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PTHrP contributes to the anti-proliferative and integrin $\alpha 6 \beta 4$ -regulating effects of 1,25-dihydroxyvitamin D₃*

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Abstract

Parathyroid hormone-related protein (PTHrP) increases the growth and metastatic potential of prostate cancer cells, making it important to control PTHrP expression in these cells. 1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] suppresses PTHrP expression and exerts an anti-proliferative effect in prostate carcinoma cells. We used the human prostate cancer cell line C4-2 as a model system to ask whether down-regulation of PTHrP expression by 1,25(OH)₂D₃ plays a role in the anti-proliferative effects of 1,25(OH)₂D₃. Since PTHrP increases the expression of the pro-invasive integrin $\alpha 6 \beta 4$, we also asked whether 1,25(OH)₂D₃ decreases integrin $\alpha 6 \beta 4$ expression in C4-2 cells, and whether modulation of PTHrP expression by 1,25(OH)₂D₃ plays a role in the effects of 1,25(OH)₂D₃ on integrin $\alpha 6 \beta 4$ expression. Two strategies were utilized to modulate PTHrP levels: overexpression of PTHrP (−36 to +139) and suppression of endogenous PTHrP expression using siRNAs. We report a direct correlation between PTHrP expression, C4-2 cell proliferation, and integrin $\alpha 6 \beta 4$ expression at the mRNA and cell surface protein level. Treatment of parental C4-2 cells with 1,25(OH)₂D₃ decreased cell proliferation and integrin $\alpha 6$ and $\beta 4$ expression. These 1,25(OH)₂D₃ effects were significantly attenuated in cells with suppressed PTHrP expression. 1,25(OH)₂D₃ regulates PTHrP expression via a negative vitamin D response element (nVDRE) within the noncoding region of the PTHrP gene. The effects of 1,25(OH)₂D₃ on cell proliferation and integrin $\alpha 6 \beta 4$ expression were significantly attenuated in cells overexpressing PTHrP (−36 to +139), which lacks the nVDRE. These findings suggest that one of the pathways via which 1,25(OH)₂D₃ exerts its anti-proliferative effects is through down-regulation of PTHrP expression.

Keywords

Steroids; PTHrP; 1,25-dihydroxyvitamin D; integrin $\alpha 6 \beta 4$; prostate cancer

Introduction

Prostate cancer is the most common solid tumor in American men and the second leading cause of cancer death. The incidence of prostate cancer is rising annually (1). Nearly 70% of prostate cancer patients develop bone lesions, and skeletal metastasis is a major cause of morbidity

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(2). The prostate is highly dependent on androgens for normal developmental and physiological functions. However, additional factors, including growth factors, neuroendocrine peptides, and cytokines also play important roles in this organ (3); one of these factors is parathyroid hormone-related protein (PTHrP). PTHrP was initially identified through its role in humoral hypercalcemia of malignancy, one of the most frequent paraneoplastic syndromes (4). Subsequently, PTHrP was found to be widely distributed in most fetal and adult tissues, including the prostate. PTHrP has been localized to normal neuroendocrine cells and the glandular epithelium of normal and benign prostatic hyperplasia (BPH) tissues (5,6). Cultured epithelial cells derived from normal and BPH tissues, and immortalized prostate cancer cell lines secrete PTHrP (7,8). The progression of normal prostate epithelium to BPH and then to carcinoma is accompanied by an increase in PTHrP expression (9,10). Several studies have demonstrated that PTHrP contributes to the pathogenesis and progression of prostate carcinoma and its preference to metastasize to bone (11,12). It is therefore clinically relevant to develop agents that control PTHrP production by these cells.

Vitamin D deficiency has been linked with increased prostate cancer incidence (reviewed in 13). 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the hormonally active form of vitamin D, inhibits cell proliferation in many cancer cell types, including prostate carcinoma cells (14–16). We and others have shown that PTHrP increases prostate cancer cell proliferation (17, 18). Here we asked whether down-regulation of PTHrP expression by 1,25(OH)₂D₃ plays a role in the observed effects of 1,25(OH)₂D₃ on cell proliferation. The C4-2 cell line, an androgen independent, second generation LNCaP subline which metastasizes to lymph nodes and bone when injected into nude mice (19), was used as a model system. C4-2 cells produce mixed lytic/blastic lesions (20), thereby mimicking the *in vivo* situation.

