Vitamin D Receptor Expression, 24-Hydroxylase Activity, and Inhibition of Growth by 1α,25-Dihydroxyvitamin D3 in Seven Human Prostatic Carcinoma Cell Lines

Gary J. Miller, Tammy E. Hedlund, and Kirsten A. Moffatt

ABSTRACT

Although prostatic cancer is often viewed as an androgen-dependent malignancy, a number of other hormones including 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) are now recognized to modulate its growth and differentiated phenotype. Seven different continuous human prostatic carcinoma cell lines were examined for the presence of bioologically active receptors for 1α,25(OH)2D3. All seven lines were found to contain mRNA for the vitamin D receptor using an RNase protection assay. Six of the seven cell lines were found to have high-affinity saturable binding sites for 1α,25(OH)2D3. The seventh line was found to contain vitamin D receptors by sucrose gradient analysis. All seven lines were found to express 24-hydroxylase activity by a HPLC assay that measures the conversion of 25-hydroxyvitamin D3 to 24,25-dihydroxyvitamin D3. 24-Hydroxylase activity was up-regulated in all seven cell lines by preincubation with 1α,25(OH)2D3. In the presence of fetal bovine serum, the growth of four of the seven cell lines was inhibited. In the majority of cell lines growth inhibition was related not only to the number of receptors per cell, but also in inverse proportion to the 24-hydroxylase activity of each cell line. The ubiquitous presence of vitamin D receptor and 24-hydroxylase activity in human prostatic carcinoma cells suggests new alternatives for the pharmacological treatment of advanced prostatic cancer and implies that chemoprevention strategies could also make use of this endocrine axis.

INTRODUCTION

The secosteroid 1α,25(OH)2D3 is now recognized to have several significant functions beyond the regulation of calcium homeostasis (1, 2). Over the last 10 years, numerous examples of effects on growth and differentiation of benign and malignant mammalian cells have been described. These actions are believed to be mediated, at least in part, through nuclear 1α,25(OH)2D3 receptors (VDr) that have homology with the nuclear receptors for sex steroids, retinoids, and the thyroid hormone (3). Unlike the sex steroid receptors that carry out their functions as homodimers for a single ligand, the VDr is also known to form heterodimers with the retinoid X receptor (4). Recent data suggest that ligand binding to the VDr actually enhances the formation of heterodimers with retinoid X receptor and alters its ability to regulate transcription (5). In addition to direct effects on transcription, it is likely that the effects of 1α,25(OH)2D3 on cell differentiation involve other signaling pathways including G proteins, phosphoinositide metabolism, and protein kinase C (6).

Prostatic cancer is now recognized as the most common cancer of males in the United States (7). Although its cause remains unknown, recent epidemiological data suggest that vitamin D may play a role in the progression of clinical disease. Schwartz and Hulka (8) have proposed that the recognized risk factors of age and race are related to prostate cancer mortality in combination with UV exposure as a form of vitamin D deficiency disease. Recent findings have supported this hypothesis and suggest that about 6% of the mortality from prostate cancer in the United States could be related to UV exposure (9). It has also been found that low serum vitamin D levels correlate with specific subgroups of patients who develop palpable or anaplastic carcinoma of the prostate (10).

We have previously shown that the androgen-responsive prostatic carcinoma cell line LNCaP contains specific, high-affinity nuclear receptors for 1α,25(OH)2D3 (11). These VDr were found to be capable of modulating the growth and differentiation (expression of prostate-specific antigen) of LNCaP cells. We have also presented preliminary evidence that other continuous human prostatic carcinoma (HPCa) cell lines (DU145 and PC-3) contain similar 1α,25(OH)2D3 binding sites (12). We have now extended these studies by characterizing the vitamin D3 receptors of these as well as four additional HPCa cell lines. In addition, we have investigated the biological activity of these VDr in the seven cell lines as a function of the induction of 24-hydroxylase activity by 1α,25(OH)2D3 and/or growth regulation in the presence of FBS. Our results indicate that the presence of VDr in prostatic carcinoma cell lines is ubiquitous, and that the regulation of growth by 1α,25(OH)2D3 may be affected by the number of VDr/cell and/or the ability of cells to metabolize this hormone.
MATERIALS AND METHODS

