Vitamin D Metabolism in Human Prostate Cells: Implications for Prostate Cancer Chemoprevention by Vitamin D

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Abstract. Background: Prostate cells can produce 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) from 25-hydroxyvitamin D3 (25(OH)D3) to regulate their own growth. Here, the questions of whether prostate cells express vitamin D-25-hydroxylase (25-OHase) and can convert vitamin D3 to 1α,25(OH)2D3 were investigated. Materials and Methods: Protein and receptor binding assays were used to determine 25(OH)D3 and 1α,25(OH)2D3, respectively. Measurements of proliferation by 3H-thymidine incorporation, and 1α,25(OH)2D-responsive gene expression by real-time qPCR and by Western blot were used as functional assays for the presence of 25-OHase activity. Results: Prostate cells metabolized vitamin D3 to 1α,25(OH)2D3. Vitamin D3 up-regulated 25(OH)D-24R-hydroxylase and IGFBP3, two 1α,25(OH)2D-responsive genes, in prostate cells. CYP2R1 was the major form of 25-OHase expressed in normal and cancerous prostate cells as determined by qPCR. Conclusion: The autocrine synthesis of 1α,25(OH)2D3 from vitamin D3 suggests that maintaining adequate levels of serum vitamin D could be a safe and effective chemo-preventive measure to decrease the risk of prostate cancer.

Vitamin D regulates over 60 genes by interacting with a specific nuclear receptor, the vitamin D receptor (VDR) (1). The genes include those associated with calcium homeostasis, immune responses, cellular growth, differentiation, apoptosis, and the enzymes involved in its own metabolism. Vitamin D (which includes both vitamin D2 and vitamin D3) requires two successive hydroxylation steps first in the liver by the 25-OHase and then in the kidneys by 1α-OHase (or CYP27B1) to form the active metabolite, 1α,25(OH)2D (2). The kidneys can also hydroxylate 25(OH)D and 1α,25(OH)2D at C-24 by 24R-OHase (or CYP24A1) to form 24, 25-dihydroxyvitamin D (24,25(OH)2D) and 1α,24,25-trihydroxyvitamin D (1α,24,25(OH)3D), respectively. The main function of CYP24A1 is to regulate the circulating concentration of 1α,25(OH)2D (2). Similarly to extra-renal 1α-hydroxylation, which occurs in many tissues including skin, intestine, colon, breast and prostate (3), a broad tissue distribution of extra-hepatic 25-OHase activity has been observed in adrenal, lung, spleen, skin, kidney, colon and small intestine (4). At the present time, there are at least six candidate 25-OHases, CYP27A1, CYP2C11, CYP2D25, CYP2F3, CYP3A4 and CYP2R1. All have been implicated in the conversion of vitamin D to 25(OH)D. Among them, only CYP27A1 is found in the mitochondria, while the rest are located in the microsomal fraction (5).

Previously, we have demonstrated that prostate cells are capable of synthesizing 1α,25(OH)2D intracellularly from 25(OH)D (6), and that the antiproliferative effect of 25(OH)D was indistinguishable from that of 1α,25(OH)2D (7). This finding has important implications for prostate cancer chemoprevention because the risk of hypercalcemia associated with the systemic administration of vitamin D and 25(OH)D is far lower than that for 1α,25(OH)2D (8). In this study, we investigated whether prostate cells have the capacity to convert vitamin D3 to 1α,25(OH)2D and examined the potential of vitamin D3 to induce 1α,25(OH)2D biological activities in prostate cells. It was demonstrated that prostate cells express 25-OHase mRNA, and are capable of metabolizing vitamin D3 to 1α,25(OH)2D, leading to the up-regulation of 24R-OHase and IGFBP3, and the inhibition of prostate cell proliferation.