PTHrP increases the expression of the pro-invasive integrin $\alpha 6 \beta 4$ in multiple cell lines (21–23). Integrin $\alpha 6 \beta 4$ is an adhesion receptor for most of the known laminins, and contributes to the malignant functions of carcinoma cells (24,25). 1,25(OH)₂D₃ decreases integrin $\alpha 6$ and $\beta 4$ expression in prostate cancer cells (26). Therefore, we also asked whether down-regulation of PTHrP expression by 1,25(OH)₂D₃ plays a role in the observed effects of 1,25(OH)₂D₃ on integrin $\alpha 6$ and $\beta 4$ levels.

Experimental

Materials

1,25(OH)₂D₃ was provided by Dr. M. Uskokovic (Hoffmann La-Roche, Nutley, New Jersey, USA), and was dissolved in ethanol at 10⁻⁴ M. Fetal bovine serum (FBS) and dialyzed FBS were obtained from Atlanta Biologicals (Norcross, GA). The phycoerythrin-conjugated anti-integrin $\alpha 6$ and $\beta 4$ antibodies, and their isotype controls were obtained from BD PharMingen (San Diego, CA). The unconjugated anti-integrin $\alpha 6$ and $\beta 4$ antibodies and the GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNAs targeting PTHrP and the corresponding non-specific siRNAs were purchased from Dharmacon (Lafayette, CO).

Plasmid constructs

A cDNA encoding human PTHrP (Genentech, South San Francisco, CA, USA) was cloned into pEGFP-N1 (Clontech, Mountain View, CA). Two independent PTHrP siRNA sequences (GAGCUGUGUCUGAACAUCAU and GAUCGCAGAAAUCCACAC AUU) targeting the open reading frame of the human PTHrP gene were each cloned into pSilencer 2.1 U6 neo (Ambion, Austin, TX). Similarly cloned scrambled siRNA sequences served as controls. All constructs were transfected into C4-2 cells by electroporation.

Cell culture and transfection

C4-2 cells were purchased from UroCor (Oklahoma City, OK) and were grown at 37 °C in humidified 95% air/5% CO₂ in RPMI 1640 medium containing 10% FBS and L-glutamine. In some experiments conventional FBS was replaced with dialyzed FBS to minimize cell exposure to endogenous steroids present in serum. After transfection, individual clones were isolated as described (23).

Treatment with 1,25-dihydroxyvitamin D₃

To measure the effects of 1,25(OH)₂D₃ on cell proliferation and on PTHrP and integrin expression, cells were maintained in medium containing 10% dialyzed FBS for 4 days. The cells were then treated with the indicated concentrations of 1,25(OH)₂D₃ for the indicated time intervals. Control cells received an equivalent volume of vehicle (ethanol; 0.01% final concentration).

Analysis of PTHrP and integrin expression

PTHrP secretion was measured using an immunoradiometric assay (IRMA; DSL, Webster, TX) (18). Expressed mRNAs were measured by reverse transcription/real-time PCR analysis, and cell surface integrin α 6 and β 4 protein levels in PTHrP siRNA transfectants were measured by Fluorescence-Activated Cell Sorting (FACS) analysis. Since the presence of Green Fluorescent Protein (GFP) interfered with FACS analysis, integrin levels in PTHrP-overexpressing cells were analyzed by Western blot analysis.

Real-time Polymerase Chain Reaction Analysis of PTHrP, Integrin α 6, and Integrin β 4 Gene Expression

Total RNA from C4-2 cells was extracted using the RNeasy[®] isolation kit (Ambion Inc.), per the manufacturer's protocol. RNA concentrations were determined by spectrophotometry.

