Antiproliferative Effects of 1,25-Dihydroxyvitamin D3 on Primary Cultures of Human Prostatic Cells

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ABSTRACT

Cultures of adult human prostatic epithelial and fibroblastic cells were established from normal, benign hyperplastic, and malignant tissues. Vitamin D receptors were detected by ligand binding of [3H]1,25-dihydroxyvitamin D3 [1,25(OH)2D3] in cytosolic extracts prepared from all types of cultures as well as from fresh prostatic tissues. Vitamin D receptor transcripts were demonstrated by Northern blot analysis. 1,25-(OH)2D3 inhibited the growth of epithelial cells with half-maximal inhibition at approximately 1 nM. The growth of fibroblasts was also inhibited by 1,25(OH)2D3 but to a lesser extent. This is consistent with the apparently lower level of vitamin D receptors in fibroblasts compared to epithelial cells determined by ligand binding and Northern analysis of RNA transcripts. The growth inhibition of epithelial cells by 1,25(OH)2D3 was irreversible even after a short 2-h exposure, but morphology and keratin expression were not appreciably altered by long-term exposure to the hormone. A physiological role for 1,25(OH)2D3 in the prostate is postulated, and the inhibitory effect of 1,25(OH)2D3 on cancer-derived prostate cells may provide a basis for new preventive or therapeutic strategies.

INTRODUCTION

Vitamin D affects a wide range of physiological functions within the body including intestinal calcium absorption, bone remodeling, and conservation of minerals at the kidney (1). Like other steroid hormones, vitamin D regulates gene expression in a variety of target cells (2) and has been shown to regulate cell proliferation and mediate differentiation of several cell types including melanoma cells, keratinocytes, and lymphocytes (3-5).

An intracellular VDR3 has been detected in numerous tissues and cultured cells ranging from mouse fibroblasts to human breast cancer cells (2, 6-7). Therefore, it appears that vitamin D may have a much broader physiological impact in the body than previously envisioned with many organs directly subject to vitamin D regulation. A role for vitamin D in the prostate has recently been addressed by the study of several established prostate cancer cell lines. Miller et al. (8) reported that LNCaP cells have VDR and are stimulated to proliferate by 1,25(OH)2D3 in medium supplemented with charcoal-stripped serum. Skowronski et al. (9) also found VDR in LNCaP cells as well as in two other prostate cell lines, PC-3 and DU 145, but observed that 1,25-(OH)2D3 was growth inhibitory in medium with untreated serum. Human prostatic tissues or primary cultures have not been previously characterized. In this study, we analyzed epithelial and fibroblastic cell strains derived from normal, benign hyperplastic and malignant prostatic tissues and found expression of the vitamin D receptor as well as cellular response to vitamin D. Our results indicate that prostatic epithelial and stromal cells are targets of vitamin D and that vitamin D is an important inhibitor of prostatic growth.

MATERIALS AND METHODS

Cell Culture. Tissue samples were dissected from histologically normal PZ, normal CZ, BPH, and cancers of specimens obtained by radical prostatectomy. None of the patients had received any prior hormonal, radiation, or chemical therapy. Following overnight collagenase digestion of the tissue, epithelial cultures were derived according to previously published protocols (10). Primary and serial cultures of epithelial cells were maintained in medium PFMFR-4A (11) supplemented with cholecalciferol (10 ng/ml), epidermal growth factor (10 ng/ml), bovine pituitary extract (10 μg/ml), insulin (4 μg/ml), hydrocortisone (1 μg/ml), phosphoethanolamine (0.1 mM), selenous acid (3 × 10-4 M), α-tocopherol (2.3 × 10-5 M), retinoic acid (3 × 10-11 M), and gentamicin (100 μg/ml). Dishes coated with type I collagen were routinely used. The epithelial nature of cultures obtained by these methods was verified by immunocytochemical labeling of keratin and prostate-associated antigens. Nomenclature for epithelial cell strains is “E” followed by the histology of origin and then the strain number (e.g., E-CZ-1).