Abbreviations: 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; 25-OHase, vitamin D-25-hydroxylase; 25(OH)D3, 25-hydroxyvitamin D3; 1α-OHase, 25(OH)D-1α-hydroxylase; 24R-OHase, 25(OH)D-24R-hydroxylase; IGFBP3, insulin-like growth factor binding protein-3; vitamin D2, ergocalciferol; vitamin D3, cholecalciferol; 24,25(OH)2D, 24, 25-dihydroxyvitamin D; 1α,24,25(OH)3D, 1α,24,25-trihydroxyvitamin D; CYP, cytochrome; VDR, vitamin D receptor; DPPD, 1, 2-dianilinoethane; qPCR, quantitative PCR.

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Key Words: Prostate cancer, vitamin D, hydroxylases, cytochrome P450, chemoprevention.
Table I. Sequence specific primers for real-time qPCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CYP27A1</td>
<td>5'-CTT TTT GCT ACA TCA TGT TCG AGA AA-3'</td>
<td>5'-CTG GAA CAT TAA CCC GAT GGA-3'</td>
</tr>
<tr>
<td>CYP2R1</td>
<td>5'-TGA CCC ATC ATC TAC TTT CTC CA-3'</td>
<td>5'-TTA GGA TAA AGG GCC ATG AAA AGA-3'</td>
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<tr>
<td>CYP3A4</td>
<td>5'-CGT GGC CCA ATC AAT TAT CTT T-3'</td>
<td>5'-GCA TCA ATT TCC TCC TGC AGT T-3'</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>5'-CAA GTT CCA CCC CCT CCA TT-3'</td>
<td>5'-TCT CTA CGG CAG GGA CCA TAT T-3'</td>
</tr>
<tr>
<td>CYP24A</td>
<td>5'-GGC CTG GAT GTA CTA TTT GC-3'</td>
<td>5'-ACA ATC CAA CAA AGA GCC AAA TGG TGA A-3'</td>
</tr>
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Materials and Methods

Cell cultures. LNCaP and PC-3 cells (ATCC, Manassas, VA, USA) were maintained in RPMI (Sigma, St Louis, MO, USA) supplemented with 5% FBS (GibcoBRL, Gaithersburg, MD, USA), whereas PZ-HPV-7 cells (ATCC) were grown in serum-free keratinocyte growth medium (9).

Cell proliferation assay. Tritiated thymidine (Perkin-Elmer, Boston, MA, USA) incorporation into DNA was used to determine the effect of vitamin D3 on PZ-HPV-7 cell proliferation (9).

Determination of 25(OH)D and 1α,25(OH)2D. A competitive protein binding assay and a radio-receptor binding assay (10, 11) were employed to measure the concentrations of 25(OH)D3 and 1α,25(OH)2D3, respectively. Briefly, cells, grown to 90% confluence on 35-mm plates, were treated with 10–6 M vitamin D3 for 2 hours in the presence of the 10 mM 1,2-dianilinoethane (DPPD). Lipid extracts were obtained with the addition of methanol to monolayer cultures after the media had been removed. The 25(OH)D and 1α,25(OH)2D fractions from the lipid extracts were collected separately from C18-OH cartridge chromatography, and their concentrations were determined as described (10, 11).

Real-time qPCR analysis. Total RNA from normal human liver (Cat#-64099-1) and normal prostate (Cat#-64108-1) tissue were purchased from BD Biosciences, Piscataway, NJ, USA. Total RNA from prostatic cell lines was isolated using the SV Total RNA kit (Promega, Madison WI, USA). For each sample, cDNA was generated with 2 mg of total RNA using Superscript RNAase H- kit (Promega, Madison WI, USA). For each sample, cDNA was generated with 2 mg of total RNA using Superscript RNAase H- kit (Promega, Madison WI, USA). Primer sequences were designed against coding sequences of human CYP27A1 (414120), CYP2R1 (33591221), CYP3A4 (13904851), IGFBP3 (40675391) and CYP24A (306703) with Primer Express software (Applied Biosystems, Forest City, CA, USA) (Table I).

