Human Prostate Cells Synthesize 1,25-Dihydroxyvitamin D₃ from 25-Hydroxyvitamin D₃

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Abstract

Epidemiological and laboratory data support a role for vitamin D in the growth and differentiation of human prostatic cells. These findings prompted us to ask whether prostatic cells could convert 25-hydroxyvitamin D₃ (25-OH-D₃), the major circulating metabolite of vitamin D₂, to 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the hormonally active metabolite, in a manner similar to cultured human keratinocytes. Therefore, we investigated three well-characterized human prostate cancer cell lines, LNCaP, DU 145, and PC-3; two primary cultures of cells derived from noncancerous human prostates (one normal and one benign prostatic hyperplasia); and primary cultures of normal human keratinocytes for their ability to synthesize 1,25(OH)₂D₃. Assays were performed in the presence of 25-OH-D₃, as the enzyme substrate and 1,2-dianilinoethane, an antioxidant and free radical scavenger, and in the presence and absence of clotrimazole, a cytochrome P450 inhibitor.

DU 145 and PC-3 cells produced 0.31 ± 0.06 pmol of 1,25(OH)₂D₃/mg protein/h, respectively. No measurable 1,25(OH)₂D₃ was detected in LNCaP cells. The normal and benign prostatic hyperplasia primary cultures and keratinocyte cultures produced 3.08 ± 1.56, 1.05 ± 0.31, and 2.1 ± 0.1 pmol of 1,25(OH)₂D₃/mg protein/h, respectively, using a calf thymus receptor binding assay to measure 1,25(OH)₂D₃ in the presence of 1,2-dianilinoethane. The identity of the analyte as 1,25(OH)₂D₃ was supported by high performance liquid chromatography using [3H]25-OH-D₃ as the enzyme substrate and a solvent system that is specific for 1,25(OH)₂D₃. The production of 1,25(OH)₂D₃ in the prostate cancer cell lines and in the primary cultures was completely inhibited in the presence of clotrimazole.

This report demonstrates that two of three human prostate cancer cell lines, as well as primary cultures of noncancerous prostatic cells, possess 1α-hydroxylase activity and can synthesize 1,25(OH)₂D₃ from 25-OH-D₃. Together with recent data indicating that 1,25(OH)₂D₃ inhibits the invasiveness of human prostate cancer cells (G. G. Schwartz et al., Cancer Epidemiol. Biomark. Prev., 6: 727–732, 1997), these data suggest a potential role for 25-OH-D₃ in the chemoprevention of invasive prostate cancer.

Introduction

Apart from its role in calcium homeostasis, 1,25(OH)₂D₃ is now known to play important roles in the regulation of cell growth and differentiation (1). The synthesis of 1,25(OH)₂D₃ begins with the cutaneous production of vitamin D after exposure to sunlight or after the intestinal absorption of vitamin D₂ or vitamin D₃ obtained from the diet. To become biologically active, vitamin D must undergo two hydroxylation steps. The first hydroxylation occurs in the liver at the 25th carbon position, forming 25-OH-D₃, the major circulating metabolite of vitamin D. The second hydroxylation occurs in the kidney at the 1α position, forming 1,25(OH)₂D₃, the hormonally active metabolite (2). Although the kidney is the major source of 1,25(OH)₂D₃, the enzyme that converts 25-OH-D₃ to 1,25(OH)₂D₃, 1α-hydroxylase, is also present in several types of nonrenal cells, e.g., activated macrophages and keratinocytes (3, 4). The production of 1,25(OH)₂D₃ in these cells suggests an autocrine/paracrine role for 1,25(OH)₂D₃ in which it locally modulates cell proliferation and differentiation (5, 6).