Fibroblastic cell strains were established by inoculating collagenase-digested tissue into MCDB 105 (Sigma Chemical Co., St. Louis, MO) supplemented with fetal bovine serum (10% v/v) and gentamicin (100 μg/ml). Cell strains derived by this method were not labeled by anti-keratin but were stained by antibodies against vimentin and fibronectin. Nomenclature for fibroblastic cell strains is “F” followed by the histology of origin and the strain number (e.g., F-BPH-1).

MCF-7 (human breast cancer cells) and J1 and J2 (skin fibroblasts derived from a patient with HVDDR and a phenotypically normal sibling, respectively) were maintained as described previously (12, 13).

Clonal Growth Assays. Previously described protocols to measure clonal growth of prostatic epithelial cells were followed (14) except that MCDB 105 replaced PFMFR-4A as the basal medium. Two hundred cells were inoculated into each 60-mm dish containing experimental medium. After 10 days of incubation, the cells were fixed and stained. The total area of each dish that was covered by cells was measured with an Artrek image analyzer (Dynatech, Chantilly, VA).

A similar protocol was used for fibroblasts except that 500 cells were inoculated per dish into MCDB 105 supplemented with 10% fetal bovine serum, and the dishes were incubated 14 days before fixation.

1,25(OH)2D3 was obtained from Biomol (Plymouth Meeting, PA). 1,28,25-(OH)3D3, 24,25(OH)2D3, and 25(OH)D3 were the generous gifts of Dr. M. Uskokovic (Hoffman La Roche Co., Nutley, NJ). Concentrated stocks were prepared in ethanol and stored at -20°C. The concentration of ethanol in media did not exceed 0.01%.

VDR Binding Analysis. Cells were grown to near-confluency, rinsed twice with ice-cold PBS, and harvested by scraping with a rubber policeman without prior trypsinization. After an initial wash in PBS, cell pellets were resuspended in KTEDM buffer (0.3 mM KCl, 10 mM Tris-Cl, pH 7.4, 1.5 mM EDTA, 1 mM dithiothreitol, and 10 mM sodium molybdate) containing a protease inhibitor cocktail (10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 μg/ml aprotinin). Cells were disrupted by sonication on ice. The sonicate was centrifuged at 207,000 g for 35 min at 4°C to obtain a soluble extract used for binding studies. In a typical, single-point saturation experiment, 200 μl of soluble extract (1-2 μg protein/ml) were incubated with 1 nM of 1α,25(OH)2[23,24(n)-3H]vitamin D3 (specific activity, 102 Ci/mmol; Amersham Chemical Co., Arlington Heights, IL) for 16-20 h at 4°C with or without a 250-fold excess of radiolabeled vitamin D3 (a gift from Dr. M. Uskokovic). For equilibrium binding experiments, increasing concentrations (0.02 to 0.8 nM) of 1α,25(OH)2[26,27 methyl-3H]vitamin D3 (specific activity, 174 Ci/mmol) were incubated with cytosolic extracts from 16-20 h at 4°C. Bound and free hormone were separated by hydroxylapatite method (12). The protein concentration of soluble extract samples was measured by the method of Lowry et al. (15).
of Bradford (15). Specific binding was calculated by subtracting non-specific binding obtained in the presence of a 250-fold excess of radioinert 1,25(OH)_2D_3 from the total binding measured in the absence of radioinert steroid. The apparent dissociation constant (K_d) and total number of binding sites (N_max) were calculated by Scatchard analysis (16).