For each real-time qPCR reaction, 20 ng of single-stranded cDNA was mixed with 2X SYBR Green PCR Master Mix (Applied Biosystems) and an optimal concentration of sequence specific primers. Samples were analyzed on an ABI Prism 7700 sequence detection system. To normalize the amount of sample cDNA added to the reaction, Taqman PDAR eukaryotic 18S rRNA, (Applied Biosystems) was used as the endogenous control. Relative quantitation of gene expression was calculated using the DDCt method (User Bulletin publication #2, Applied Biosystems).

Western blot analysis. Cells lysates in a buffer containing 100 mM Tris (pH 8.5), 250 mM NaCl, 1% NP40, 1 mM EDTA and 5 mM DTT were sonicated, boiled and centrifuged at 14,000 rpm for 10 minutes to remove particulate matter. A sample of 50 μg of protein extracts was separated on a 10% SDS-PAGE gel, transferred to nitrocellulose paper and probed with a rabbit polyclonal antibody against human IGFBP3 (sc-9028, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a secondary antibody using goat anti-rabbit IgG conjugated to horseradish peroxidase. The paper was then developed with chemiluminescent reagents (Amersham Biosciences, Piscataway, NJ, USA).

Results

Inhibition of cellular proliferation by vitamin D3 in normal prostate cells, an indication of prostatic 25-OHase activity. In order to determine if prostate cells have 25-OHase activity and the ability to hydroxylate vitamin D to 25(OH)D before being activated to 1α,25(OH)2D, we investigated whether vitamin D3 would inhibit the growth of PZ-HPV-7 cells (9). We found that cells treated with vitamin D3 at 10–6 M for 18 hours reduced 3H-thymidine incorporation by 40% compared to the ethanol vehicle control. For a positive control, PZ-HPV-7 cells were treated with 1α,25(OH)2D3 at 10–6 M for 18 hours which reduced the cell proliferation by 80% of the control, similar to previously published studies (7). These results indicate that vitamin D3 elicits a similar biological effect as 1α,25(OH)2D3 on prostate cell growth, which may reflect that vitamin D3 has been metabolized to 25(OH)D3 by a prostatic 25-OHase.

Conversion of vitamin D3 to 1α,25(OH)2D3 in normal prostate cells. To further investigate the presence of 25-OHase in PZ-HPV-7 cells, we measured 25(OH)D3 and 1α,25(OH)2D3 formation in prostate cells treated with vitamin D3 (10, 11). Although we were unable to detect 25(OH)D3 in the lipid extracts of PZ-HPV-7 cells incubated for 2 hours with 10–6 M of vitamin D3, which could result from the high endogenous 1α-OHase activity, we detected 50±3 fmols of 1α,25(OH)2D3 in the same cultures (Figure 1). We also incubated heat-inactivated cells with either vitamin D3 (10–6 M) or 1α,25(OH)2D3 (10–8 M) for 2 hours in the presence of DPPD as controls. We were able to detect 1α,25(OH)2D3 in heat-inactivated cells treated with...
1α,25(OH)₂D₃, but not those treated with vitamin D₃ (Figure 1). This indicates that the 1α,25(OH)₂D₃ detected in live PZ-HPV-7 cells treated with vitamin D₃ was derived from the enzymatic hydroxylation of vitamin D₃ to 25(OH)D₃ catalyzed by endogenous 25-OHase prior to the hydroxylation by 1α-OHase.

Up-regulation of 1α,25(OH)₂D₃-inducible genes by vitamin D₃ in PZ-HPV-7 cells, indicating prostate cells contain all the necessary hydroxylases involved in activation of vitamin D.