Relative quantification of gene expression—Separate tube (singleplex) real-time PCR was performed using 0.5 μ g of RNA as template to detect PTHrP, integrin α 6 and integrin β 4 mRNA transcripts and 18S rRNA (endogenous control for normalization purposes). PTHrP primers (Assays-on-Demand[™], P/N 4331182, 20X assay mix of primers), the PTHrP TaqMan MGB probe (FAM[™] dye-labeled, PTHLH (parathyroid hormone-like hormone), NM 002820, Hs00174969 m1: CGCCGCCTCAAAAGAGCTGTGTCTG), integrin α 6 primers (ITGA6, NM 000210, X59512, X53586, BC007002 Hs00173952_m1 AAGCGGCTGTTGCTCGTGGGGGCC), integrin β 4 primers (ITGB4, NM_001005619, NM_001005731, NM_000213, X51841, X52186, AF011375, Hs00173995_m1 GGTTCCTCTGCAATGACCGAGGACG) and the pre-developed 18S rRNA primers (VIC[™]-dye labeled probe, TaqMan[®] assay reagent, P/N 4319413E) were obtained from Applied Biosystems, as was the universal PCR master mix reagent kit (P/N 4304437). The cycling parameters for real-time PCR were: UNG activation at 50 °C for 2 min, AmpliTaq activation at 95 °C for 10 min, then denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min (repeated 40 times) on an ABI7000 real-time PCR machine. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalization to the endogenous reference (18S). Real-time PCR was performed using the facilities of UTMB's Sealy Center for Cancer Cell Biology's Real-Time PCR core facility; <http://www.utmb.edu/sccc/pcr/index.htm>. As negative controls, we carried out PCR reactions without RNA.

FACS analysis

The cell surface expression of the integrin $\alpha 6$ and $\beta 4$ subunits was measured by staining cells with the phycoerythrin-labeled antibodies to these subunits. As control, cells were stained with the respective isotype control antibodies. For these experiments, 2×10^5 cells were trypsinized, washed with PBS, and incubated on ice with the specific antibody for 20 min. The cells were then washed twice with PBS containing 2% newborn calf serum (NCS) and 0.02% sodium azide, and the mean fluorescence intensity (MFI) was measured with a FACS Scan flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ). Viable cells were electronically gated using forward and side scatter parameters. Relative integrin expression was calculated after subtracting the log MFI of the corresponding isotype control antibody-stained cells. Flow cytometry data were analyzed using the CellQuest program (Becton Dickinson and Co.).

Western blot analysis

Cells were grown to 70–80% confluence in 100 mm dishes. For whole cell extracts, cells were washed twice with cold PBS on ice and lysed in RIPA buffer containing a Protease Inhibitor cocktail (Santa Cruz Biotechnology). To enrich for cytosol and membrane fractions, cells were washed and lysed as described for whole cells extracts. The cell lysates were centrifuged twice at $1000 \times g$ for 5 min at 4 °C. The supernatants from each step were pooled and centrifuged at $13,000 \times g$ for 30 min at 4 °C. The supernatant (cytosol fraction) was collected, and the membrane material in the pellet was resuspended in 1:1 RIPA buffer and MB-EGTA (210 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 10 mM HEPES) (27). Total protein was estimated using the Bio-Rad protein assay.

For Western analysis, 60 μ g of protein were resolved on 8% SDS polyacrylamide gels and subsequently transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Sanford, ME). The membranes were incubated with the anti-integrin $\alpha 6$ or $\beta 4$ antibodies (Santa Cruz Biotechnology) diluted in blocking buffer [5% non-fat dry milk in TBST]. After three washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The signals were detected using the SuperSignal West Pico Substrate kit (Pierce Biotechnology, Rockford, IL). The membranes were then stripped with Restore Western Blot Stripping buffer (Pierce), washed 4 times with TBST buffer, and re-probed with an anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology).

Cell Proliferation

Cell proliferation was measured using the Quick Cell Proliferation Assay kit (Biovision Inc., Mountain View, CA). Cells (1×10^4) were plated in 96-well dishes in a final volume of 100 μ l/well of medium containing 10% FBS. At various time points (24 to 96 h), WST-1/ECS solution (20 μ l), prepared by dissolving the WST-1 reagent with Electro-Coupling solution (ECS), was added to each well. After incubating at 37 °C in a humidified 95% O₂-5% CO₂ atmosphere for 1 h, the plates were shaken thoroughly for 1 min, and absorbance was measured using a SpectraMax M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 450 nm. Culture medium plus WST-1/ECS solution was used as the empty blank control.

Statistics

Numerical data are presented as the mean $\pm$ SEM. Data were analyzed by ANOVA, followed by a Bonferroni post-test to determine the statistical significance of differences. All statistical analyses were performed using Instat Software (GraphPad Software, Inc., San Diego, CA).

