Regulation of insulin-like growth factor (IGF) II and IGF binding protein 3 autocrine loop in human PC-3 prostate cancer cells by vitamin D metabolite 1,25(OH)_{2}D_{3} and its analog EB1089

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Abstract. Prostate cancer and benign prostate hyperplasia (BPH) are major public health problems. Prostate epithelial cell proliferation is regulated by insulin-like growth factor I (IGF-I) which is mitogenic and anti-apoptotic, and IGF binding protein 3 (IGFBP-3) which is an apoptotic agent in these cells. We demonstrate that the 1,25(OH)_{2}D_{3} and its analog EB1089-induced growth inhibition was associated with increased IGFBP-3 mRNA abundance, IGFBP-3 mRNA stability, IGFBP-3 protein accumulation, and decreased IGF-II gene expression. Anti-IGF-II antibody and exogenous recombinant human IGFBP-3 inhibit PC-3 cell proliferation. The results document the inhibitory effects of 1,25(OH)_{2}D_{3} and EB1089 on the IGF system of mitogen in prostate cancer cells, and suggest a potential therapeutic use of EB1089 in treatment of BPH and prostate cancer.

Introduction

The biologically active metabolite of vitamin D, 1,25(OH)_{2}D_{3}, is a principal mediator of bone and mineral metabolism (1), and also serves as a modulator of cell proliferation and differentiation in both normal and malignant cells (26). The vitamin D receptor (VDR) has been detected in the intestine, kidney, bone (7,8) (reviewed in ref. 6), and in many neoplastic cell lines (3,4,9) as well as in primary cultures of stromal and epithelial cells derived from normal and malignant prostate tissues (10). 1,25(OH)_{2}D_{3} deficiency may be associated with an increased risk of prostate cancer (11). Vitamin D-related compounds have been shown to decrease tumour size, number, and lethality when given in vivo to animals in which colon and skin tumours were chemically induced (12-14) or grafted (5,15,16). 1,25(OH)_{2}D_{3} has been shown to inhibit prostate cancer cell proliferation (17) and induce apoptosis in prostate cancer cells (18), however clinical exploitation of this antineoplastic activity has been limited by hypercalcemia which is associated with the administration of high doses of 1,25(OH)_{2}D_{3}. Recently, several new vitamin D analogs with potent antineoplastic activity in vitro and in vivo, but little effect on calcium homeostasis, have been described (19-26). Investigation into the molecular mechanisms underlying growth inhibition induced by these vitamin D analogs is incomplete.

The proliferation of epithelial cells in the prostate is influenced by factors such as EGF, TGF-α, TGF-β, NGF and members of the insulin-like growth factor (IGF) and FGF family (27,28). The prostate stroma secretes IGF-I, and the epithelial cells respond to IGF-I and IGF-II through the interaction of these growth factors with the type I IGF receptor (28,29). Several studies have indicated that IGF-I is mitogenic in prostate cells (reviewed in refs. 27 and 30). IGF bioavailability is controlled by a family of seven IGF binding proteins (IGFBPs) (31,32) that bind to IGF-I and IGF-II with very high affinity. Normal prostate epithelial cells have been shown to secrete IGFBP-2 and IGFBP-4, while the stromal fibroblasts produce IGFBP-2, 3 and 4 (28,33,34). In the adult prostate, IGFBP-2 is expressed while IGFBP-5 expression is repressed (27). The physiological actions of IGFBPs in prostate cells are not known. Some IGFBPs have intrinsic bioactivity independent of IGF (18,35,36).

Our in vitro experiments demonstrate that 1,25(OH)_{2}D_{3} and EB1089 inhibit PC-3 prostate cancer cell proliferation in part by enhancing IGFBP-3 expression and suppressing IGF-II gene expression.

Materials and methods

Cell culture and proliferation assay. PC-3 cells were maintained as monolayer cultures in RPMI media (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). Confluent stock cultures were trypsinized and plated at 2.5x10^{4} cells per well in 24-well dishes in RPMI supplemented with 5% FCS. After 24 h, cell monolayers were rinsed twice with serum-free RPMI and incubated for a further 24 h in serum-free RPMI. Cells were then incubated for 7 days in the presence or absence of various concentrations of 1,25(OH)_{2}D_{3} (Biomol Research Laboratories, Plymouth}
Meeting, PA) or EB1089 (kindly provided by LEO Pharmaceutical Product, Ballerup, Denmark). Fresh media was replaced every 2 days. The cell number was determined as described (37). Experiments were carried out in triplicate.