Considerable evidence indicates that 1,25(OH)₂D₃ modulates the growth and differentiation of prostatic cells (7). This evidence includes the ubiquitous presence of receptors for 1,25(OH)₂D₃ (vitamin D receptors) in human prostatic cells (8–10), and the antiproliferative and prodifferentiating effects of 1,25(OH)₂D₃ on these cells in vitro and in vivo (11–14). These findings led us to evaluate whether prostatic cells also possess 1α-hydroxylase activity. We investigated three well-characterized human prostate cancer cell lines, LNCaP, DU 145, and PC-3, and two primary cultures of cells derived from noncancerous human prostates. We report that two of these cell lines, DU 145 and PC-3, as well as the primary cultures, can synthesize 1,25(OH)₂D₃ from its precursor, 25-OH-D₃.

Materials and Methods

Cell Lines. DU 145, PC-3, and LNCaP cell lines were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were tested and found to be free of Mycoplasma contamination.

Culture Conditions. Prostate cancer cell lines were cultured following several passages in vitro. Cells were routinely cultured in complete medium [RPMI 1640 supplemented with...
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Cambridge, MA) at 1 x 10⁶ cells/dish in complete medium. Medium was changed to a serum-free medium (RPMI 1640 containing 10 μg/ml insulin, 10 μg/ml transferrin, and 1 ng/ml selenious acid) 24 h before adding 25-OH-D₃ (15).

Primary cultures of human prostatic epithelial cells were established and characterized as described by Lokeshwar et al. (15). Prostatic epithelial cells cultured in a serum-free defined medium (Mammary Epithelial Growth Medium; Clontech, San Diego, CA) express luminal epithelium-specific cytokeratins (cytokeratins 8 and 18) as detected immunohistochemically using an anti-cytokeratin antibody, CAM 5.2 (Becton Dickinson, Mountain View, CA). The serum-free medium contains MCDF170 supplemented with 25 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 1 x 10⁻⁴ methanolamine, 5 μg/ml insulin, 5 μg/ml transferrin, and 70 μg/ml white bovine pituitary extract (16).

Prostatic cells used for this study were at their first passage in vitro and were cultured in the serum-free RPMI 1640 during incubation with vitamin D metabolites. We investigated two primary cultures: NP96-5, cultured from the histologically normal prostate of a 23-year-old Caucasian donor; and BPH96-11, cultured from an open prostatectomy specimen of a 56-year-old Caucasian with BPH. Tissues were obtained according to a human subjects protocol approved by a University Institutional Review Board. Histological examination of adjacent tissue sections taken from specimens used for cultures confirmed their identity as normal or BPH cultures.

Keratinocyte Culture. Because 1-hydroxylase activity has been well-established in keratinocytes, we used cultured human keratinocytes for comparison with the prostatic cultures. Keratinocytes were grown in culture following a modification of the method of Rheinwald and Green (17), as described in detail previously (18, 19). Briefly, keratinocytes were obtained from neonatal foreskin after trypsinization at 4°C. Keratinocytes were plated and grown on lethally irradiated 3T3 fibroblast feeder cells in a serum-free basal medium containing 0.15 msi calcium and supplemented with growth factors including bovine pituitary extract (16).

Results

Table 1 demonstrates the production of 1,25(OH)₂D₃ by three human prostatic cancer cell lines and primary cultures of prostatic cells and keratinocytes in the presence and absence of the cytochrome P450 inhibitor, clotrimazole.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>1,25(OH)₂D₃ produced (pmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P450 inhibitor absent</td>
</tr>
<tr>
<td>DU 145</td>
<td>0.31 ± 0.06 (n = 6)</td>
</tr>
<tr>
<td>PC-3</td>
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</tr>
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</tr>
<tr>
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* n. number of replications; errors are SE.

The 1α-hydroxylase activity was also determined in the primary cultures of prostatic cells by using radioactive 25-OH-D₃ (20 ng of nonradioactive 25-OH-D₃ and 0.91 μCi of [³H]25-OH-D₃) instead of only nonradioactive 25-OH-D₃ as a substrate. The incubation medium, time, temperature, extraction procedure, and C-18 OH cartridge chromatography were the same as described for the thymus receptor method, except that the fraction eluted from C-18 OH cartridge with 10% methylene chloride in hexane (25-OH-D₃ fraction) and with 6% isopropanol in n-hexane [1,25(OH)₂D₃, and 24,25(OH)₂D₃ fraction; Ref. 25] was dried down under nitrogen and redissolved in 200 μl of ethanol. Two 40-μl aliquots were taken for 1,25(OH)₂D₃ analysis by thymus receptor binding assay as described by Chen et al. (24).

