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1,25-Dihydroxyvitamin D_3 regulates PTHrP expression via transcriptional, post-transcriptional and post-translational pathways

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ABSTRACT

Parathyroid hormone-related protein (PTHrP) increases the growth and osteolytic potential of prostate cancer cells, making it important to control PTHrP expression. PTHrP expression is suppressed by 1,25-dihydroxyvitamin D_3 (1,25D). The aim of this study was to identify the pathways via which 1,25D exerts these effects. Our main findings are that 1,25D regulates PTHrP levels via multiple pathways in PC-3 and C4-2 (human prostate cancer) cell lines, and regulation is dependent on VDR expression. The human PTHrP gene has three promoters (P); PC-3 cells preferentially utilize P2 and P3, while C4-2 cells preferentially utilize P1. 1,25D regulates PTHrP transcriptional activity from both P1 and P3. The 1,25D-mediated decrease in PTHrP mRNA levels also involves a post-transcriptional pathway since 1,25D decreases PTHrP mRNA stability. 1,25D also suppresses PTHrP expression directly at the protein level by increasing its degradation. Regulation of PTHrP levels is dependent on VDR expression, as using siRNAs to deplete VDR expression negates the 1,25D-mediated downregulation of PTHrP expression. These results indicate the importance of maintaining adequate 1,25D levels and VDR status to control PTHrP levels.

1. Introduction

Prostate cancer is the second-leading cause of cancer-related death in men in the United States (Jemal et al., 2007). The most common site of prostate cancer metastasis is the bone (Bubendorf et al., 2000; Rana et al., 1993). Histologic evidence shows that these metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions (Berruti et al., 1996; Guise et al., 2006; Keller et al., 2001). The metastatic process requires that cells acquire new capabilities, including an increased ability to migrate and invade surrounding tissues to reach the vasculature and lymphatics (Hanahan and Weinberg, 2005). This process is accompanied by neoangiogenesis (Eccles, 2005).

Androgens play a pivotal role in the development and physiologic function of the prostate, as well as in prostate cancer. However, additional factors such as growth factors, neuroendocrine peptides and cytokines are also involved in prostate physiology and pathology (Deftos, 2000). Several studies have demonstrated a role for parathyroid hormone-related protein (PTHrP) in the pathogenesis and progression of prostate carcinoma and its tendency to metastasize to the bone (Hall et al., 2005, 2006). Both normal and neoplastic prostate epithelial cells express PTHrP (Iwamura et al., 1994; Kramer et al., 1991). PTHrP enhances prostate cancer cell proliferation, survival, migration, invasion and anchorage-independent cell growth *in vitro* (Bhatia et al., 2009; Dougherty et al., 1999; Tovar Sepulveda and Falzon, 2002), and increases growth of prostate and colon cancer cells in a nude mouse model (Bhatia et al., 2009; Shen et al., 2007a). The *in vivo* effects of PTHrP are accompanied by increased angiogenesis and decreased apoptosis (Bhatia et al., 2009). Moreover, PTHrP overexpression in prostate cancer cells decreases the latency and increases the severity of bone lesions. PTHrP also changes the bone lesion profile from predominantly osteoblastic to osteolytic (Bhatia et al., 2009). PTHrP expression also directly correlates with prostate cancer differentiation. In well-differentiated prostate cancer, expression is predominantly confined to the basal layer. As the cancer progresses to poorly-differentiated, intense cytoplasmic and nuclear PTHrP immunoreactivity is observed (Bhatia et al., 2009). These studies underlie the critical role of PTHrP in prostate cancer.

There are limited options for the treatment of metastatic prostate cancer. Although prostate cancers initially respond to androgen ablation therapy, they eventually become androgen independent. Biological response modifiers such as vitamin D analogs may be effective in slowing prostate cancer progression. Epidemiological studies have shown that vitamin D deficiency is linked with increased prostate cancer incidence (reviewed in Bouillon et al., 2008). 1,25-Dihydroxyvitamin D_3 (1,25D), the hormonally active

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form of vitamin D, regulates cell proliferation, differentiation, apoptosis, immune responses and angiogenesis in many cancer cell types (De Luca, 2004; Holick, 2003; Mantell et al., 2000). 1,25D also regulates PTHrP expression in a number of human cell types, including prostate cancer cell lines (El Abdaimi et al., 1999; Haq et al., 1993; Tovar Sepulveda et al., 2006).

The human PTHrP gene is complex, with nine exons spanning more than 15 kb of genomic DNA (Broadus and Stewart, 1994). PTHrP transcription may initiate at three promoters (P). P1 and P3 are canonical TATA promoters, and P2 is a high GC-element promoter (Vasavada et al., 1993). Promoter usage is cell type-specific (Cataisson et al., 2002; Hamzaoui et al., 2007; Luparello et al., 1999). The regulation of PTHrP expression by 1,25D has focused predominantly on suppression of PTHrP mRNA levels at the transcriptional level, mediated via a negative vitamin D response element (nVDRE) located within P1 (Abe et al., 1998; Nishishita et al., 1998; Tovar Sepulveda and Falzon, 2003). In this study, we asked whether alternative pathways independent of the nVDRE within P1 contribute to transcriptional regulation of the PTHrP gene by 1,25D, and whether post-transcriptional and post-translational components are also involved. Since VDR levels may be altered in cancer cells, we also investigated the effect of 1,25D on PTHrP levels in cells with decreased VDR expression. The C4-2 and PC-3 cell lines were used as model systems. The C4-2 cell line is a second-generation LNCaP subline that is androgen-independent and metastasizes to the lymph node and bone when injected orthotopically into nude mice (Thalmann et al., 1994; Wu et al., 1994). C4-2 cells produce mixed lytic/blastic lesions (Vinholes et al., 1996). The androgen-independent PC-3 cell line was initiated from a bone metastasis and produces predominantly lytic lesions. PTHrP plays both proliferative and metastatic roles in prostate cancer. Thus, identifying the pathways via which its expression is suppressed by 1,25D may lead to new therapeutic approaches.