To test the effects of exogenous IGFBP-3 and IGF-II antibody on cell proliferation, PC-3 cells were plated as described above. Cells were incubated with serum free RPMI containing recombinant IGFBP-3 (Upstate Biotechnology, Lake placid, NY) for 72 h. [3H]-thymidine incorporation was determined as described (36). For effects of anti-IGF-II antibody on PC-3 cell proliferation, PC-3 cells were plated as described above. Cells were treated with various concentrations of monoclonal antibodies against IGF-II (Upstate Biotechnology) for 4 days. The proliferation rate was determined by the cell number. For Western, ligand, and Northern blotting, cells were seeded 5 x 10^5 cells per 100 mm dish and were grown and treated as described above. Forty-eight hours conditioned media was collected, clarified and frozen until assayed for IGFBPs by Western ligand blotting.

**Ligand blotting.** IGF-I was radioionated using Na[^3][H] (ICN Biochemical Inc., Irvine, CA) and the chloramine-T method to a specific activity of 350-500 μCi/μg. One ml of conditioned medium was concentrated 20X by a Centricron 10 microconcentrator (Amicon, Beverly, MA). Total proteins were resolved by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and electrophoretically on nitrocellulose membranes. Ligand blotting for secreted IGFBPs was performed as described (38).

**Western blotting.** Proteins from conditioned media were separated under reducing conditions and blotted as described (38). Immunoblot analysis with polyclonal antibodies against IGFBP-3 (UBI, Lake Placid, NY), and horseradish peroxidase-conjugated anti-rabbit antiserum (Amersham, Oakville, Ont.) was performed using standard protocols. Western blots were visualized by a chemiluminescence-based photoblot system (ECL; Amersham).

**Northern analysis.** Total RNA was extracted as previously described (39) using the RNAzol B method (Tel-Test, Friendswood, Texas). Total RNA (50 μg) was fractionated and transferred, and prehybridization, hybridization and washing were performed as described (39). Blots were hybridized with either human IGFBP-3 or IGF-II cDNAs (ATCC) (40). To control for equal loading of wells, we compared total amounts of RNA present in different lanes hy reblooding the blots with β-actin cDNA (41). Quantitative analysis of gene expression was accomplished by scanning autoradiograms densitometrically.
Figure 3. Western immunoblotting of IGFBP-3 from PC-3 cell conditioned media. PC-3 cells were treated with vehicle (C) or various concentrations of EB1089 (EB) and 1,25(OH)\(_2\)D\(_3\) (vit D) as indicated for 48 h. Conditioned media were collected and Western blotting was performed as described in Materials and methods. A representative Western blot of IGFBP-3 accumulation in PC-3 cell conditioned media is shown in (A). A representative densitometric scanning of IGFBP-3 protein bands is shown in (B). IGFBP-3 levels significantly increased in the presence of 1,25(OH)\(_2\)D\(_3\) or EB1089 compared to vehicle alone (P<0.01). The experiments were repeated three times with similar results. Means ± SEM of triplicate experiments are plotted.

Figure 4. Effects of 1,25(OH)\(_2\)D\(_3\) and EB1089 on IGFBP-3 gene expression. Cells were treated with vehicle (C) or various concentrations of EB1089 (EB) and 1,25(OH)\(_2\)D\(_3\) (vit D) as indicated for 48 h. Total RNA was extracted and Northern blotting was performed as described (45). Blots were hybridized with human IGFBP-3 (40) (A) and human β-actin (ATCC) (B) cDNA probes. A representative densitometric scanning of the band corresponding to IGFBP-3 mRNA is shown in (C). IGFBP-3 mRNA levels significantly increased in the presence of 1,25(OH)\(_2\)D\(_3\) or EB1089 compared to vehicle alone (P<0.01). The experiments were repeated three times with similar results. Means ± SEM of triplicate experiments are plotted.