Nonlabeled 25(OH)D$_3$, 1α,25(OH)$_2$D$_3$, and 24,25(OH)$_2$D$_3$ were generous gifts from Dr. M. Uskokovic (Hoffman LaRoche, Nutley, NJ). [26,27-methyl-3H]1α,25(OH)$_2$D$_3$ (specific activity, 178–180 Ci/mmol), [26,27-methyl-3H]25(OH)$_2$D$_3$ (specific activity, 175 Ci/mmol), and R1881 were obtained from New England Nuclear (Boston, MA).

Cell Lines and Culture. LNCaP cells (13, 14) were obtained from Dr. J. Horoszewicz (Roswell Park Memorial Institute, Buffalo, NY) at the 14th passage and were used at approximately passage 34. The DU145 (15) and PC-3 (16) cell lines were obtained from American Type Culture Collection (Rockville, MD) and were used at passages 144 and 29, respectively. ALVA-31 cells (17) were obtained from Dr. S. Loop (American Lake Veterans Administration Hospital, Tacoma, WA) and were used at passage 35. PPC-1 cells (18) were obtained from Dr. A. Brothman (University of Utah, Salt Lake City, UT) and used at passage 15. TSU-Pr1 cells (19) were obtained from Dr. J. Isaacs (The Johns Hopkins Cancer Center, Baltimore, MD) and were used at passage 33. JCA-1 cells (20) were obtained from Dr. J. Chiao (New York Medical College, Valhalla, NY) and were used at passage 27. All of the cell lines were maintained by serial passage in RPMI 1640 medium containing 10% FBS and were grown for an additional 4 days. The total DNA content of the cells were plated in triplicate in medium containing 10% FBS 0.26 X 10$^3$/cm$^2$; and were transferred into multiwell plates at the following densities: comparison to the original published karyotypes.

Material and Methods

were maintained by serial passage in RPMI 1640 medium contain-

growth inhibition was calculated as the percentage of difference of the treated cells

culminated using linear gradients of 5–20% sucrose in 300 mM KCl, 1 mM EDTA, 5 mM DTT, 10 mM Tris-HCl, and 10 mM NaMo (pH 7.4). Aliquots of cytosol (0.2 ml) were incubated with 1.5 nm $[^{3}H]$1α,25(OH)$_2$D$_3$ for 3 h at 4°C in the dark. The labeled cytosol was treated with dextran-coated charcoal to remove unbound ligand. Labeled VDRs were applied to the tops of gradients and were centrifuged in a SW55Ti rotor at 45,000 rpm (234,000 × g) for 18 h at 4°C. Fractions (0.2 ml) were collected from the bottoms of the centrifuge tubes and were added directly to 5 ml Ecolume (ICN Biochemical, Costa Mesa, CA) for scintillation counting. Competition controls were performed by adding a 200-fold molar excess of 1,25(OH)$_2$D$_3$ to the tubes during the incubation with the labeled ligand. Gradient shift assays were performed using the 9A7γ (22) rat monoclonal antichicken VDR antibody (a generous gift from Dr. J. W. Pike, Ligand Pharmaceuticals, San Diego, CA) that recognizes a 17-amino acid epitope located near the binding domain of the VDr (23). After labeling the receptors with the radiolabeled ligand, 20 µg 9A7γ were added to the cytosol and incubated for another 2.5 h at 4°C. The receptor/antibody complexes were then subjected to centrifugation as described above.

Ribonuclease (RNase) Protection Assay. Initial attempts to demonstrate the presence of hVDR mRNA by Northern blot analysis using our previous approach (11) revealed only questionable reactions in some of the cell lines examined (data not shown). In view of this, it was decided to carry out further analysis using RNase protection assays.