Results

PTHrP up-regulates integrin $\alpha 6$ and $\beta 4$ expression

To measure the effects of PTHrP on integrin $\alpha 6$ and $\beta 4$ expression, we modulated PTHrP expression in C4-2 cells using two strategies: PTHrP-overexpressing cells were established by stable transfection with a PTHrP (−36 to +139) expression vector, and suppression of PTHrP expression was achieved by stable transfection with a construct expressing an siRNA targeting the open reading frame of the human PTHrP gene. For PTHrP-overexpressing cells, the data presented were obtained using three independent clones. In these cells, PTHrP was expressed as a PTHrP-GFP fusion protein, to allow visualization of the subcellular localization of PTHrP. To eliminate site-specific effects of the PTHrP siRNA, two siRNA sequences targeting different regions of the PTHrP gene were used to generate two independent cell lines with suppressed PTHrP expression. Transfection with these constructs produced the expected changes in PTHrP mRNA and secreted protein levels (Fig. 1, A and B). In PTHrP-overexpressing cells, PTHrP was localized in both cytosolic and nuclear compartments (data not shown). As control, cells were transfected with the empty vector or with constructs expressing the scrambled siRNA sequences.

We observed that increasing PTHrP expression results in elevated integrin $\alpha 6$ and $\beta 4$ mRNA levels. Conversely, decreasing PTHrP expression results in decreased integrin $\alpha 6$ and $\beta 4$ mRNA levels (Fig. 1A). Integrins exert their biological effects at the cell surface membrane. Integrin $\alpha 6$ and $\beta 4$ protein levels in PTHrP-overexpressing cells co-expressing GFP were measured by Western blot analysis, since the GFP fluorescence interferes with FACS. Overexpressing PTHrP in C4-2 cells produced a significant increase in the total cellular and membrane protein levels of these integrins when compared to control cells (Fig. 2, A and B). Since the integrin $\beta 4$ subunit undergoes proteolytic processing (28,29), multiple bands are evident (Fig. 2, A and B). The cytosol fraction contains fewer post-translational products (Fig. 2C). Interestingly, in extracts enriched in the cytosolic fraction, integrin $\beta 4$ levels are still elevated in PTHrP-overexpressing vs. control cells. However, integrin $\alpha 6$ levels are lower (Fig. 2C). Transfection with the PTHrP siRNA-expressing construct significantly decreased integrin $\alpha 6$ and $\beta 4$ cell surface protein levels to ~ 40% of control (Fig. 2, D and E), as measured by FACS. In all FACS analysis data, there was no significant difference in the MFI of the siRNA and scrambled RNA transfectants stained with the isotype control antibodies (data not shown).

PTHrP expression is required for the 1,25(OH) $_2$ D $_3$ –mediated down-regulation of integrin $\alpha 6$ and $\beta 4$ expression

Fig. 3, A and B shows that treatment with 1,25(OH) $_2$ D $_3$ produces a concentration- and time-dependent decrease in integrin $\alpha 6$ and $\beta 4$ mRNA levels. The decrease in integrin mRNA levels lags behind that of PTHrP; while PTHrP mRNA levels were significantly decreased after a 24 h treatment with 10^{−8} M 1,25(OH) $_2$ D $_3$, no significant effect on integrin $\alpha 6$ and $\beta 4$ levels was observed until 48 h of treatment (Fig. 3B). Down-regulation of integrin $\alpha 6$ and $\beta 4$ mRNA levels is accompanied by a decrease in the cell surface protein expression (Fig. 3C).

Since 1,25(OH) $_2$ D $_3$ down-regulates PTHrP expression, and PTHrP up-regulates integrin $\alpha 6$ and $\beta 4$ expression, we asked whether down-regulating PTHrP expression by 1,25(OH) $_2$ D $_3$ plays a role in the observed effects of this compound on integrin expression. 1,25(OH) $_2$ D $_3$ (10^{−8} M) produced a significant and comparable decrease in PTHrP mRNA levels (Fig. 4A) and secretion (Fig. 5A) in cells with suppressed PTHrP expression and in control cells (~ 65% decrease), indicating that this steroid produces comparable decreases in target protein levels when these proteins are expressed at differing levels in the cell, as is the case in PTHrP siRNA and scrambled siRNA transfectants. However, the 1,25(OH) $_2$ D $_3$ - mediated decrease in integrin mRNA levels was significantly lower in PTHrP siRNA transfectants (~ 25% decrease) than in

control cells (~ 65% decrease) (Fig. 4, B and C). Similarly, treatment with $1,25(\text{OH})_2\text{D}_3$ produced a significantly greater decrease in cell surface integrin $\alpha 6$ and $\beta 4$ expression in control cells (~ 65% decrease) than in PTHrP siRNA transfectants (~ 20% decrease) (Fig. 5, B and C). Differences in the response of PTHrP siRNA transfected and control cells to $1,25(\text{OH})_2\text{D}_3$ were also observed at concentrations of 10^{-10} M and 10^{-9} M (data not shown).