Figure 5. Effects of 1,25(OH)\(_2\)D\(_3\) and EB1089 on IGF-II gene expression. Cells were treated with vehicle (C) or various concentrations of EB1089 (EB) and 1,25(OH)\(_2\)D\(_3\) (vit D) as indicated for 48 h. Total RNA was extracted and Northern blotting was performed as described in Materials and methods. Blots were hybridized with human IGF-II (ATCC) (A) and human β-actin (ATCC) (B) cDNA probes. A representative densitometric scanning of the sum of all the bands (8.5, 6 and 1.4 kb) corresponding to IGF-II mRNA is shown in (C). IGF-II mRNA levels significantly decreased in the presence of 1,25(OH)\(_2\)D\(_3\) or EB1089 compared to vehicle alone (P<0.01). The experiments were repeated three times with similar results. Means ± SEM of triplicate experiments are plotted.
IGFBP-3 mRNA half-life. Transcription of cellular mRNA was inhibited by treating cells with 25 μg/ml of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Calbiochem, La Jolla, CA) as described (42). Total RNA was extracted from the cells every 3 h after DRB treatment and analysed by Northern blotting using IGFBP-3 cDNA (40). These blots were rehybridized with a human IGFBP-3 and β-actin cDNA probe as a control for inhibition of transcription as described (43).

In vitro transcription assay. Cells were cultured in the absence or presence of EB1089 for 48 h, harvested by scraping, and pelleted at 800 g for 5 min. Nuclei isolation, RNA labelling, binding of human β-actin and IGFBP-3 cDNAs to nitrocellulose membrane, hybridization of labelled RNA to DNA immobilized on strips of nitrocellulose and washes were performed as described (43).

Results

Treatment of PC-3 cells with 1,25(OH)₂D₃ or EB1089 for 7 days resulted in a dose-dependent decrease in the cell number (Fig. 1) which is in agreement with prior reports (19,21-23). To determine whether there is a modulation in the cellular microenvironment as a consequence of 1,25(OH)₂D₃ and EB1089 treatment, Western ligand blots were performed on the conditioned media from control and treated cells. Fig. 2 shows the effects of EB1089 and 1,25(OH)₂D₃ on IGFBP accumulation in PC-3 cell conditioned media. IGFBP-3 was considered to be the main candidate of the IGFBP species affected by EB1089 and 1,25(OH)₂D₃ treatments on the basis of its molecular weight. IGFBP-4 and IGFBP-2 were less affected by these treatments. To confirm this observation, Western blots were performed. Immunobots with an antisem to IGFBP-3 showed EB1089 and 1,25(OH)₂D₃-induced accumulation of IGFBP-3 (Fig. 3).

To determine whether IGFBP-3 protein accumulation was associated with IGFBP-3 mRNA abundance, Northern blot analysis of total cellular RNA was performed. As shown in Fig. 4, a cDNA probe for human IGFBP-3 (40) hybridized to a 2.6 kb mRNA species. Treatment of cells with 1,25(OH)₂D₃ and EB1089 resulted in an increase in IGFBP-3 mRNA.

Since IGF-II has been implicated in regulation of PC-3 cell growth (34), it is important to determine if EB1089 and 1,25(OH)₂D₃ also inhibit IGF-II gene expression. Fig. 5 shows that both EB1089 and 1,25(OH)₂D₃ significantly (p<0.01) suppressed IGF-II gene expression.

Limited proteolysis of IGFBPs is one of the mechanisms regulating the bioavailability of IGFs at the cellular level. It has been shown that IGFBP-3 accumulation can be influenced by changes in both synthesis and proteolysis (34), but no evidence for a major effect of 1,25(OH)₂D₃ and EB1089 on IGFBP-3 proteolysis was seen (Fig. 3). Furthermore, affinity labelling experiments showed no significant influence of 1,25(OH)₂D₃ and EB1089 on PC-3 cell IGF-I receptor levels (data not shown).

To determine if 1,25(OH)₂D₃ and EB1089-induced IGFBP-3 accumulation and inhibition of IGF-II gene expression are functionally related to their growth inhibitory action, we carried out proliferation experiments with IGFBP-3 and anti IGF-II monoclonal antibodies. Fig. 6A shows that exogenous IGFBP-3 (0.5 and 1 μg) significantly (p<0.05) inhibited [3H]thymidine incorporation into PC-3 cells. Furthermore, the addition of IGF-II antibodies to PC-3 cells for 4 days resulted in a significant reduction in cell number, while control IgG had no effect (Fig. 6B).

