Human Prostate Cells Synthesize 1,25-Dihydroxyvitamin D$_3$ from 25-Hydroxyvitamin D$_3$

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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 D$_3$ (25-OH-D$_3$), the major circulating metabolite of vitamin D$_3$, to 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), 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)$_2$D$_3$. Assays were performed in the presence of 25-OH-D$_3$ 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 and 0.07 ± 0.01 pmol of 1,25(OH)$_2$D$_3$/mg protein/h, respectively. No measurable 1,25(OH)$_2$D$_3$ 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)$_2$D$_3$/mg protein/h, respectively, using a calf thymus receptor binding assay to measure 1,25(OH)$_2$D$_3$ in the presence of 1,2-dianilinoethane. The identity of the analyte as 1,25(OH)$_2$D$_3$ was supported by high performance liquid chromatography using $^{[1]}$H$25$OH-D$_3$ as the enzyme substrate and a solvent system that is specific for 1,25(OH)$_2$D$_3$. The production of 1,25(OH)$_2$D$_3$ 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)$_2$D$_3$ from 25-OH-D$_3$. Together with recent data indicating that 1,25(OH)$_2$D$_3$ 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-D$_3$ in the chemoprevention of invasive prostate cancer.

Introduction
Apart from its role in calcium homeostasis, 1,25(OH)$_2$D$_3$ is now known to play important roles in the regulation of cell growth and differentiation (1). The synthesis of 1,25(OH)$_2$D$_3$ begins with the cutaneous production of vitamin D after exposure to sunlight or after the intestinal absorption of vitamin D$_2$ or vitamin D$_3$ 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-D$_3$, the major circulating metabolite of vitamin D. The second hydroxylation occurs in the kidney at the 1α position, forming 1,25(OH)$_2$D$_3$, the hormonally active metabolite (2). Although the kidney is the major source of 1,25(OH)$_2$D$_3$, the enzyme that converts 25-OH-D$_3$ to 1,25(OH)$_2$D$_3$, 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)$_2$D$_3$ in these cells suggests an autocrine/paracrine role for 1,25(OH)$_2$D$_3$, in which it locally modulates cell proliferation and differentiation (5, 6).

Considerable evidence indicates that 1,25(OH)$_2$D$_3$ modulates the growth and differentiation of prostatic cells (7). This evidence includes the ubiquitous presence of receptors for 1,25(OH)$_2$D$_3$ (vitamin D receptors) in human prostatic cells (8–10), and the antiproliferative and prodifferentiating effects of 1,25(OH)$_2$D$_3$ on these cells in vitro (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)$_2$D$_3$ from its precursor, 25-OH-D$_3$.

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 selenous 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 Tor, 0.5 pg/ml hydrocortisone, 1

Cells were seeded in 35-mm culture dishes (Corning-Costar, 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 selenous acid) 24 h before adding 25-OH-D3 (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 MCD170 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 whole 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 organ 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 mm calcium and supplemented with growth factors including bovine pituitary extract (3 μg/ml), epidermal growth factor (25 ng/ml), insulin (5 μg/ml), and prostaglandin E1 (50 ng/ml). To enhance the plating efficiency, 0.1 μg/ml chola toxin and 200 ng/ml hydrocortisone were added into the medium during the initial plating of the primary culture and the subsequent subcultures. Cells were fed and maintained without chola toxin and hydrocortisone and used for enzyme assay.

25-Hydroxyvitamin D-1α-hydroxylase (1α-Hydroxylase) Assay. 1α-Hydroxylase activity was determined in monolayer cultures of the cell lines and primary cultures. The assays were performed in the presence of 50 nm 25-OH-D3 as the enzyme substrate (20) and DPPD (Sigma-Aldrich, Allentown, PA), an antioxidant and a known inhibitor of free radical-generated 1,25(OH)2D3 (21, 22). Assays were also performed in the presence and absence of the cytochrome P450 inhibitor, clotrimazole (20 μM; Sigma Chemical Co., St. Louis, MO; Ref. 23).