**Northern Blot Analysis.** Total cellular RNA was isolated by the 4 ml LiCl-8 m urea precipitation method of Auffray and Rougeon (17). Ten μg of each RNA sample were fractionated by electrophoresis in 1% agarose/0.66 m formaldehyde gels. An RNA ladder (0.24-9.5 kilobases; Bethesda Research Laboratories, Gaithersburg, MD) was used to determine the size of the RNA bands. Fractionated RNA was transferred to nylon (Hybond-N; Amersham) by capillary action in 10× standard saline citrate (1× standard saline citrate = 150 mM sodium chloride and 15 mM sodium citrate, pH 7) at room temperature. After transfer, the nylon blots were probed with a 2.1 kilobase EcoRI cDNA fragment containing the entire coding sequence for the human VDR (18) labeled with [32P]dCTP by the random primed method (Boehringer Mannheim Biochemicals, Indianapolis, IN). To control for RNA sample loading and transfer, Northern blots were also probed with a [32P] labeled 0.9 kilobase EcoRI fragment of the human cDNA for the ribosomal protein gene L7 (19). Blots were exposed to Kodak XAR film at —80°C and developed.

**Immunoblot Analysis.** Cell extracts were prepared in lysis buffer containing 0.1 M Tris (pH 6.8), 1% sodium dodecyl sulfate, 5% glycerol, 1% β-mercaptoethanol, 0.005% bromphenol blue, 0.005% pyronin Y, and 0.01% aprotonin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (20) using a gel of 10% acrylamide. After separation, proteins were transferred to nitrocellulose filter according to Towbin et al. (21) using a Transblot unit (Hoefer Scientific Instruments, San Francisco, CA) operated at 1.5 A for 1.25 h. Strips of nitrocellulose were blocked overnight with 10% horse serum in PBS before incubating with primary antibodies. Anti-keratin antibody 902 (specific for keratin 8) was obtained from Enzo Diagnostics (New York, NY). Antibody against keratin 10 (8.60) was purchased from ICN (Lisle, IL), and antibody against keratin 18 (CKS) was obtained from Sigma. Bound antibodies were detected using biotinylated secondary antibody and the ABC reagent (Vector Laboratories, Burlingame, CA). The substrate chloronapthol was used as a color reagent.

**RESULTS**

**Binding of [3H]1,25(OH)_2D_3 by Prostate Tissues and Cells.** The presence of VDR in prostate tissue freshly removed at surgery was studied by radioligand binding methods using 1 nM of [3H]1,25(OH)_2D_3 in a single-point saturation binding assay. Each tissue sample exhibited specific binding at levels ranging from 10 to 22 fmole/mg of protein (Table 1). VDR were also analyzed in primary cell cultures. Sixteen epithelial and four fibroblastic cell strains derived from normal PZ, normal CZ, BPH, or cancer also specifically bound [3H]1,25(OH)_2D_3. The extent of binding among epithelial cell strains varied from 13 to 79 fmole/mg of protein, but there was no discernible association between extent of binding and histology of origin. The fibroblastic cell strains bound 6 to 19 fmole/mg of protein, which was somewhat lower binding overall than that exhibited by the epithelial cell strains. No binding was detectable in one fibroblastic cell strain.

**Scatchard Analysis.** We next further characterized VDR in prostate cell strains by Scatchard analysis (16). The data from equilibrium binding studies using various concentrations of [3H]1,25(OH)_2D_3 are shown in Fig. 1. Fig. 1A illustrates dose-response saturation plots obtained after correction for nonspecific binding. In two representative epithelial cell strains, specific binding reached saturation at about 0.4 nM of [3H]1,25(OH)_2D_3. Scatchard plots of specific binding data are shown in Fig. 1B. Linear regression analysis shows a single class of specific, high affinity, saturable receptors with similar apparent dissociation constants (K_d) of 1.05 X 10^-10 M and 1.33 X 10^-10 M for E-PZ-2 and E-CA-3 strains, respectively. These values are in excellent agreement with VDR measurements in classical target organs (1, 7). Corresponding binding capacity (N_max) for the two epithelial strains were 62 and 41 fmol/mg of protein, respectively, confirming the VDR abundance range in other epithelial cell strains screened by one-point