To examine the mode of IGFBP-3 mRNA regulation by EB1089, IGFBP-3 message stability was estimated using the mRNA specific transcription inhibitor DRB, which was added after the cells were cultured for 48 h in the absence or in the presence of 10⁻⁷ M EB1089. As shown in Fig. 7, the rate of IGFBP-3 mRNA degradation in EB1089-treated cells decreased in comparison to control cells. The half-life for IGFBP-3 mRNA was estimated to be approximately 6 h for
Figure 7. Half-life of IGFBP-3 mRNA in response to EB1089 treatment. PC-3 cells were grown and treated with 10^{-7} M EB1089 for 48 h. The half-life of IGFBP-3 mRNA and β-actin mRNA were determined as described in Materials and methods. The half-lives of IGFBP-3 mRNA and β-actin were estimated by densitometric scanning of Northern blots. The half-life for IGFBP-3 mRNA was estimated to be approximately 6 h for the control cells and 18 h for the EB1089 treated cells. The half-life for β-actin was approximately 3 h under all conditions. The experiments were repeated three times with similar results. Means ± SEM of triplicate experiments are plotted.

The control cells and approximately 18 h for the EB1089 treated cells. The half-life for β-actin was approximately 3 h under all conditions, in agreement with a previous study (42). To determine if EB1089 also affects the transcription rate of the IGFBP-3 gene, a nuclear run-off assay was performed. The transcription rate of the IGFBP-3 gene was not altered by EB1089 treatment (data not shown). The data suggest that variations in the stability of IGFBP-3 mRNA make a major contribution to the effects of EB1089 and 1,25(OH)_{2}D_{3} on IGFBP-3 mRNA abundance.
Discussion

We demonstrate that 1,25(OH)₂D₃ and EB1089 inhibited human PC-3 prostate cancer cell proliferation in part by disrupting the autocrine IGF-II loop and enhancing IGFBP-3 expression. Since both IGF-I and IGF-II are mitogenic (27,30) and anti-apoptotic factors (27) and IGFBP-3 is an apoptotic agent (18) involved in prostate epithelial cell proliferation, these observations provide insights into the mechanisms of 1,25(OH)₂D₃ action. This may be relevant to potential therapeutic use of 1,25(OH)₂D₃ analogs in benign prostate hyperplasia and prostate cancer treatment. The data also provides an explanation for observations that patients with VDR positive tumours experience significantly longer disease-free survival than those with VDR negative breast tumours (5,44), and that tumour size, number and lethality were reduced when vitamin D analogs were given in vivo to animals in which colon and skin tumours were chemically induced (12-14) or grafted (5,15,16).

IGF autocrine activity is itself closely regulated by IGFBPs that display a central role in regulating IGF availability and bioactivity. In the PC-3 cell model studied here, we propose that the maintenance of these cells in the proliferative state, even in the absence of serum, is due to low levels of IGFBP-3 biosynthesis and the presence of endogenous IGF-II. Since endogenous IGF-II was detected in PC-3 cell conditioned media (34) and IGF-I mRNA was undetected in the cells, we postulate that IGFBP-3 prevents the autocrine/paracrine regulatory function of endogenous IGF-II and/or possibly inhibits DNA synthesis by a mechanism independent of IGF, rather than reducing the bioavailability of IGF-II for cell surface receptor binding as previously reported (18,35,36). Another possibility is that an interaction between IGF-II and IGFBP-3 would prevent the binding of IGFBP-3 to its putative receptor, thus reducing direct growth inhibition of IGFBP-3.

Our results show that the regulation of IGFBP-3 accumulation by EB1089 is via the modulation of the IGFBP-3 mRNA half-life. EB1089 extends the half-life of IGFBP-3 mRNA from 6 to approximately 18 h, although the precise mechanism of post-transcriptional control of the IGFBP-3 mRNA in our experimental system remains uncharacterized. We observed, however, that inhibition of protein synthesis by cyclohexamide resulted in increased IGFBP-3 mRNA half-life (data not shown). This observation indicates that protein synthesis is required for IGFBP-3 mRNA degradation and EB1089 may suppress the synthesis of the postulated regulatory protein, thereby increasing the IGFBP-3 mRNA that is available for translation.

It has been reported that IGFBP-3 induced apoptosis in PC-3 cells (18). In our experimental system, exogenous IGFBP-3 also induced apoptosis if incubation was prolonged (data not shown). IGF-II antibodies reduced growth of these cells with minimal apoptosis (data not shown).

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References