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HPLC. A 30-μl aliquot was mixed with 10 μl each of standard nonradioactive 25-OH-D₃, and 1,25(OH)₂D₃ and was applied to an Econosphere silica column (5-μm particle size, 250 x 4.6 mm) with a flow rate of 0.5 ml/min using a methylene chloride isopropanol (19:1) solvent system as the mobile phase (26, 28). Thirty fractions were collected at 1-minute intervals from each HPLC. Fractions were allowed to evaporate by air to dryness, followed by the addition of scintillation fluid and counting with a beta counter. The retention volume for 25-OH-D₃, 24,25(OH)₂D₃, and 1,25(OH)₂D₃ was calibrated by applying standard 25-OH-D₃, 24,25(OH)₂D₃, and 1,25(OH)₂D₃ to the HPLC column before, during, and after unknown sample application. The protein concentration in each 35-mm dish was determined as described by Bradford (27). The enzyme activity was expressed as pmol of 1,25(OH)₂D₃/mg protein/h.

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Table 1. Synthesis of 1,25(OH)₂D₃ by three human prostatic cancer cell lines and primary cultures of prostatic cells and keratinocytes in the presence and absence of the cytochrome P450 inhibitor, clotrimazole.

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<td></td>
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The abbreviations used are: BPH, benign prostatic hyperplasia; DPPD, 1,2-dianilinoethane; HPLC, high-performance liquid chromatography.

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pmol of 1,25(OH)_{2}D_{3}/mg protein/h, respectively. The production of 1,25(OH)_{2}D_{3} was completely inhibited in the presence of clotrimazole. Conversely, no measurable 1,25(OH)_{2}D_{3} was detected in LNCaP cells. We also grew primary cultures of prostatic cells from two patients, NP96-5 (normal prostate) and BPH96-11 (BPH), and determined their enzyme activities in the presence of DPPD and in the presence and absence of clotrimazole. The NP96-5 and BPH96-11 cultures produced 3.08 ± 1.56 pmol/mg protein/h and 1.05 ± 0.31 pmol/mg protein/h 1,25(OH)_{2}D_{3}, respectively, in the presence of DPPD and in the absence of cytochrome P450 inhibitor. The 1a-hydroxylase activity found in the two primary cultures of prostatic cells was comparable with that found in normal human keratinocytes (Table 1). As in the two cell lines, the production of 1,25(OH)_{2}D_{3} in the primary cultures of prostatic cells and keratinocytes was completely inhibited in the presence of clotrimazole (Table 1).

The enzyme activity detected by thymus receptor binding assay in the primary cultures of prostatic cells was further supported by HPLC analysis using a solvent system that specifically separates 1,25(OH)_{2}D_{3} from another metabolite of 25-OH-D_{3}, 10-oxo-19-nor-25-OH-D_{3}. This metabolite, which is present in significant quantity in kidney homogenates of rats and chicken, is known to comigrate with 1,25(OH)_{2}D_{3} on normal phase HPLC with the n-hexane:isopropanol (9:1) solvent system, the traditional chromatographic system for isolating 1,25(OH)_{2}D_{3}. Therefore, to ensure that 1,25(OH)_{2}D_{3} was separated from any 10-oxo-19-nor-25-OH-D_{3} present, we used the methylene chloride:isopropanol (19:1) normal phase solvent system. In addition, 1a-hydroxylase activity was determined by using [3H]25-OH-D_{3} as substrate.

Fig. 1 demonstrates typical HPLC chromatograms of the two primary prostate cell cultures in the presence and absence of clotrimazole.