The plasmid RPVDr (a generous gift from Dr. S. R. T. Evans, Georgetown University, Washington, DC) containing cDNA sequences to the human 1α,25(OH)$_2$D$_3$ receptor (24) was propagated in Escherichia coli DH1. The plasmid was purified using a Wizard kit (Promega, Madison, WI) and linearized with NcoI. A radiolabeled riboprobe of 350 bp corresponding to a 319-bp protected fragment of the 3’ coding region of the hVDR was synthesized using a MaxScript kit (Ambion, Inc., Austin, TX) and T7 RNA polymerase. This corresponds to amino acids 289 through 424 of the hVDR.

Total cellular RNA from each of the cell lines and normal human liver was isolated using a guanidinium thiocyanate method (25). Liver was chosen as a negative control (26). RNase protection assays were carried out using an RPAII kit (Ambion, Inc.). The 32P-labeled riboprobe and a 32P-radiolabeled GAPDH probe (Ambion, Inc.) were incubated with whole RNA extracts overnight at 45°C. The GAPDH probe was used as an internal loading control. The samples were incubated with a cocktail of RNase A/T1 at 50°C for 45 min to degrade the unprotected sequences. The product of the reaction was run on a 5% polyacrylamide/urea gel. Autoradiography was performed for 4 days at −70°C to detect the hVDR. The assays were performed in triplicate.

25(OH)D$_3$-24-Hydroxylase Assays. The activity of the mitochondrial enzyme 24-steroid hydroxylase (P450 c24) was quantitated by measuring the conversion of $[^{3}H]25$(OH)$_2$D$_3$ into $[^{3}H]24,25$(OH)$_2$D$_3$ essentially as described by Gamblin et al. (27). Cultured cells were preincubated with varying concentrations of unlabeled 1α,25(OH)$_2$D$_3$ (10$^{-10}$ M or 10$^{-8}$ M) or ethanol vehicle for 15 h at 37°C. The cells were then trypsinized

20,000 rpm. The homogenates were ultracentrifuged at 200,000 × g at 4°C for 30 min. Sedimentation coefficients were estimated using linear gradients of 5–20% sucrose in 300 mM KCl, 1 mM EDTA, 5 mM DTT, 10 mM Tris-HCl, and 10 mM NaMo (pH 7.4). Aliquots of cytosol (0.2 ml) were incubated with 1.5 nm $[^{3}H]$1α,25(OH)$_2$D$_3$ for 3 h at 4°C in the dark. The labeled cytosol was treated with dextran-coated charcoal to remove unbound ligand. Labeled VDRs were applied to the tops of gradients and were centrifuged in a SW55Ti rotor at 45,000 rpm (234,000 × g) for 18 h at 4°C. Fractions (0.2 ml) were collected from the bottoms of the centrifuge tubes and were added directly to 5 ml Ecolume (ICN Biochemical, Costa Mesa, CA) for scintillation counting. Competition controls were performed by adding a 200-fold molar excess of 1,25(OH)$_2$D$_3$ to the tubes during the incubation with the labeled ligand. Gradient shift assays were performed using the 9A7γ (22) rat monoclonal antichicken VDR antibody (a generous gift from Dr. J. W. Pike, Ligand Pharmaceuticals, San Diego, CA) that recognizes a 17-amino acid epitope located near the binding domain of the VDr (23). After labeling the receptors with the radiolabeled ligand, 20 µg 9A7γ were added to the cytosol and incubated for another 2.5 h at 4°C. The receptor/antibody complexes were then subjected to centrifugation as described above.

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and washed three times with medium A (Eagle's MEM, 0.584 g/liter l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mm Tricine-HCl, pH 7.4) by centrifugation. The cells were then resuspended in medium A containing 1% fetal bovine serum. Two × 10^6 cells in 0.2 ml were added to a glass tube containing 100 μl of radiolabeled 25(OH)D3 (specific activity, 177 Ci/mmol). Incubation was carried out at 37°C for 30 min with periodic vortexing. Nonlabeled 25(OH)D3 (3.2 × 10^-5 M), 24,25(OH)_{2}D_3 (3.2 × 10^-4 M), and 1α,25(OH)_{2}D_3 (3.2 × 10^-4 M) were added as reference standards. The reaction was stopped by adding 0.62 ml chloroform:methanol (2:1) and vortexing. After 1 h at 24°C, the reaction mixtures were centrifuged to remove insoluble debris. The supernatants were added to tubes containing 0.2 ml chloroform, 0.1 ml H2O, and vortexed. After centrifugation, the lower organic phase was removed and saved. The aqueous phase was reextracted with an additional 0.4 ml chloroform, vortexed, and centrifuged. The organic phase was recovered and pooled with that from the first extraction. The pooled organic phases were reduced to dryness with a stream of nitrogen gas. The residue was redissolved in methanol, and subjected to periodate oxidation as described above.