After 2 h of incubation at 37°C, cultures were placed on ice, and media were removed. Immediately afterward, 1 ml of methanol was added to extract 25-OH-D3 and 1α,25(OH)2D3. A 10-μl aliquot of 1α,25-dihydroxy[26,27-methyl-3H]vitamin D3 containing 1000 cpm radioactivity in ethanol was also added to each well for calculating the recovery. After extraction at room temperature for 15 min, the methanol extract was transferred to a glass test tube, and the cells were washed with an additional 0.5 ml of methanol. The extract and wash were combined, dried down with a stream of nitrogen, and redissolved in 1 ml of acetonitrile, followed by the addition of 1 ml of 0.4 M K2HPO4 (pH 10.0). The mixture was then applied to a C-18-OH reversed-phase cartridge (24, 25). The fraction containing 1,25(OH)2D3 was then dried down under a stream of nitrogen and reconstituted 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).

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 methylene chloride isotopropanol (19:1) for high performance liquid chromatographic analysis as described below

HPLC. A 30-μl aliquot was mixed with 10 μl of each 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.

Results
Table 1 demonstrates the production of 1,25(OH)2D3 in three cultured human prostate cancer cell lines, two primary cultures of human prostatic cells, and cultured normal human keratinocytes. DU 145 and PC-3 produced 0.31 ± 0.06 and 0.07 ± 0.01 pmol of 1,25(OH)2D3/mg protein/h.

| Table 1: Synthesis of 1,25(OH)2D3 by three human prostatic cancer cell lines and primary cultures of prostate cells and keratinocytes in the presence and absence of the cytochrome P450 inhibitor, clotrimazole. |
|---|---|---|---|
| Cell type | 1,25(OH)2D3 produced (pmol/mg protein/h)*a | P450 inhibitor present | P450 inhibitor absent |
| DU 145 | 0.31 ± 0.06 (n = 6) | Undetectable | Undetectable |
| PC-3 | 0.07 ± 0.01 (n = 6) | Undetectable | Undetectable |
| LNCap | Undetectable | Undetectable | Undetectable |
| NP96-5 | 3.08 ± 1.56 (n = 3) | Undetectable | Undetectable |
| BPH96-11 | 1.05 ± 0.31 (n = 3) | Undetectable | Undetectable |

*a. number of replications; errors are SE.

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

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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)2D3 from another metabolite of 25-OH-D3, 10-oxo-19-nor-25-OH-D3. This metabolite, which is present in significant quantity in kidney homogenates of rats and chicken, is known to comigrate with 1,25(OH)2D3 on normal phase HPLC with the n-hexane-isopropanol (9:1) solvent system, the traditional chromatographic system for isolating 1,25(OH)2D3. Therefore, to ensure that 1,25(OH)2D3 was separated from any 10-oxo-19-nor-25-OH-D3 present, we used the methylene chloride-isopropanol (19:1) normal phase solvent system. In addition, 1α-hydroxylase activity was determined by using [3H]25-OH-D3 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-D3, DU 145 and PC-3 prostate cancer cell lines and the two primary cultures produced comparable levels of 1,25(OH)2D3 (Table 1). There are several reasons for believing that the product is authentic 1,25(OH)2D3: (a) the radioactive metabolite generated from [3H]25-OH-D3 comigrated with authentic standard 1,25(OH)2D3 in an HPLC solvent well-established for the separation and identification of 1,25(OH)2D3 (26, 28). Not only was 1,25(OH)2D3 well-separated from 24,25(OH)2D3 (Fig. 1), but it was also separated from a commonly found metabolite, 10-oxo-19-nor-25-OH-D3; (b) the enzyme product was detected by using a specific calf...
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 [1H]25-OH-D3 to [1H]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 D, 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 of primary human prostate cells, 3.08 ± I.56 and 1.05 ± 0.31 pmol/mg protein/h (normal and BPH, respectively), are comparable with those produced in cultured human keratinocytes of primary human prostate cells. 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 10−11 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. 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 antimetastatic 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 antimetastatic 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.

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References
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