To further address the role of PTHrP in the $1,25(\text{OH})_2\text{D}_3$ -mediated down-regulation of integrin $\alpha 6$ and $\beta 4$ expression, C4-2 cells overexpressing PTHrP (−36 to +139) were treated with $1,25(\text{OH})_2\text{D}_3$. Down-regulation of PTHrP expression by $1,25(\text{OH})_2\text{D}_3$ is mediated via a negative vitamin D response element (nVDRE) at position −546 to −517 relative to the transcriptional start site of the PTHrP gene (30,31). Since this and other promoter elements are not present within the transfected PTHrP sequence, PTHrP expression is not regulated by $1,25(\text{OH})_2\text{D}_3$ in cells overexpressing this PTHrP isoform (Fig. 4A). In control (vehicle-treated) cells, $1,25(\text{OH})_2\text{D}_3$ produced a concentration-dependent decrease of 60% to 70% in PTHrP mRNA levels (Fig. 4A, data not shown). This was accompanied by an ~ 60% decrease in integrin $\alpha 6$ and $\beta 4$ mRNA levels (Fig. 4, B and C). However, under the same conditions, the effect of $1,25(\text{OH})_2\text{D}_3$ on integrin $\alpha 6$ and $\beta 4$ expression in PTHrP-overexpressing cells was significantly attenuated (~10% to 15% decrease, Fig. 4, B and C). Therefore, these data re-enforce those obtained with the PTHrP siRNA transfectants, and show that regulation of PTHrP expression by $1,25(\text{OH})_2\text{D}_3$ plays a major role in the regulation of integrin $\alpha 6$ and $\beta 4$ expression by $1,25(\text{OH})_2\text{D}_3$.

PTHrP plays a role in the anti-proliferative effects of $1,25(\text{OH})_2\text{D}_3$

We observed a direct correlation between PTHrP expression and C4-2 cell proliferation, measured over a 5-day period (Fig. 6A). Conversely, $1,25(\text{OH})_2\text{D}_3$ decreases C4-2 cell proliferation [Fig. 6B; (32)]. However, this compound exerts a significantly smaller effect on the proliferation of C4-2 cells transfected with the PTHrP siRNA (Fig. 6B). Thus, a 5-day treatment with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ decreased the proliferation of scrambled siRNA-transfected cells to 15% of control (Fig. 6B). The proliferation rate of cells transfected with the PTHrP siRNA was decreased to only 60% of control (Fig. 6B).

As presented in Fig. 4A, $1,25(\text{OH})_2\text{D}_3$ does not down-regulate transfected PTHrP (−36 to +139) expression in C4-2 cells. Here we also investigated the effect of $1,25(\text{OH})_2\text{D}_3$ on the proliferation of cells overexpressing PTHrP (−36 to +139). Treatment of control cells with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ for 5 days decreased cell proliferation to ~ 15% of control (Fig. 6B). Under the same conditions, the proliferation of PTHrP-overexpressing cells by $1,25(\text{OH})_2\text{D}_3$ was only decreased to ~ 60% of control (Fig. 6B).

Discussion

There is considerable evidence supporting a link between vitamin D deficiency and an increased incidence of prostate cancer (reviewed in 13). $1,25(\text{OH})_2\text{D}_3$, the hormonally active form of vitamin D, regulates cell growth and differentiation (33). Pathways which contribute to the anti-proliferative effects include the induction of cell cycle arrest through increased p21 and p27 expression, and regulation of growth factors such as insulin-like growth factor binding protein-3, transforming growth factor- β and cyclooxygenase-2 (34–37). $1,25(\text{OH})_2\text{D}_3$ also inhibits tumor cell migration, metastasis, and angiogenesis (34,35).