### Discussion

After incubation with 25-OH-D_{3}, DU 145 and PC-3 prostate cancer cell lines and the two primary cultures produced detectable levels of 1,25(OH)_{2}D_{3} (Table 1). There are several reasons for believing that the product is authentic 1,25(OH)_{2}D_{3}: (a) the radioactive metabolite generated from [3H]25-OH-D_{3} comigrated with authentic standard 1,25(OH)_{2}D_{3} in an HPLC solvent well-established for the separation and identification of 1,25(OH)_{2}D_{3} (26, 28). Not only was 1,25(OH)_{2}D_{3} well-separated from 24,25(OH)_{2}D_{3} (Fig. 1), but it was also separated from a commonly found metabolite, 10-oxo-19-nor-25-OH-D_{3}; (b) the enzyme product was detected by using a specific calf...
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thymus receptor binding assay, which has very poor binding affinity for 24,25(OH)₂D₃ and 25,26(OH)₂D₃ (25). In addition, possible contamination by 25-OH-D₃ was eliminated because the fraction used for analysis of 1,25(OH)₂D₃ contained no 25-OH-D₃ (24, 25); (c) the 100% inhibition of 1,25(OH)₂D₃ production by the specific cytochrome P450 inhibitor, clotrimazole, further suggests that a cytochrome P450-dependent 1α-hydroxylase similar to that found in kidney proximal tubular cells is present in these prostatic cells (29); and (d) the identity of the analyte as [³H]1,25(OH)₂D₃ from the radioactive substrate was supported by including DPPD in the presence and absence of clotrimazole during HPLC analysis. The addition of the P450 inhibitor almost totally inhibited the conversion of [³H]25-OH-D₃ to [³H]1,25(OH)₂D₃ (Fig. 1, B and D). Thus, DU 145 and PC-3 human prostate cancer cells, as well as two primary cultures derived from noncancerous human prostates, possess 1α-hydroxylase activity and are capable of converting the major circulating metabolite of vitamin D₃, 25-OH-D₃, to the hormonally active vitamin D metabolite, 1,25(OH)₂D₃.

No detectable 1,25(OH)₂D₃ was produced by LnCaP cells. 24-Hydroxylase activity has also been reported to be low or undetectable in LnCaP and highest in DU 145 (8, 9). However, for reasons discussed above, it is unlikely that the present findings for 1α-hydroxylase activity are contaminated by 24,25(OH)₂D₃.

Both primary cultures of noncancerous prostate cells produced 1,25(OH)₂D₃ at levels 10-40-fold higher than the cell lines. Very high levels of 1,25(OH)₂D₃ were observed from the culture derived from the 23-year-old organ donor. The quantities of 1,25(OH)₂D₃ produced by the two primary noncancerous human prostate cells, 3.08 ± 1.56 and 1.05 ± 0.31 pmol/mg protein/h (normal and BPH, respectively), are comparable with those produced in cultured human keratinocytes (2.1 ± 0.1 pmol/mg protein/h) and to the HEP 62 hepatoma cell line (2.3 pmol/mg protein/h; Ref. 30) and human T-lymphotrophic virus-transformed lymphocytes (1.6 pmol/mg protein/h; Ref. 31). These values are at least 10-fold higher than those reported in other extrarenal sites such as human bone cells (0.068 pmol/mg protein/h; Ref. 32).

The enzyme activity found in the primary cultures of prostate cells is comparable with that found in primary cultures of renal proximal tubular cells, 5.3–5.6 pmol/h/mg protein (33). Because it is established that renal 1α-hydroxylase is the major (and probably the sole) source of the enzyme responsible for maintaining the circulating concentration of 1,25(OH)₂D₃ under normal physiological conditions, we believe that the amount of 1,25(OH)₂D₃ produced by prostatic cells is likely to be physiologically significant, at least for the microenvironment of prostatic cells. We have shown recently that levels of 1,25(OH)₂D₃ as low as 10⁻¹⁰ M can significantly inhibit the invasiveness of human prostate cancer cells through an artificial basement membrane composed of human amnions (34).