Prostate cells express 24R-OHase (12) and IGFBP3 (13), which are inducible by 1α,25(OH)₂D₃ but not vitamin D₃ or 25(OH)D₃ per se (14). Therefore, we examined the up-regulation of 24R-OHase and IGFBP3 expression in response to vitamin D₃ as indicators of the two-step activation of vitamin D₃ via 25-hydroxylation and subsequent 1α-hydroxylation to form 1α,25(OH)₂D₃ in PZ-HPV-7 cells. PZ-HPV-7 cells at 90% confluence were treated with vitamin D₃ (10⁻⁶, 10⁻⁷ and 10⁻⁶ M) or 1α,25(OH)₂D₃ (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) for 24 hours. As a positive control, cells were also treated with 1α,25(OH)₂D₃. The expression of 24R-OHase and IGFBP3 mRNAs were analyzed by real-time qPCR. Figure 2A demonstrates that 24R-OHase expression was up-regulated in a dose-dependent manner by vitamin D₃ (32-, 241- and 1688-fold) and 1α,25(OH)₂D₃ (2-, 69- and 930-fold) versus controls, at the indicated doses. Similarly, IGFBP3 expression was up-regulated by both vitamin D₃ and 1α,25(OH)₂D₃ as indicated in Figure 2B. We also evaluated the IGFBP3 protein by Western blot analysis in vitamin D₃ and 1α,25(OH)₂D₃-treated PZ-HPV-7 cells. As demonstrated in Figure 2C, the IGFBP3 protein was up-regulated by pretreatment of PZ-HPV-7 cells with vitamin D₃ (10⁻⁶ M) and 1α,25(OH)₂D₃ (10⁻⁷ M). These results demonstrate that
vitamin D3 modulated VDR responsive genes, and reflect the two-step activation of vitamin D3 via 25-hydroxylation then 1α-hydroxylation to form 1α,25(OH)2D3.

Expression profile of candidate 25-OHases - CYP27A, CYP2R1 and CYP3A4 in human liver and non-cancerous and cancerous prostate cells. Three 25-OHases, including the mitochondrial CYP27A1 and the two microsomal enzymes, CYP3A4 and CYP2R1, have been found in several species including humans (4,5). In order to determine which p450s might be responsible for the observed 25-OHase activity in prostate cells, the expression of these three candidate 25-OHases were investigated by real-time qPCR. Figure 3 shows that CYP2R1 expression was two-fold greater in normal human prostate tissue, three-fold greater in PZ-HPV-7 cells and six-fold greater in LNCaP prostate cancer cells than in normal liver tissue, whereas CYP27A1 was expressed ten-fold more in normal liver tissue than in normal prostate tissue. Very little or no expression of CYP27A1 was found in the PZ-HPV-7, LNCaP and PC-3 cells. PC-3 cells also expressed very little or no CYP2R1. CYP3A4 was expressed only in normal liver tissue and not in any prostate cells examined. The data suggest that CYP2R1 represents the major 25-OHase in prostate cells, and is probably responsible for the conversion of vitamin D3 to 25(OH)D3.

