Regulation of proliferation of prostate epithelial cells by 1,25-dihydroxyvitamin D₃ is accompanied by an increase in insulin-like growth factor binding protein-3

C C Sprenger¹, A Peterson², R Lance², J L Ware³, R H Drivdahl¹ and S R Plymate^{1,4}

¹Geriatric Research Education and Clinical Center, VAPSHCS, Tacoma, Washington, USA

²Department of Urology, Madigan Army Medical Center, Tacoma, Washington, USA

³Department of Pathology, MCV/VCU, Richmond, Virginia, USA

⁴Department of Medicine, University of Washington, Seattle, Washington, USA

(Requests for offprints should be addressed to S R Plymate, GRECC (182 B), Bldg 85, Room 128, American Lake VAMC, Tacoma, Washington 98493, USA; Email: splymate@u.washington.edu)

Abstract

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) has been shown to regulate the proliferation of human prostate epithelial cell lines. Since the insulin-like growth factor (IGF) system is involved in the transformation process of epithelial cells, the following study was undertaken to determine if the IGF system, in particular IGF binding protein-3 (IGFBP-3), is altered by 1,25-(OH)₂D₃ in normal prostate epithelial cells as part of a mechanism for inhibition of transformation. Two cell systems were used in this study: (1) primary cultures of benign human prostate epithelial cells (PECs) and (2) an SV40-T immortalized prostate epithelial cell line (P153) that is non-tumorigenic. 1,25-(OH)₂D₃ was added to parallel sets of PECs and P153 cells

in addition to the presence or absence of IGF-I or des(1-3)IGF-I. Treatment with 1,25-(OH)₂D₃ resulted in significant growth inhibition of both PECs and P153 cells. Furthermore, 1,25-(OH)₂D₃ inhibited IGF-induced proliferation, but this was partially reversed by high concentrations of IGF-I. Western ligand blots of condition media demonstrated a significant increase in IGFBP-3; likewise Northern blots demonstrated an increase in mRNA for IGFBP-3. Proliferation assays using an antibody designed to block the IGF-independent effects of IGFBP-3 failed to reverse the inhibitory effect of 1,25-(OH)₂D₃. Thus, IGFBP-3 acts in an IGF-dependent manner to inhibit cell growth of benign prostate epithelial cells.

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Introduction

Epidemiological studies suggest that vitamin D deficiency may be an underlying risk factor in the development of prostate cancer, since men with elevated serum levels of vitamin D are at a decreased risk for developing prostate cancer (Schwartz & Hulka 1990, Corder *et al.* 1995, Konety *et al.* 1996). The classic role of vitamin D is to regulate calcium and phosphorous homeostasis, by conversion to the active metabolite, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃). This conversion is stringently controlled by a person's calcium needs (Reichel *et al.* 1989). However, the ubiquitous nature of the vitamin D receptor beyond its classical target organs (intestines, kidney and bone) has led investigators to explore other possible roles of 1,25-(OH)₂D₃ (Walters 1992).

1,25-(OH)₂D₃ regulates growth and differentiation in various cell lines, including malignant cultures (Gross *et al.* 1996, Konety *et al.* 1996, Taylor *et al.* 1996, Feldman

1997, Ingles *et al.* 1997, Rozen *et al.* 1997, Schwartz *et al.* 1997, Wang *et al.* 1997, Zhuang & Burnstein 1998, Moffatt *et al.* 1999). Treatment with 1,25-(OH)₂D₃ *in vivo* increased the survival time of mice inoculated with myeloid leukemia cells, inhibited tumor growth in immunosuppressed mice with human malignant melanoma, colonic cancer cell, and breast cancer cell xenographs, and decreased the number of lung metastases in athymic mice injected with Lewis lung carcinoma cells (Sato *et al.* 1983, Eisman *et al.* 1987, 1989). In prostate cancer, 1,25-(OH)₂D₃ is growth inhibitory for many established lines, including LNCaP, PC-3, ALVA-31 and DU-145 (Hedlund *et al.* 1996, Chen *et al.* 1997, Zhuang & Burnstein 1998). Furthermore, Nickerson and Huyhn (1999) demonstrated a 25% loss in ventral prostate weight when rats were given a 1,25-(OH)₂D₃ analog for 14 days. Peehl *et al.* (1994) also demonstrated that 1,25-(OH)₂D₃ treatment of primary prostate epithelial and fibroblast cells inhibits growth.