The synthesis of 1,25(OH)₂D₃ by prostatic cells in vitro may clarify an important question concerning the epidemiology of prostate cancer with respect to vitamin D. Hanchette and Schwartz (35) have shown that prostate cancer mortality rates per county in the contiguous United States are inversely correlated with levels of UV radiation. They interpreted these findings to suggest that 1,25(OH)₂D₃ maintains the differentiated phenotype of prostatic cells and that low levels of 1,25(OH)₂D₃ may increase the risk for fatal prostate cancer (36). However, although systemic levels of 25-OH-D₃ are known to be dependent on exposure to UV radiation, systemic levels of 1,25(OH)₂D₃ in normal individuals are very tightly regulated and generally are not correlated with systemic levels of 25-OH-D₃ (37, 38). Thus, a mechanism by which UV radiation or vitamin D could result in increased exposure of prostatic cells to 1,25(OH)₂D₃ was unclear. This apparent paradox would be resolved if, like prostatic cells in vitro, prostatic cells in vivo synthesize 1,25(OH)₂D₃, locally from 25-OH-D₃.

These findings also have implications for the design and interpretation of biomarker studies of prostate cancer with respect to vitamin D. For example, if the prostate gland synthesizes 1,25(OH)₂D₃ in vivo, then systemic levels of 1,25(OH)₂D₃ measured in serum (39, 40) may not reflect levels of 1,25(OH)₂D₃ at the level of the target cell. Thus, the risk of prostate cancer may be influenced by intraprostatic as well as systemic levels of vitamin D. A dissociation between systemic and intraprostatic hormone levels in men has been demonstrated for another steroid hormone that is activated enzymatically by prostatic cells, dihydrotestosterone (41).

Finally, the synthesis of 1,25(OH)₂D₃ by prostatic cells may have implications for the use of vitamin D metabolites in prostate cancer chemoprevention. It is now well-established that 1,25(OH)₂D₃ exerts antiproliferative and pro-differentiating effects on normal and cancerous prostate cells. Moreover, we have shown that in vitro, physiological levels of 1,25(OH)₂D₃ significantly inhibit the invasiveness of DU 145 cells through an artificial basement membrane. This inhibition of invasiveness is correlated with a decrease in the secreted levels of type IV collagenase (matrix metalloproteinases 2 and 9; Ref. 34). We have shown that 1,25(OH)₂D₃ also exhibits impressive antimitastatic effects on prostate cancer cells in vivo (42). These findings support a role for 1,25(OH)₂D₃ in the chemoprevention of invasive prostate cancer. However, 1,25(OH)₂D₃ may not be suitable for use as a chemopreventive agent because of the risk of hypercalcemia (43). Our present findings raise the possibility that, by increasing the available substrate, supplementation of men with 25-OH-D₃ could promote the local synthesis of 1,25(OH)₂D₃ by prostatic cells. However, whether 25-OH-D₃ supplementation of men who are already vitamin D sufficient would promote the synthesis of physiologically significant levels of 1,25(OH)₂D₃ by prostatic cells in vivo remains to be demonstrated.

In summary, we report that DU 145 and PC-3 human prostate cancer cells and noncancerous human prostate cells in primary culture can convert 25-OH-D₃ to the hormonally active metabolite, 1,25(OH)₂D₃. Together with recent data indicating that vitamin D receptors are ubiquitous in prostatic cells and that 1,25(OH)₂D₃ exerts antiproliferative, pro-differentiating, and antimitastatic effects in these cells, our data suggest that 1,25(OH)₂D₃ may exert an autocrine/paracrine role in the prostate. These findings may provide a mechanism for the observed north-south gradient in prostate cancer mortality and support the potential use of 25-OH-D₃ in the chemoprevention of invasive prostate cancer.

Acknowledgments

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References


Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25-hydroxyvitamin D3.