**Table 1 Binding of 1,25(OH)_2D_3 to prostatic tissues or cells**

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>No. of specimens</th>
<th>Mean (fmole/mg protein)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tissue</td>
<td>4</td>
<td>17</td>
<td>10-22</td>
</tr>
<tr>
<td>E-PZ or CZ</td>
<td>3</td>
<td>18</td>
<td>15-55</td>
</tr>
<tr>
<td>E-BPH</td>
<td>9</td>
<td>24</td>
<td>24-51</td>
</tr>
<tr>
<td>F-CA</td>
<td>2</td>
<td>18</td>
<td>9-27</td>
</tr>
<tr>
<td>F-BPH</td>
<td>6</td>
<td>19</td>
<td>0-11</td>
</tr>
<tr>
<td>F-CA</td>
<td>1</td>
<td>19</td>
<td>0-19</td>
</tr>
</tbody>
</table>

* Measurements were made by single-point saturation binding assay with 1 nM [3H]-1,25(OH)_2D_3 as described in "Materials and Methods."
* Two samples were obtained from normal PZ, one sample was from an area containing about 40% cancer (Gleason pattern 3/3), and the other was of unknown histology.
* Binding was undetectable in one specimen.
METHODS. A, Lane 1, F-BPH-3; Lane 2, E-PZ-8; Lane 3, MCF-7; Lane 4, Ji; Lane 5, J2. B, the same filter was stripped and rehybridized to an L7 ribosomal protein cDNA extracted. After size fractionation and Northern transfer, the blot was probed with [32P]-labeled 2.1-kilobase human VDR cDNA fragment as described in "MATERIALS AND METHODS." A, Lane 1, F-BPH-3; Lane 2, E-PZ-8; Lane 3, MCF-7; Lane 4, Ji; Lane 5, J2. B, the same filter was stripped and rehybridized to an L7 ribosomal protein cDNA probe as a control for RNA loading and transfer.

VDR Transcripts. Northern blot analysis was performed with total RNA extracted from prostatic epithelial and fibroblastic cell strains and hybridized with a human VDR cDNA probe. A hybridization band of approximately 4.6 kilobases was noted in the RNA from each cell strain (Fig. 2). A similar VDR transcript was demonstrated in MCF-7 human breast carcinoma cells, a positive control known to express normal levels of biologically active VDR (13), and J2, fibroblasts from an unaffected sibling of a patient with HVDRR (12). In contrast, VDR mRNA was not detected in a negative control sample from J1, fibroblasts from an individual with HVDRR (12). The level of VDR expression appeared lower in the prostatic fibroblast cell strain compared to the epithelial strain when loading and transfer were controlled for by expression of the ribosomal protein gene L7 (Fig. 2). This is in agreement with the lower levels of VDR abundance found in fibroblastic strains in the ligand binding experiments (Table 1).

Growth Inhibitory Effects of 1,25(OH)2D3. Prostatic fibroblasts were inoculated at 500 cells/dish into MCDB 105 supplemented with 10% fetal bovine serum and concentrations of 1,25(OH)2D3 ranging from 0.025 to 25 nm. After 14 days of incubation, growth was quantitated and compared to that in medium without added hormone (Fig. 3). Fibroblasts derived from normal, BPH, or malignant tissues were similarly inhibited by 1,25(OH)2D3 with maximal inhibition of about 50% of growth occurring at 25 nm. 1,24,25(OH)3D3 and 24,25(OH)2D3 were not as inhibitory as 1,25(OH)2D3 and only inhibited growth by about 40% at 25 nm, whereas 25(OH)D3 was with no effect even at 25 nm (not shown).

Clonal growth assays in serum-free medium were used to measure the effect of 1,25(OH)2D3 on the proliferation of prostatic epithelial cells. Fig. 4 shows that the growth of cell strains derived from normal, benign hyperplastic, and malignant tissues was inhibited by 1,25(OH)2D3. The half-maximal inhibitory dose did not vary depending on the histology of origin and was approximately 0.25–1 nm. At 25 nm of 1,25(OH)2D3, growth was completely inhibited. 1,24,25(OH)3D3 inhibited cells similarly to 1,25(OH)2D3 with half-maximal inhibition also at approximately 1 nm and complete inhibition at 25 nm, but 24,25(OH)2D3 and 25(OH)D3 were without effect even at 25 nm (data not shown). The relative potency of these different compounds is compatible with a VDR-mediated process.