The insulin-like growth factor (IGF) system has been shown to contribute to the transformation of prostate epithelial cells and to the development of prostate cancer in men (Pietrzkowski *et al.* 1993, Plymate *et al.* 1996, Damon *et al.* 1998). The role of the insulin-like growth factor binding proteins (IGFBP), especially IGFBP-3, can be growth inhibitory or stimulatory (Schmid *et al.* 1991, Oh *et al.* 1995, Angeloz-Nicoud *et al.* 1996, Rajah *et al.* 1997). Several studies have demonstrated that IGFBP-3 expression increases in response to 1,25-(OH)₂D₃, or its analogs, in established prostate cancer cell lines (Huynh *et al.* 1996, Nickerson *et al.* 1998, Goossens *et al.* 1999, Nickerson & Huynh 1999). Most studies have correlated the increase in IGFBP-3 with a decrease in cell growth but none have determined if the 1,25-(OH)₂D₃-induced increase in IGFBP-3 alters the response of the cells to IGF ligands. The purpose of this study was to determine if the increased IGFBP-3 expression ascribed to 1,25-(OH)₂D₃ in transformed prostate cancer cell lines also occurs in primary prostate epithelial cells and is therefore a potential explanation for the putative association of increased vitamin D levels and decreased incidence of prostate cancer.

Materials and Methods

Materials

Tissue culture media, RPMI-1640, F12 nutrient mixture (Ham's) powder, HEPES, gentamicin, fungizone, geneticin (G418), and deoxyribonuclease were obtained from Life Technologies (Gibco-BRL) (Grand Island, NY, USA). Epidermal growth factor (EGF), dexamethasone, and the additive ITS (insulin, transferrin, selenium) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Bovine pituitary extract (BPE) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Nonradioactive 1,25-(OH)₂D₃ was purchased from Calbiochem (La Jolla, CA, USA). Insulin-like growth factors-I and -II were gifts from Eli Lilly and Co. (Indianapolis, IN, USA). IGFBP-3 was purchased from Upstate Biotechnology, Inc. Anti-IGFBP-3 was obtained from Diagnostic Systems Laboratories (Houston, TX, USA). The Cell Titer 96 Aqueous cell proliferation kit was obtained from Promega (Madison, WI, USA). Nitrocellulose and electrophoresis reagents were purchased from BioRad Laboratories (Richmond, CA, USA); nylon membranes (GeneScreen) were obtained from New England Nuclear (Boston, MA, USA). [¹²⁵I]IGF-II (2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA). ³²P-dCTP was obtained from NEN-DuPont (Wilmington, DE, USA). The IGFBP-3 cDNA was obtained from Dr S Shimazaki (Whittier Institute for Diabetes and Endocrinology, Scripps Memorial Hospital,

San Diego, CA, USA). Each experiment was performed at least three separate times.

Cell culture

P153 cells were derived from benign prostate epithelial cells obtained from a radical prostatectomy specimen and cells were immortalized with the early region SV40-T genes as previously described (Bae *et al.* 1994). P153 cells were cultured in RPMI-1640 medium supplemented with 10 ng/ml EGF, 0.02 mM dexamethasone, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, fungizone and gentamicin at 37 °C under 5% CO₂. All cells used in these experiments were mycoplasma free, as determined by the Mycoplasma PCR Primer Set (Stratagene, La Jolla, CA, USA).

Tissue biopsies obtained during radical prostatectomies were digested overnight at 37 °C with 0.1% collagenase (Type 1) containing fungizone and gentamicin. The epithelial and stromal cells were then separated by growing the cells in F12/HEPES medium supplemented with 10 ng/ml EGF, 0.02 mM dexamethasone, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 50 µg/ml BPE, fungizone and gentamicin at 37 °C under 5% CO₂. The resulting primary epithelial cells (PECs) are composed predominantly of basal epithelial cells.