We and others have shown that PTHrP increases the proliferation of cell lines derived from various tissues, including prostate cancer (17,18,22,38). Conversely, $1,25(\text{OH})_2\text{D}_3$ inhibits cell proliferation (14–16) and decreases PTHrP expression at both the mRNA and secreted protein level (16,32). Our study demonstrates that one of the pathways via which $1,25(\text{OH})_2\text{D}_3$ exerts its anti-proliferative effects is through down-regulation of PTHrP expression, since the anti-

proliferative effects of $1,25(\text{OH})_2\text{D}_3$ are significantly attenuated in cells with suppressed PTHrP expression and in cells where the transfected PTHrP is not under $1,25(\text{OH})_2\text{D}_3$ regulation. PTHrP may act as an intermediate in the observed effects of $1,25(\text{OH})_2\text{D}_3$ on cell cycle control, and may exert its effects either directly and/or indirectly by regulating proteins that are known to affect cell proliferation, such as p21, p27, and transforming growth factor- β (35,37).

In this and previous studies (21–23), we show a direct correlation between PTHrP, and integrin $\alpha 6$ and $\beta 4$ expression at both the mRNA and cell surface protein level. Integrins are comprised of $\alpha\beta$ heterodimers that function as transmembrane receptors. They play a major role in cancer cell behavior, and activate intracellular signaling pathways that regulate cell proliferation, differentiation, apoptosis, motility, and gene expression (39,40). Integrin $\alpha 6\beta 4$ expression has been linked to the progression of breast, thyroid, bladder, colorectal, and gastric tumors, among others (41). Relatively less is known about the role of integrin $\alpha 6\beta 4$ role in prostate cancer, although it has been shown that decreased expression of this integrin is associated with reduced adhesion to laminin, and suppressed Matrigel invasion (42). Since integrin $\alpha 6\beta 4$ exerts a positive effect on cell proliferation (43,44), the PTHrP-mediated up-regulation of integrin expression by PTHrP may play an important role in the observed proliferative effects of PTHrP. In support of this, it has been shown that $\beta 4$ signaling promotes ErbB2-mediated mammary tumor cell proliferation (45), and that ErbB2 is required for C4-2 cell growth (46). In addition, $1,25(\text{OH})_2\text{D}_3$ decreases ErbB2 activity in C4-2 cells (47).

We observed that the increase in membrane-associated integrin $\alpha 6$ expression in PTHrP-overexpressing cells is accompanied by a decrease in cytosolic levels. Integrin $\beta 4$ expression is elevated at both the membrane and cytosol level. The PTHrP-mediated increase in integrin $\alpha 6\beta 4$ cell surface protein expression may thus primarily result from up-regulation by PTHrP of the $\beta 4$ subunit. The increased cell surface $\alpha 6$ levels in PTHrP-overexpressing cells may be dependent on $\alpha 6$ mobilization through heterodimerization with $\beta 4$. However, PTHrP also up-regulates integrin $\alpha 6$ and $\beta 4$ mRNA levels, indicating a direct and/or indirect effect of PTHrP at the transcriptional and/or post-transcriptional level for both subunits.

We also report a direct link between $1,25(\text{OH})_2\text{D}_3$, PTHrP and integrin $\alpha 6\beta 4$ expression. $1,25(\text{OH})_2\text{D}_3$ significantly decreases integrin $\alpha 6\beta 4$ expression in parental C4-2 cells. This effect is significantly attenuated in cells with suppressed PTHrP expression, indicating that down-regulation of PTHrP expression by $1,25(\text{OH})_2\text{D}_3$ plays a major role in the observed effects of $1,25(\text{OH})_2\text{D}_3$ on integrin $\alpha 6$ and $\beta 4$ expression. In support of this, the $1,25(\text{OH})_2\text{D}_3$ -mediated down-regulation of integrin $\alpha 6$ and $\beta 4$ mRNA levels in DU145 and PC-3 cells shows a delayed time-course profile, implying an indirect mechanism of action (26). These data indicate that inhibition of integrin $\alpha 6$ and $\beta 4$ expression by $1,25(\text{OH})_2\text{D}_3$ occurs downstream of PTHrP inhibition - PTHrP is first down-regulated by $1,25(\text{OH})_2\text{D}_3$, leading to a decrease in integrin $\alpha 6$ and $\beta 4$ levels. These observations do not preclude a direct role for $1,25(\text{OH})_2\text{D}_3$ in the regulation of one or both of these integrins. In support, we observe that $1,25(\text{OH})_2\text{D}_3$ does decrease integrin $\alpha 6$ and $\beta 4$ expression in PTHrP siRNA transfectants.