### RESULTS

Using a whole-cell binding assay, we have previously reported (11) that LNCaP cells contain high-affinity (K_d = 1.4 × 10^-9 M) receptors for 1α,25(OH)_{2}D_3. The same assay confirmed the presence of similar, saturable, and specific binding sites in five of the other six cell lines (Fig. 1). Addition of a 200-fold molar excess of nonlabeled 1α,25(OH)_{2}D_3 consistently reduced binding to less than 10%. However, as previously found for LNCaP cells, a 250-fold excess of R1881 produced no significant competition. Scatchard rearrangement of the data revealed varying numbers of binding sites ranging from approximately 1000 to 8000/cell with K_d ranging from 6.3 × 10^-9 to 5 × 10^-10 M (Table 1). Specific binding of 1α,25(OH)_{2}D_3 to the nuclei of JCA-1 cells could not be demonstrated with the whole-cell binding assay. The final nuclear pellets from these cells were much smaller than those obtained from the other six cell lines, apparently due to nuclear lysis, which could account for our inability to detect binding.

The results of a sucrose gradient analysis of JCA-1 cell cytosol incubated with [3H]1α,25(OH)_{2}D_3 are presented in Table 1. Inset, Scatchard rearrangement of [3H]1α,25(OH)_{2}D_3 binding in the absence of R1881.

### Table 1 1α,25(OH)_{2}D_3 Receptors in human prostatic carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>K_d (mean ± SE)</th>
<th>Receptor no./cell (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC-1</td>
<td>3.2 ± 3.0 × 10^-9</td>
<td>600 ± 300</td>
</tr>
<tr>
<td>PC-3</td>
<td>1.3 ± 0.3 × 10^-9</td>
<td>800 ± 100</td>
</tr>
<tr>
<td>DU145</td>
<td>0.6 ± 0.1 × 10^-9</td>
<td>1100 ± 100</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>1.2 ± 0.7 × 10^-9</td>
<td>1200 ± 200</td>
</tr>
<tr>
<td>LNCaP</td>
<td>1.4 ± 0.1 × 10^-9</td>
<td>2900 ± 300</td>
</tr>
<tr>
<td>ALVA-31</td>
<td>0.5 ± 0.1 × 10^-9</td>
<td>9700 ± 1400</td>
</tr>
<tr>
<td>JCA-1</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

*Means represent multiple (2–8) determinations/cell line, each performed in triplicate.

* N/D, not detected.
out of proportion to the measured numbers of receptors. There was a weak signal for the presence of hVDR mRNA in human liver extracts. This could represent either very low levels of expression of hVDR by hepatocytes or the contribution of contaminating stromal cells.

Isocratic HPLC was consistently capable of resolving the vitamin D metabolite standards and comigrating radioactive metabolites as seen in Fig. 4. All of the seven prostatic carcinoma cell lines were found to express baseline levels of 24-

hydroxylase activity as presented in Table 2. Addition of 10^{-8} M 1α,25(OH)_{2}D_{3} to the cultures for 15 h prior to enzyme assay caused an increase in activities in each of the cell lines ranging from 98 to 1005%. In addition, LNCaP cells were found to increase their 25(OH)D_{3}-24-hydroxylase activity in response to 1α,25(OH)_{2}D_{3} in a dose-dependent fashion. Periodate treatment of the putative 24,25(OH)_{2}D_{3} peak identified from an LNCaP cell extract abolished its radioactivity following rechromatography consistent with the gem-diol structure of 24,25(OH)_{2}D_{3} (Fig. 5). Loss of the radioactive peak was not seen in the aliquot pretreated with water alone.