Thus benign as well as malignant prostatic epithelial cells were sensitive to the effects of vitamin D. Although it is difficult to directly compare the growth inhibition by 1,25(OH)2D3 of epithelial versus fibroblastic cells due to the necessity of using different culture conditions, it appeared that 1,25(OH)2D3 was a more potent inhibitor of epithelial than fibroblastic growth. This finding possibly relates to the lower level of VDR in the prostatic fibroblasts.

Irreversible Growth Inhibition by 1,25(OH)2D3. Prostatic epithelial cells were inoculated into culture dishes at clonal densities and were exposed to 25 nm of 1,25(OH)2D3 for 2, 4, 8, or 24 h or for 10 days. As expected from our previous studies, no growth occurred in dishes containing cells which were exposed to 1,25(OH)2D3 continuously for 10 days. In fact, even limited duration of exposure to 1,25(OH)2D3 severely inhibited the ability of the cells to recover and proliferate after removal of the hormone. After 2 h in medium containing 1,25(OH)2D3, no growth resulted even if the medium was changed to hormone-free medium for the remainder of the 10-day clonal assay (Fig. 5). This lack of growth was not due to overt apoptosis or cell lysis since individual cells from the original inoculum were still visible in the culture dishes at the end of the 10-day assay. Rather, 1,25(OH)2D3 appeared to put the cells into a state from which they could not re-enter the proliferative cycle.

Effect of 1,25(OH)2D3 on Morphology. Semiconfluent cultures of prostatic epithelial cells were exposed to medium containing 25 nm of 1,25(OH)2D3 for 3 days. At the end of that period, the cells were fixed and stained, and their morphology was compared to that of control cultures grown in medium without hormone. No obvious differences in morphology were perceived between the cultures maintained with or without 1,25(OH)2D3 (Fig. 6). This was in contrast to
striking morphological changes which are induced in prostatic epithelial cells by other growth inhibitory factors such as transforming growth factor-β, vitamin A, or suramin (22). Therefore, despite the fact that 1,25(OH)2D3 was a potent and irreversible inhibitor of prostate cell growth, neither cell death nor morphological changes accompanied this antiproliferative effect.

Evaluation of Differentiation by Analysis of Keratin Expression. Growth inhibition may be mediated by many mechanisms. One means would be by the induction of a terminally differentiated phenotype in the exposed target cell. To examine the possibility that this mechanism was involved in the growth inhibition of prostate cells by 1,25(OH)2D3, we examined the pattern of keratin expression in epithelial cells by immunoblot analysis. Keratins 8, 10, and 18 were chosen as particularly interesting because of their associations with certain phenotypes. Keratin 10 is expressed in terminally differentiated squamous epithelial cells (23), whereas keratins 8 and 18 are expressed in the differentiated secretory luminal cells of the prostatic epithelium (24).

Fig. 7 shows that the pattern of keratin expression in normal, BPH, or malignant prostatic epithelial cells was unchanged by exposure to 25 nm of 1,25(OH)2D3 for 3 days. Keratins 8 and 18 were visible in immunoblots derived from cells growing in the absence of vitamin D, and the intensity of either keratin band was not appreciably altered by exposure of cells to 1,25(OH)2D3. Keratin 10 was generally faint or undetectable in cultures growing in standard medium, and 1,25(OH)2D3 did not induce increased expression of this keratin. These results suggest that 1,25(OH)2D3 did not inhibit growth of prostatic epithelial cells by enhancing secretory differentiation or inducing squamous differentiation.

**DISCUSSION**

We have demonstrated that cultured human prostatic epithelial and fibroblastic cells, as well as fresh whole prostatic tissues, specifically bound [3H]1,25(OH)2D3. Further evidence of VDR in prostate cells was provided by Northern blot detection of VDR transcripts. These results indicate that the VDR is present in both the epithelial and stromal compartments of the prostate. However, by all analyses, VDR appeared to be present at higher concentrations in the epithelial cells, perhaps indicating a more prominent role for 1,25(OH)2D3 in the prostatic epithelium compared to the stroma.