Prostate cancer has a high propensity to metastasize to bone and PTHrP expression by prostate cancer cells facilitates this process (11,12). Prostate cancer metastasis to the bone is comprised of an initial osteolytic phase, followed by a transition phase and an osteoblastic phase (20,48, 49). Since PTHrP plays a positive role in both processes (20,49), down-regulating PTHrP expression effectively inhibits both the lytic and blastic phases of prostate cancer metastasis to the bone. During the osteolytic phase, PTHrP also mediates the release from the bone matrix of growth factors, which in turn provide a major stimulus for cancer cell proliferation (50). Integrin $\alpha 6\beta 4$ also enhances cancer cell migration, invasion and metastasis *in vitro* and *in vivo* (42,51). Since these integrins are regulated downstream of PTHrP, they likely also

contribute to the PTHrP-mediated pathogenesis and progression of prostate and other cancers. These studies therefore stress the potential clinical significance of the observed attenuation of PTHrP expression by 1,25(OH)₂D₃.

In conclusion, in this study we show that one of the pathways via which 1,25(OH)₂D₃ exerts its anti-proliferative effects is through down-regulation of the expression of PTHrP, which is known to increase the proliferation of cells derived from prostate and other tissues. We also show that inhibition of pro-invasive integrin $\alpha\beta 4$ expression by 1,25(OH)₂D₃ occurs downstream of PTHrP inhibition. Since PTHrP plays a major role in prostate cancer growth and metastasis, and most prostate cancers show slow growth initially, these studies underlie the chemopreventive potential of vitamin D to decrease PTHrP expression and its ensuing proliferative effects. Moreover, noncalcemic vitamin D analogues may also be useful in the prevention and treatment of prostate cancer.

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Figure 1. Characterization of C4-2 cells overexpressing PTHrP (–36 to +139) or with suppressed PTHrP expression

(A) PTHrP and integrin $\alpha 6$ and $\beta 4$ mRNA levels were measured by reverse transcription-real-time PCR, as described in *Materials and Methods*. Values are expressed relative to the corresponding V value, set arbitrarily at 1.0. (B) Secreted PTHrP levels, measured using an immunoradiometric assay. + = PTHrP-overexpressing cells; V = empty vector or scrambled siRNA transfectants; – = PTHrP siRNA transfectants. Each bar is the mean $\pm$ SEM of three independent experiments for each of three individual PTHrP-overexpressing clones (+), or each of two C4-2 cell lines transfected with PTHrP siRNA-expressing constructs (–). The V bar is the mean $\pm$ SEM of three independent experiments with pEGFP-N1 (empty vector) or the pSilencer 2.1 U6 neo/scrambled siRNA constructs. * = Significantly different from V ($P < 0.001$).

Figure 2. Integrin $\alpha 6$ and $\beta 4$ protein levels in C4-2 cells overexpressing PTHrP (–36 to +139) or with suppressed PTHrP expression

Levels of the integrin $\alpha 6$ and $\beta 4$ subunits in whole cell (A), membrane (B), and cytosol (C) fractions from PTHrP-overexpressing and control cells. Data for two individual PTHrP-overexpressing (+) and empty vector-transfected (V) clones are presented. Each figure is representative of three independent experiments. (D and E). Cell surface protein expression of integrins $\alpha 6$ and $\beta 4$ in cells transfected with one of two independent PTHrP siRNA expression vectors (si1 and si2). Control cells were transfected with a construct expressing the relative scrambled siRNA sequence (sc1 and sc2). Cells were incubated with phycoerythrin-conjugated anti-integrin $\alpha 6$ or anti-integrin β antibodies, and analyzed by FACS as described in *Materials and Methods*. Each value is expressed relative to the sc value, set at 100%, after subtracting the isotype control background value. Each bar is the mean $\pm$ SEM of three independent experiments. * = Significantly different from sc ($P < 0.001$).