The nasopharyngeal carcinoma cell line COLO 2650 was originally reported to contain no measurable 1α,25(OH)_{2}D_{3} nuclear receptors (28). Baseline 25(OH)D_{3}-24-hydroxylase activity in this cell line was 10-fold less than that measured in the prostatic carcinoma cell lines. Likewise, preincubation of COLO 2650 cells with 10^{-8} M 1α,25(OH)_{2}D_{3} resulted in stimulation of 25(OH)D_{3}-24-hydroxylase activity, although the stimulated levels remained less than the activity/cell found in nearly all of the prostatic carcinoma cell lines tested.

In addition to the labeled 24,25(OH)_{2}D_{3} peak, the chromatograms for each of the seven cell lines also revealed the presence of two other labeled peaks. The first was eluted between authentic 24,25(OH)_{2}D_{3} and 1α,25(OH)_{2}D_{3}. This peak most likely represents a more distal metabolite of 24,25(OH)_{2}D_{3} such as 24-oxo-25(OH)_{2}D_{3}. The second comigrated with 1α,25(OH)_{2}D_{3}. While it is possible that this represents 1α,25(OH)_{2}D_{3}, it is more likely that it represents 19-nor-10-keto-25-hydroxyvitamin D_{3}, which comigrates with 1α,25(OH)_{2}D_{3} in this solvent system (29).

Finally, each of the seven cell lines was tested for growth sensitivity to 1α,25(OH)_{2}D_{3} in the presence of FBS (Table 3). Only LNCaP, ALVA-31, PPC-1, and DU145 were found to exhibit statistically significant growth inhibition following addition of 10^{-8} M 1α,25(OH)_{2}D_{3}. The other three cell lines (JCA-1, PC-3, and TSU-Pr1) showed neither inhibition nor stimulation of growth.
DISCUSSION

The results presented here confirm our earlier reports (11, 12) regarding the presence of high-affinity VDr in the LNCaP, PC-3, and DU145 prostatic cancer cell lines. In addition, we have now found that four additional lines (PPC-1, ALVA-31, TSU-Pr1, and JCA-1) also contain receptors with similar properties. Recently, Skowronska et al. (30) confirmed the presence of VDr in PC-3 and DU145 cells. In addition, Peehl et al. (31) found that benign hyperplastic epithelial cells and prostate tissue extracts contained comparable receptors. Taken together with our findings, these results are consistent with the hypothesis that VDr are a common feature of benign and malignant prostatic epithelium and could modulate the malignant phenotype of prostatic carcinoma in vivo. The growth inhibitory effects of vitamin D on prostatic carcinoma cells are consistent with the epidemiological data (8–10), and further suggest that the inclusion of vitamin D analogues into chemoprevention strategies (32) is justified.

The results described above provided somewhat conflicting data regarding the JCA-1 cell line. Although specific binding could not be demonstrated in whole-cell binding assays, (a) an appropriate VDr could be demonstrated by sucrose gradient analysis and (b) induction of 25(OH)D3-24-hydroxylase was obtained in these cells. As an additional confirmation of the presence of VDr in JCA-1 cells, RNase protection assays were used to detect the presence of low levels of hVDr mRNA. As in previous studies (11, 30), hVDr mRNA was again detected in LNCaP, PC-3, and DU145 cells. In addition, the other four cell lines were also found to contain this message. In some cell lines (i.e., ALVA-31, LNCaP, TSU-Pr1, and PC-3), there was good correlation between the amount of hVDr mRNA and the measured numbers of receptors per cell. However, DU145 and PPC-1 both appeared to have fewer receptors than would have been anticipated from the amounts of mRNA detected. Several factors could account for this finding including receptor and/or mRNA stability. Additional studies are under way to determine the nature of these differences. The amounts of hVDr mRNA detected in the present study are remarkably less than those reported by others. Skowronska et al. (30) demonstrated intense bands on Northern blot analyses using whole RNA. We could not reproduce such findings, even using poly(A)-enriched mRNA, which is consistent with the fact that the hVDr message is believed to be a very rare message in mammalian cells (33).

