and growth factors (epidermal growth factor, TGFα, and TGFβ) have been demonstrated to influence migration and invasion of human transformed cells, at least in part, by up- or down-regulating type IV collagenases (35). Interestingly, it has been reported that extracellular organelles produced by cells during matrix mineralization contain metalloproteinases that are regulated by vitamin D metabolites (36). This suggests that vitamin D may play a fundamental role in cell-matrix interactions.

Our observations in human prostate cancer cells are similar to those of Hansen *et al.* (37), who demonstrated a dose-dependent inhibition of both migration and invasive ability of MDA-MB-231 human breast cancer cells *in vitro* in the presence of 1,25 D over a 4-day period in which no antiproliferative effect was noted (37). Similarly, Young *et al.* (38) have shown that 1,25 D reduces tumor metastasis and recurrence in mice carrying Lewis lung carcinoma (38). Further, they presented evidence that this was accomplished through modulation of the myelopoiesis-associated immunosuppressor cascade (39). This is not to say that 1,25 D is not antiproliferative. In fact, at least six recent publications have presented evidence of such an effect (11, 13, 15, 16). Rather, it was the specialized circumstances of experimental conditions linked to short-term exposures that allowed the separation of anti-invasive from antiproliferative effects to be examined in this study.

A logical next step from this *in vitro* study would be to use vitamin D analogues in an *in vivo* model of metastatic prostate cancer. Such a study is presently underway in the authors' laboratory (40). However, this work is tempered by the knowledge that the clinical use of oral 1,25 D (1.5 µg p.o.) in the treatment of metastatic prostate cancer has been explored in a Phase II trial (41). No objective responses were observed (defined as a 50% sustained decrease in serum prostate-specific antigen). However, two men experienced transient decreases in prostate-specific antigen of 25 and 45%. The major toxicity was hypercalcemia. These findings suggest that, in at least some men, 1,25 D is bioactive in advanced prostate cancer and that a "noncalcemic" analogue of 1,25 D may be an attractive candidate for future clinical trials.

In summary, we report that 1,25 D and a vitamin D analogue significantly inhibit the invasion of DU 145 human prostate cancer cells in a dose-dependent fashion. Consistent findings demonstrating inhibition of prostate cancer cell invasion in Matrigel by 1,25 D has recently been reported (in an abstract) by other investigators.[4] The anti-invasion effect we observed is apparent at physiological levels of 1,25 D (*i.e.*, 10^{-10} M) and is correlated with a decreased ability of prostate cancer cells to manufacture matrix-degrading proteases. We conclude that the antitumor actions of 1,25 D in human prostate cancer involves, in part, the inhibition of cell invasiveness. These findings support the hypothesis that 1,25 D reduces the risk of invasive prostate cancer and suggest a potential role for vitamin D compounds in the chemoprevention of invasive prostate cancer (42).

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