Cell proliferation assays

Cell proliferation was assessed by a colorimetric MTT assay for quantification of viable cells (Cell Titer 96 Aqueous kit, Promega). PECs and P153 cells were plated in 96-well plates at a density of 5000 cells/well in RPMI medium containing 5% serum. The following day, experimental media conditions were added (all serum free). The treatments included des(1–3) IGF-I in addition to IGF-I because it has a fivefold decreased affinity for IGFBP-3 compared with IGF-I, and thus should give an indication of the effect of IGFBP-3 on sequestration of intact IGF-I from the type I IGF receptor (IGF-IR) (Salahifar *et al.* 2000). The plates were incubated for 96 h, after which the tetrazolium salt and dye solution was added and color development was allowed to proceed for 4 h at 37 °C, 5% CO₂. Each plate was then read at an absorbance of 490 nm; each cell line was tested three times. The correlation between cell number and the MTT assay in our laboratory is $r=0.97$. Statistical analyses were performed using the unpaired *t*-test with a 95% confidence interval.

Cell counts

Cell counts were performed on the above treatments using the hemocytometer method (Sprenger *et al.* 1999). Each count was performed in triplicate.

IGFBP-3 studies

Proliferation assays were conducted using the treatments in *Cell proliferation assays* with the addition of anti-IGFBP-3. Because the antibody against IGFBP-3 contained sodium azide as a preservative, the toxicity of this reagent was first evaluated by cell proliferation assays in the presence or absence of 0.00009% sodium azide. Proliferation studies with exogenous IGFBP-3 (500 ng/ml) added to medium containing IGF-I (10 ng/ml) were also performed.

IGFBP expression studies

PEC and P153 cells were grown to 80% confluence in 60-mm tissue culture dishes and treated with the following: control (0.1% ethanol) or 1,25-(OH)₂D₃ (10 nM) in RPMI supplemented with 5 µg/ml transferrin and 5 ng/ml selenium. After 24 h, medium and total cytoplasmic RNA were collected for Western ligand or immunoblots (see *Western ligand and immunoblots*) or Northern blots (see *mRNA analysis*) respectively.

Western ligand and immunoblots

Media from cells were collected and concentrated by filtration through nitrocellulose; the amount of media used to prepare each sample was based on cell number of each culture (Birnbaum *et al.* 1994). Western immunoblots and ligand blots were performed as previously described (Birnbaum *et al.* 1994, Sprenger *et al.* 1999).

mRNA analysis

Cells were grown and treated with growth factors as described above and total cytoplasmic RNA was extracted using an acid guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski & Sacchi 1987). Northern blot analysis was performed as previously described (Sprenger *et al.* 1999).

Results

Dose-responses to IGF-I, des(1-3)IGF-I, and 1,25-(OH)₂D₃

The effects of increasing concentrations of IGF-I (0, 1, 10, 100 ng/ml), des(1-3)IGF-I (0, 1, 10, 100 ng/ml), and 1,25-(OH)₂D₃ (1, 5, 10 nM) on cell proliferation were tested on both PECs and P153 cells. All concentrations of IGF-I and des(1-3)IGF-I significantly increased proliferation of the PECs (Fig. 1A, B). The growth of these cells was significantly inhibited by 10 nM 1,25-(OH)₂D₃ (Fig. 1C, D). In the P153 cells, the 10 and 100 ng/ml concentrations of IGF-I and all concentrations of des(1-3)IGF-I significantly increased proliferation (Fig. 2A, B). The P153

cells were significantly inhibited at the 5 and 10 nM concentrations of 1,25-(OH)₂D₃, with 10 nM having the greatest inhibition (Fig. 2C, D). Therefore, the 1 and 50 ng/ml concentrations of IGF-I and des(1-3)IGF-I as well as the 10 nM concentration of 1,25-(OH)₂D₃, were used for the remaining experiments for both cell types.