Discussion

Although the existence of extra-hepatic 25-OHases has been known for some times, its physiological significance is currently not entirely clear. The best studied extra-hepatic 25-OHase system is the one described in human keratinocytes (15-17). Using 3H-vitamin D3 as substrate, Schuessler et al. (15) observed continuous slow formation of 3H-25(OH)D3 in cultured human keratinocytes, following a linear time course and yielding only 1.5% of the product after 4 hours of incubation. This finding is in agreement with the low but constant levels of CYP27A1 mRNA expression found in human keratinocytes by qPCR (15, 16). The low but constant formation of 3H-25(OH)D3 resulted in a sustained up-regulation of 24R-OHase after the addition of physiological doses of vitamin D3. Since both vitamin D3 and 25(OH)D3 lack intrinsic 24-OHase-inducing capacity (18), up-regulation of 24-OHase has to be the consequence of the two-step activation process via 25-hydroxylation and subsequent 1α-hydroxylation. The data, therefore, suggest that the local cutaneous reservoir of vitamin D3 together with 25-hydroxylation at the rate limiting step of the vitamin D3 cascade would provide the low, but constant levels of 1α,25(OH)2D3 that could be sufficient to exert hormonal activity in the target cells. In this report, evidence was provided that 1α,25(OH)2D3 was detected by the thymus receptor binding assay after the addition of 10–6 M vitamin D3 into the PZ-HPV-7 cells (Figure 1), suggesting the presence of 25-OHase, in addition to 1α-OHase, in PZ-HPV-7 prostate cells. Its presence was further confirmed by three functional assays. First, treatment of PZ-HPV-7 cells with vitamin D3 at 10–6 M caused a 40% inhibition of [3H]thymidine incorporation into DNA. Second, the addition of vitamin D3 to PZ-HPV-7 cells caused a dose-dependent up-regulation of 24-OHase (CYP24A1) (Figure 2A). Third,
the addition of vitamin D3 to PZ-HPV-7 cells caused a dose-dependent up-regulation of IGFBP3 mRNA expression (Figure 2B). CYP24A1 and IGFBP3 are two genes known to be sensitive to 1α,25(OH)2D3 regulation in prostate cells (12, 13). The vitamin D3-induced IGFBP3 expression was further supported by the detection of IGFBP3 protein using Western blot analysis (Figure 2C). Thus, the data indicate that vitamin D3 was first converted to 25(OH)D3 and then to 1α,25(OH)2D3 before exerting biological actions in prostate cells. It is likely that prostate cells work similarly to keratinocytes, producing sufficient and constant levels of 1α,25(OH)2D to exert hormonal activity in the cells from the local pool of vitamin D3, together with 25-hydroxylation at the rate limiting step of the vitamin D3 cascade.

We are interested to know which form of 25-OHase is predominately expressed in prostate cells. The mRNAs for CYP27A1, CYP3A4 and CYP2R1 have previously been found in some human tissues. We therefore compared their expression in prostate cells to that in the liver by qPCR. Our qPCR data demonstrated that essentially no expression of CYP27A1 and CYP3A4 was found in prostate cells. On the contrary, CYP27A1 and CYP3A4 were expressed at least 10-fold greater in normal liver tissue than in normal prostate tissue and cells. This is in agreement with the finding that the function of CYP3A4 is mainly for the metabolism and detoxification of xenobiots and carcinogens rather than hydroxylation of vitamin D (19). Our qPCR data also show that CYP2R1 was expressed to a much greater extent in normal human prostate tissue and cultured prostate cells than in normal liver tissue. It has been reported that CYP2R1 is an evolutionarily conserved enzyme and its mRNA was more highly expressed in the testis than the liver (20). Most importantly, a homozogous mutation in exon 2 of the CYP2R1 gene on chromosome 11p15.2, which causes the substitution of a proline for an evolutionarily conserved leucine at amino acid 99 in the CYP2R1 protein, has been identified to be responsible for the elimination of 25-OHase activity in a patient with the classic symptoms of vitamin D deficiency with low circulating levels of 25(OH)D (21). It was therefore concluded that CYP2R1 is a biologically relevant vitamin D 25-OHase in humans. Our data demonstrating that CYP2R1 is the major form of 25-OHase expressed in prostate tissue and cultured prostate cells strongly support this conclusion.

Despite the encouraging data of 1α,25(OH)2D as a potential chemotherapeutic agent for prostate cancer (22, 23), it is not suitable for clinical use because systemic administration of 1α,25(OH)2D can cause hypercalcemia and hypercalciuria (22, 23). Thus, in order for vitamin D metabolites to be useful in prostate cancer chemoprevention, a method must be devised to safely expose prostate cells to 1α,25(OH)2D without systemic side-effects. The presence of the two activating enzymes (CYP2R1/25OHase and 1α-OHase) and the catabolic enzyme (24R-OHase) for the vitamin D system in prostate cells suggest that local production of 1α,25(OH)2D3 could provide an important cell growth regulatory mechanism. In conclusion, our results suggest that maintaining adequate levels of serum vitamin D or 25(OH)D by oral supplementation or sun exposure can be a safe and effective chemo-preventive measure to decrease the risk of prostate cancer.

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