Functional VDRs were indicated by the growth responses of epithelial and fibroblastic cells to 1,25(OH)2D3. Vitamin D was a potent inhibitor of prostatic epithelial cell growth with a half-maximal inhibitory dose of approximately 1 nm in clonal growth assays with serum-free medium and complete inhibition at 25 nm. Cancer-derived and benign cells were equally inhibited by 1,25(OH)2D3. No stimulatory effects of 1,25(OH)2D3 were noted. This is in contrast to the finding of Miller et al. (8) that growth of LNCaP cells in medium supplemented with charcoal-stripped serum was slightly increased by 1 nm of 1,25(OH)2D3. However, Skowronska et al. (9) found that LNCaP, as well as a second prostate cancer cell line PC-3, were substantially growth-inhibited by 1 nm of 1,25(OH)2D3 in standard culture conditions with medium containing 5 to 10% untreated serum. These differences in charcoal-stripped serum cultured LNCaP cells may be explained by the interaction of 1,25(OH)2D3 with other growth factors and inhibitors and must be further explored. Nevertheless, the predominant effect of 1,25(OH)2D3 treatment of primary cell cultures (this study) and prostate cancer cell lines (9) is growth inhibition.

Fibroblasts were apparently less sensitive to the growth-inhibitory effects of 1,25(OH)2D3 with a 50% reduction in growth occurring at approximately 25 nm of vitamin D. The seemingly lesser response of fibroblasts compared to epithelial cells could partially be explained by the presence of vitamin D binding protein in the serum-supplemented medium in which the fibroblast assay was carried out. In addition, the lower number of VDR in fibroblasts compared to epithelial cells probably more importantly contributes to the lesser response of fibroblasts.

Exposure of prostatic epithelial cells to 1,25(OH)2D3 for varying periods of time followed by lack of recovery in medium without vitamin D indicated that the action of 1,25(OH)2D3 was not reversible. After even a brief exposure of 2 h, the cells could not recover and grow in clonal assays. However, the inhibitory effect of vitamin D was not accompanied by dramatic phenotypic changes or cell death. Even
after 3 days of exposure to 25 nM of 1,25(OH)2D3, morphology was not altered, and keratins 8 and 18, associated with the phenotype of secretory cells of the prostatic epithelium, were not increased. Metaplasia was also not induced in prostate cells in response to vitamin D because keratin 10, associated with squamous differentiation, did not become more prominent.

In other studies, we have noted that altered keratin expression does not consistently accompany growth inhibition but is specific for particular growth inhibitory factors. For example, transforming growth factor-β and suramin inhibit prostatic epithelial growth but do not alter keratin expression (22, 25). Retinoic acid increases keratins 8 and 18 and prevents the development of squamous metaplasia in superconfluent cultures, while absence of glucose or growth factor supplements induce squamous metaplasia (25, 26). Nevertheless, keratins are just one measure of cellular phenotype and induction of other aspects of differentiation by 1,25(OH)2D3 should not be ruled out. In this regard, Miller et al. (8) and Skowronski et al. (9) noted that the secretion of PSA by LNCaP cells was increased by 1,25(OH)2D3. Primary cultures of prostatic epithelial cells, in contrast to LNCaP cells, secrete very low levels of PSA, and ultrasensitive methods to measure PSA, which are under development, will probably be required before we can determine whether 1,25(OH)2D3 enhances PSA expression in primary cultures.

Prostatic epithelial and stromal cells thus join the growing ranks of different cell types which possess VDR and respond to vitamin D treatment. As for other steroid hormones, the mechanism of action of 1,25(OH)2D3 is very complex, and further studies will be required to elucidate the means by which vitamin D regulates the growth of prostate cells. The finding that cells derived from prostatic adenocarcinomas were inhibited by vitamin D may be of particular interest and may indicate a potential role for 1,25(OH)2D3 or analogues with less hypercalcemic potency (27) in therapy.

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