Proliferation studies with IGFs and 1,25-(OH)₂D₃

In PECs, the proliferative effect of IGF-I at a concentration of 1 ng/ml was completely inhibited by 1,25-(OH)₂D₃. The inhibition of 1,25-(OH)₂D₃ was partially overcome by an IGF-I concentration of 50 ng/ml, although there was still a significant ($P < 0.001$) decrease in proliferation when compared with IGF-I alone (Fig. 3A). Similar results were obtained for the low and high concentrations of des(1-3)IGF-I in the presence of 1,25-(OH)₂D₃ (Fig. 3B). In P153 cells, the addition of IGF-I or des(1-3)IGF-I completely overcame the inhibition by 1,25-(OH)₂D₃ (Fig. 3C, D). In fact, the combination of 1,25-(OH)₂D₃ and des(1-3)IGF-I resulted in a greater proliferative response than to either des(1-3)IGF-I concentration alone.

Effect of 1,25-(OH)₂D₃ on IGF-binding proteins

In PECs treated with 1,25-(OH)₂D₃, IGFBP-3 protein levels increased by 3.9-fold on Western ligand blots of media, and IGFBP-3 mRNA levels increased 3.3-fold (Fig. 4). When P153 cells were treated with 1,25-(OH)₂D₃, both ligands and immunoblots showed increases in IGFBP-3 protein (3.3-fold) (Fig. 5A, B). The ligand blots also showed a small increase in IGFBP-2. IGFBP-3 mRNA levels in P153 cells increased fivefold in response to 1,25-(OH)₂D₃ (Fig. 5C). Thus, the PECs had higher basal levels of IGFBP-3 mRNA and protein than the P153 cells and greater expression of IGFBP-3 in response to 1,25-(OH)₂D₃.

IGFBP-3 antibody studies

In order to determine if IGFBP-3 mediated growth inhibition by 1,25-(OH)₂D₃, an IGFBP-3 antibody was added to the treatment medium and rates of cell proliferation determined as before. However, no effect on the inhibitory action of 1,25-(OH)₂D₃ was seen when an IGFBP-3 antibody was added; results were corrected for the presence of sodium azide (data not shown). This antibody has been reported to inhibit the IGF-independent effect of IGFBP-3 by preventing it from interacting with the putative cell surface receptor (Rajah *et al.* 1997). However, IGFBP-3 is still able to bind IGF-I in the presence of antibody (Rajah *et al.* 1997). Therefore, the antibody's inability to change the inhibitory effect of 1,25-(OH)₂D₃ suggests that the effects of IGFBP-3 are IGF-dependent in the case of the P153 cells.

Figure 1 Differences in cell proliferation of primary epithelial cultures (PECs) treated with various concentrations of IGF-I (0, 1, 10, 100 ng/ml), des(1–3)IGF-I (des; 0, 1, 10, 100 ng/ml) or 1,25-(OH)₂D₃ (0, 1, 5, 10 nM). Panels A and C show proliferation assays by MTT (described in the Materials and Methods section). Panels B and D show results of cell counts (■, IGF-I treatment; ▲, des(1–3)IGF-I treatment). ****P*<0.001 when compared with control treatment.

Effects of adding exogenous IGFBP-3

The addition of 500 ng/ml IGFBP-3 did not inhibit PEC or P153 cell proliferation. However, when IGFBP-3

protein was added exogenously to either cell type in the presence of IGF-I, the stimulatory response of these mitogens was decreased significantly (Fig. 6). As with 1,25-(OH)₂D₃, the exogenous IGFBP-3 had

Figure 2 Differences in cell proliferation of P153, a non-tumorigenic, but immortalized, prostate epithelial cell line, to various concentrations of IGF-I (0, 1, 10, 100 ng/ml), des(1-3)IGF-I (des; 0, 1, 10, 100 ng/ml) or 1,25-(OH)₂D₃ (0, 1, 5, 10 nM). Panels A and C show proliferation assays by MTT (described in the Materials and Methods section). Panels B and D show results of cell counts (■, IGF-I treatment; ▲, des(1-3)IGF-I treatment). **P*<0.05, ***P*<0.01, ****P*<0.001 when compared with control treatment.

a greater inhibitory effect on the PECs than on the P153 cells.