Similar to others studying breast (34), colon (35), or prostatic carcinoma (11, 30, 31) cells in vivo, we also find that the
growth of some prostatic carcinoma cell lines is inhibited by 1α,25(OH)2D3 in the presence of a growth medium containing 10% whole FBS. In our previous studies, we found the growth of LNCaP cells to be stimulated by 1α,25(OH)2D3 in a biphasic pattern in the presence of charcoal-stripped, sulfatase-treated FBS (11). The molecular mechanisms through which 1α,25(OH)2D3 alters the growth of prostatic carcinoma cells remain to be elucidated. However, from our results, it would seem that the negative growth regulation seen in the presence of whole FBS involves the action of another growth factor or factors that are removed from serum by charcoal striping.

Two potential variables can be identified from our data that could contribute to the observed differences of growth regulation between cell lines by 1α,25(OH)2D3 in the presence of whole serum. These include: (a) the number of VDRs/cell and (b) the ability of cells to metabolize vitamin D. It has recently been shown that in breast carcinomas, the suppression of growth by 1α,25(OH)2D3 is proportional to the number of receptors/cell (36). Likewise, our studies reveal that the most sensitive cell lines in terms of growth suppression (LNCaP and ALVA-31) also have the highest numbers of receptors/cell. Contrary to this conclusion, however, the remaining lines all have similar numbers of VDRs/cell. Two of these (DU145 and PPC-1) are inhibited to a statistically significant extent, whereas the other three (PC-3, JCA-1, and TSU-Prl) are not. This is particularly interesting since the PC-1 cell line is now believed to be a subclone of PC-3 (37). One possible explanation for these apparently contradictory behaviors between lines might reside in the instability of the rat and human genes (40). In our hands, those of Skowronska et al. (30), who found that DU145 and PC-3 cells could up-regulate the mRNA for 25(OH)D2-24-hydroxylase but LNCaP could not. We have even found LNCaP cells to up-regulate 25(OH)D2-24-hydroxylase activity in response to exogenous 1α,25(OH)2D3 in a dose-dependent fashion. One potential explanation for the differences between our results and those of Skowronska et al. (30) lies in the use of a rat-derived cDNA probe for 24-hydroxylase in their assays, which could have led to poor sensitivity due to known differences in sequence between the rat and human genes (41). In our hands, even the COLO 2650 nasopharyngeal carcinoma cell line exhibited small but detectable induction of 25(OH)D2-24-hydroxylase activity in response to exogenous 1α,25(OH)2D3 stimulation. This would indicate that very small numbers of receptors are capable of regulating such a response.

Differences in growth inhibition between the various cell lines could also be regulated by the ability of each cell line to metabolize 1α,25(OH)2D3 via 24-hydroxylation. Those cell lines that are the least growth inhibited by 1α,25(OH)2D3 (JCA-1, TSU-Prl, and PC-3) all have either the highest constitutive expression and/or induced levels of 25(OH)D2-24-hydroxylase activity. Likewise, the cell lines that are most growth inhibited (LNCaP, ALVA-31, and PPC-1) have the lowest baseline and/or stimulated levels of 24-hydroxylase activity. From this association it would appear that if cells are capable of inactivating 1α,25(OH)2D3 through metabolism to 1α,24,25(OH)3D3, they are less affected in terms of growth inhibition. It is clear that growth regulation of prostatic carcinoma cells by 1α,25(OH)2D3 is a complex phenomenon involving receptor expression, ligand metabolism, and other signaling pathways.

We have shown that without exception, several prostatic carcinoma cell lines contain biologically active receptors for the secosteroid hormone 1α,25(OH)2D3. In these cell lines, inhibition of growth by this hormone is partially proportional to the number of VDRs/cell and to the expression of the catabolic enzyme 25(OH)D2-24-hydroxylase. The latter finding suggests that analogues specifically designed to be less easily metabolized could be more active in their ability to inhibit growth and/or induce differentiation. Studies using such compounds are currently under way. In any event, the presence of these receptors remains an interesting aspect of the neoplastic phenotype of prostatic carcinoma cells that merits further investigation.

REFERENCES


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