Figure 3. Effect of 1,25-dihydroxyvitamin D₃ on PTHrP, integrin $\alpha 6$, and integrin $\beta 4$ expression in C4-2 cells

(A) Cells cultured in medium containing 10% dialyzed FBS were treated with the indicated concentrations of 1,25(OH)₂D₃ for 4 days. (B) Cells cultured in 10% dialyzed FBS were treated with 10⁻⁸ M 1,25(OH)₂D₃ for the indicated time intervals (days). In (A and B), PTHrP, integrin $\alpha 6$ and integrin $\beta 4$ mRNA levels were measured by reverse transcription/real-time PCR as described in *Materials and Methods*. (C) Cell surface protein expression of the integrin $\alpha 6$ and $\beta 4$ subunits in cells cultured in 10% dialyzed FBS and treated with 10⁻⁸ M 1,25(OH)₂D₃ for 4 days. Cells were stained with phycoerythrin-conjugated antibodies against the $\alpha 6$ or $\beta 4$ integrins, and analyzed by FACS as described in *Materials and Methods*. Each value is expressed as a percentage of the corresponding value in cells treated with ethanol (vehicle control; set at 100%). Each point is the mean $\pm$ SEM of three independent experiments. * = Significantly different from the corresponding vehicle control value ($P < 0.001$).

Figure 4. Effect of 1,25-dihydroxyvitamin D₃ on PTHrP (A), integrin $\alpha 6$ (B), and integrin $\beta 4$ (C) mRNA levels in C4-2 cells with enhanced or suppressed PTHrP expression

Cells cultured in medium containing 10% dialyzed FBS were treated with 10^{-8} M 1,25 (OH) $_2$ D $_3$ for 4 days. + = overexpressing PTHrP; V = empty vector transfectants; si1 and si2 = transfected with each of two independent PTHrP siRNA expression vectors; sc = transfected with a scrambled siRNA expression vector. Treated with: + 1,25D = 1,25(OH) $_2$ D $_3$, - = ethanol (vehicle control). Each value is expressed relative to the V value (empty vector transfectants treated with ethanol), set at 1.0. Each bar is the mean $\pm$ SEM of three independent experiments. Significantly different from the corresponding control (-1,25D) value at: * = $P < 0.001$, and ** = $P < 0.05$.

Figure 5. Effect of 1,25-dihydroxyvitamin D₃ on secreted PTHrP levels (A) and on integrin α6 (B) and integrin β4 (C) cell surface protein expression in C4-2 cells with suppressed PTHrP expression Cells cultured in medium containing 10% dialyzed FBS were treated with 10^{-8} M 1,25 (OH)₂D₃ for 4 days. (A) Secreted PTHrP was measured by an immunoradiometric assay. (B and C) Cells were stained with phycoerythrin-conjugated antibodies against the α6 or β4 integrins, and analyzed by FACS, as described in *Materials and Methods*. Transfected with: si1 and si2 = PTHrP siRNA-expressing constructs; sc = scrambled siRNA expression vector. 1,25D = 1,25(OH)₂D₃. Each value is expressed as a percentage of the corresponding level in cells treated with ethanol (set at 100%). Each bar is the mean ± SEM of three independent experiments. Significantly different from the corresponding control (-1,25D) value at: * = $P < 0.001$, and ** = $P < 0.05$.

Figure 6.

(A) Proliferation of C4-2 cells with enhanced or suppressed PTHrP expression. Cells (1×10^4) were plated in 96-well dishes in medium containing 10% FBS. Cell proliferation was measured at the indicated time intervals [in days] as described in *Materials and Methods*, and is expressed as Absorbance_{450 nm}. + = PTHrP-overexpressing cells; V = cells transfected with the empty vector or the scrambled siRNA expression vector; si = PTHrP siRNA-transfected cells. Where no error bar is shown, the SEM is smaller than the symbol. **(B) Effect of 1,25-dihydroxyvitamin D₃ on the proliferation of C4-2 cells with increased or suppressed PTHrP expression.** Cells cultured in medium containing 10% dialyzed FBS were treated for 72 h with 10^{-8} M 1,25(OH)₂D₃. Cells (1×10^4) were then plated in 96-well dishes in the presence of 10^{-8} M 1,25(OH)₂D₃ for a further 48 h. Cell proliferation was measured as in (A), and is expressed as a percentage of the proliferation of empty vector transfectants. + = overexpressing PTHrP; V = empty vector transfectants; si1 and si2 = PTHrP siRNA transfected; sc = transfected with a scrambled siRNA expression vector. + 1,25D = treated with 1,25(OH)₂D₃, - = treated with ethanol (vehicle control). (A and B). For PTHrP-overexpressing cells, each point or bar is the mean $\pm$ SEM of three independent experiments for each of three independent clones. For PTHrP siRNA transfectants, each point or bar is the mean $\pm$ SEM of three independent transfections for each of two individual PTHrP siRNA-expressing constructs. * = Significantly different from the corresponding vehicle control value ($P < 0.001$).