Discussion

1,25-(OH)₂D₃ and its analogs decrease the proliferation of prostate cancer cell lines *in vitro* (Peehl *et al.* 1994, Gross *et al.* 1996, Chen *et al.* 1997, Rozen *et al.* 1997, Moffatt

et al. 1999). In animal models, 1,25-(OH)₂D₃ administration prevents the development of prostate cancer, and in epidemiological studies, higher 1,25-(OH)₂D₃ levels are associated with a decreased incidence of prostate cancer (Schwartz & Hulka 1990, Corder *et al.* 1995, Konety *et al.* 1996). These activities may be mediated by an up-regulation of the IGF system, especially IGF-I, which has been suggested to be a risk factor for prostate

Figure 3 The effect of 1,25-(OH)₂D₃ (D₃; 10 nM) in combination with either IGF-I (1 and 50 ng/ml) or des(1-3)IGF-I (des; 1 and 50 ng/ml) on cellular proliferation of PECs or P153 cells. **P*<0.05, ***P*<0.01, ****P*<0.001 when compared with control treatment and C=*P*<0.001 when 1,25-(OH)₂D₃ IGFs are compared with IGFs alone.

cancer in men (Chan *et al.* 1998). Increases in the stimulatory components of the system – IGF-I ligand and type 1 IGF receptor – have been shown to be important

events in the initiation of cancer in the transgenic mouse model of prostate cancer (TRAMP) (Kaplan *et al.* 1999).

Figure 4 Western ligand blot of media (left panel) and Northern blot (right panel) from PECs before (C=control) and after a 10 nM 1,25-(OH)₂D₃ treatment (D=1,25-(OH)₂D₃). Note the increase in both IGFBP-3 protein and mRNA following treatment. On autoradiograms exposed for shorter time periods, there was no significant increase noted in IGFBP-2 on the ligand blot. Loading of RNA was controlled for by labeling with an 18S cDNA.

Figure 5 (A) Western ligand blot of media from P153 cells before (C=control) and after a 10 nM 1,25-(OH)₂D₃ treatment (D=1,25-(OH)₂D₃). (B) Western immunoblot of media from P153 cells before and after 10 nM 1,25-(OH)₂D₃ treatment. (C) Northern blot of total RNA collected from the same cells as the media in A and B. Loading of RNA was controlled for by labeling with an 18S cDNA (not shown).

Although 1,25-(OH)₂D₃ has been shown to decrease tumor proliferation, the interaction of 1,25-(OH)₂D₃ with the IGF system in benign human prostate epithelial cell systems has not been reported previously. Modulation of the IGF system and increased IGFBP-3 expression in human cancer cell lines have been correlated with inhibition of tumor cell growth by 1,25-(OH)₂D₃. If such

Figure 6 Exogenous IGFBP-3 (BP-3; 500 ng/ml) was added alone or to media containing IGF-I (10 ng/ml) and cell proliferation of PECs (hatched bars) and P153 cells (solid bars) was assessed by MTT. *** $P < 0.001$ when compared with the control treatment and $a = P < 0.05$ or $c = P < 0.001$ when compared with IGF-I alone treatment.

changes are a mechanism for the prevention of prostate cancer, then the effects of 1,25-(OH)₂D₃ on the IGF system should also be demonstrable in the non-transformed prostate epithelial cell.

We have shown that 1,25-(OH)₂D₃ increases expression of IGFBP-3 in primary cultures of prostate epithelial cells as well as in an immortalized, but non-tumorigenic, prostate epithelial cell line. We propose that the increase in IGFBP-3 functions to suppress IGF-I activity in an IGF-dependent manner; an IGF-independent effect of IGFBP-3 on these cells does not appear to play a significant role since there was no effect when an antibody to IGFBP-3 was added. The antibody used in this study has previously been demonstrated to inhibit IGF-independent actions of IGFBP-3 but does not interfere with ligand binding to IGFBP-3 (Rajah *et al.* 1997).