2. Materials and methods

2.1. Materials

1,25D was kindly provided by Dr. M. Uskokovic (Hoffmann LaRoche, Inc., Nutley, New Jersey), and was dissolved in ethanol at 10^{-3} M. Fetal bovine serum (FBS) and dialyzed FBS were obtained from Atlanta Biologicals (Norcross, GA) and HyClone (Logan, UT), respectively. Tissue culture supplies were purchased from Gibco (Carlsbad, CA). Antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The small interfering RNAs (siRNAs) targeting the VDR and the corresponding non-target control (NTC) siRNA sequences were purchased from Dharmacon (Lafayette, CO).

2.2. Cell culture

PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown at 37 °C in a humidified 95% O_2/5% CO_2 atmosphere in RPMI-1640 medium supplemented with 8% FBS and L-glutamine. C4-2 cells were purchased from UroCor, Inc. (Oklahoma City, OK), and were grown under the same conditions in RPMI-1640 supplemented with 10% FBS and L-glutamine. At 48 h before treatment with 1,25D, conventional FBS was replaced with dialyzed FBS in order to minimize the exposure of the cells to endogenous steroids present in the serum.

2.3. Plasmid constructs

Constructs containing regions from P1, P2 or P3 from the human PTHrP gene, cloned in the luciferase reporter plasmid pGL-2 (Promega, Madison, WI), were obtained from Dr. Z. Bouizar. These constructs have been described (Cataisson et al., 2002). These constructs, as well as the empty vector control, were transfected into PC-3 and C4-2 cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA).

2.4. Cell transfections and 1,25D treatment

To ask if 1,25D alters transcription from the PTHrP gene, we measured promoter activity of each of the three PTHrP promoters in the presence and absence of 1,25D. Cells were plated in 24-well plates at 1×10^5 cells/well in medium containing dialyzed FBS (HyClone). After 48 h, the cells were transfected with the promoter constructs using Lipofectamine Plus, per the manufacturer's instructions (Invitrogen). Cells transfected with the empty vector were used as controls. Cells were co-transfected with a construct expressing Renilla luciferase for standardization purposes. After 4 h, the transfection medium was removed and replaced with fresh medium containing 1,25D (10^{-9} to 10^{-7} M). Ethanol was used as the vehicle control (final volume 0.01% v/v). After 24 h, cell lysates were prepared and promoter activity was assayed using the Dual Luciferase assay kit (Promega). Empty vector control values were subtracted from the respective firefly and Renilla luciferase values. The firefly luciferase activity corresponding to each PTHrP promoter was normalized to Renilla luciferase activity, and the fold differences were plotted as the firefly/Renilla ratio.

For siRNA transfections, cells were plated as described above. After 48 h, they were transfected by electroporation with ON-Target plus siRNAs directed against the VDR (100 nM; Dharmacon). To eliminate the potential for off-target effects, two independent siRNAs were used. As a control, cells were transfected with ON-Target-plus non-target control (NTC) siRNAs.

To determine the effect of 1,25D on PTHrP protein levels, PC-3 and C4-2 cells were plated in 100 mm plates at 5×10^5 cells/plate in medium containing dialyzed FBS (HyClone). When the cells were ~70% confluent, they were treated with 1,25D (10^{-9} to 10^{-7} M) or ethanol (vehicle control). Cell extracts were processed for Western blot analysis after 48 h of treatment.

2.5. Analysis of PTHrP and VDR mRNA levels

Total RNA was extracted using the RNAqueous® isolation kit (Ambion Inc., Austin, TX), per the manufacturer's protocol. RNA concentrations were determined by spectrophotometry. PTHrP, CYP24a1, and VDR mRNA levels were analyzed by reverse transcription/real-time PCR as described (Shen et al., 2007b). The following TaqMan inventoried products were used: PTHrP, Hs00174969_m1; VDR Hs01045840_m1; CYP24a1, Hs00167999_m1; and the pre-developed 18S rRNA primers (VIC™-dye labeled probe, TaqMan® assay reagent, P/N 4319413E), and were obtained from Applied Biosystems, as was the universal PCR master mix reagent kit (P/N 4304437).

2.6. Western blot analysis

Cells were grown to 70–80% confluence in 100 mm plates. To prepare whole cell extracts, cells were washed twice with cold PBS on ice and lysed in RIPA buffer containing a Protease Inhibitor cocktail and Phosphatase Inhibitor cocktails A and B (Santa Cruz Biotechnology). Nuclear extracts were prepared using the NE-PER Nuclear Protein Extraction Kit (Pierce Biotechnology Inc.), according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS containing phosphatase inhibitors. After scraping the cell monolayer and centrifugation, the pellet was resuspended and incubated in 1 x hypotonic buffer for 15 min on ice. Detergent was then added. After centrifugation, the pellet (nuclear fraction)