Further evidence that IGFBP-3 inhibits proliferation by a ligand-dependent mechanism is suggested by the differences in the results between the PECs and P153 cell lines as well as their responses to intact IGF-I or des(1–3)IGF-I. The difference in response to 1,25-(OH)₂D₃ may, in part, be due to the threefold higher vitamin D receptor (VDR) number in the PECs compared with P153 cells (Peehl *et al.* 1994, Gross *et al.* 1996). In PECs, in which neither IGF-I nor des(1–3)IGF-I completely reverses the 1,25-(OH)₂D₃ growth inhibition, the concentration of intact IGFBP-3 is markedly greater than in similarly treated P153 cells. IGF-I did not completely reverse the effect

of 1,25-(OH)₂D₃ in either cell type. However, des(1–3)IGF-I, which binds to intact IGFBP-3 with fivefold less affinity than intact IGF-I, completely reverses the suppressive effect of 1,25-(OH)₂D₃ in P153 cells, in which the upregulation of IGFBP-3 is markedly less than in the PECs. Since the PECs produce more IGFBP-3 protein, they would be expected to successfully sequester the des(1–3)IGF-I from the IGF-IR, whereas the P153 cells would not (Salahifar *et al.* 2000).

Further evidence that the effects of IGFBP-3 are ligand-dependent is indicated by the ineffectiveness of exogenous IGFBP-3, in the absence of additional IGF-ligand, on PEC or P153 cell proliferation. However, the absence of an inhibitory action on cell proliferation, compared with that observed with 1,25-(OH)₂D₃, suggests that 1,25-(OH)₂D₃ can suppress prostate epithelial cell proliferation by non-IGFBP-3 mechanisms as well (Chen *et al.* 1997, Rozen *et al.* 1997, Zhuang & Burnstein 1998, Agarwal *et al.* 1999, Elstner *et al.* 1999, Ly *et al.* 1999, Feldman 2000).

These data suggest that the action of 1,25-(OH)₂D₃ on the prostate epithelial cell is mediated, at least partially, through an increase in IGFBP-3. Regulation of prostate growth, especially the growth of malignant prostate epithelium, has been demonstrated to occur through an interaction of steroid and peptide hormones (Marcelli *et al.* 1995). The present study demonstrates an interaction of the steroid hormone, 1,25-(OH)₂D₃, with the IGF system in non-cancerous prostate epithelium, which may prevent progression to malignant disease. Sequestration of IGF-I from the IGF-IR would prevent receptor activation, an important event in the transformation process since the antiapoptotic activity of the IGF-IR can prevent p53-induced death of cells containing mutations (Baserga 1995, Buckbinder *et al.* 1995, LeRoith *et al.* 1995, Sell *et al.* 1995, Resnicoff & Baserga 1997, Valentinis *et al.* 1997, Baserga 1999). Additionally, specific tyrosine phosphorylation sites of the β -subunit of the IGF-IR have transforming properties separate from those of the antiapoptotic domains (O'Connor *et al.* 1997, Resnicoff & Baserga 1997). Up-regulation of IGFBP-3, by 1,25-(OH)₂D₃, would suppress activation of the IGF-IR, allowing apoptosis to occur (Valentinis *et al.* 1995). This inhibitory effect of IGFBP-3 could provide one explanation for the epidemiological association of increased serum vitamin D levels and putative decreased prostate cancer incidence (Schwartz & Hulka 1990, Corder *et al.* 1995, Correa-Cerro *et al.* 1999, Feldman 2000). In addition to the IGF-dependent activity of IGFBP-3 demonstrated in this study, IGF-independent activities have also been attributed to IGFBP-3, which could be associated with tumor prevention (Buckbinder *et al.* 1995, Valentinis *et al.* 1995, Rajah *et al.* 1997, 1999). Clinically, results of 1,25-(OH)₂D₃ treatment of patients with prostate cancer are consistent with those *in vitro* studies demonstrating suppression of growth (Feldman 2000).

In summary we have demonstrated in primary cultures of human prostate epithelial cells and non-transformed, but immortalized, human primary prostate epithelial cells that there is an increase in IGFBP-3 in response to 1,25-(OH)₂D₃. The 1,25-(OH)₂D₃-induced increase in IGFBP-3 significantly inhibits IGF-I-induced mitogenesis in PECs and P153 cells. This activity would potentially decrease the development of prostate cancer by inhibiting the anti-apoptotic activity of the IGF-IR.

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