Journal of Cellular Biochemistry 88:315–322 (2003)

Prostatic 25-Hydroxyvitamin D-1α-Hydroxylase and Its Implication in Prostate Cancer

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Abstract Evidence suggests that vitamin D may have a protective role for prostate cancer. 1α,25-Dihydroxyvitamin D [1α,25$(OH)_2$D] inhibits growth and induces differentiation of prostate cells. 25-Hydroxyvitamin D-1α-hydroxylase [1α-OHase], the enzyme that is responsible for the synthesis of 1α,25$(OH)_2$D, is expressed in cultured prostate cells. We observed a marked decrease in 1α-OHase activity in prostate cancer cells, suggesting some defect of the 1α-OHase in these cells. To investigate whether the defect was due to dysregulation of the enzyme at the promoter level, a series of deletion constructs of the promoter was synthesized and incorporated upstream into the luciferase reporter gene. Two regions were identified with high basal activity in transfected normal prostate cell line (PZHPV-7), −1100 bp (AN2), and −394 bp (AN5) upstream of ATG start site of the 1α-OHase gene. When the reporter gene with either AN2 or AN5 was transfected into prostate cancer cell lines, we observed a lower basal promoter activity in PC-3 cells and DU145 cells than that found in PZHPV-7 cells for both constructs, and a loss of promoter activity in LNCaP cells. Thus, the results suggest that the defect in enzyme activity may result from the decreased promoter activity in prostate cancer cells. J. Cell. Biochem. 88: 315–322, 2003. © 2002 Wiley-Liss, Inc.

Key words: vitamin D; promoter activity; cell cultures; luciferase; reporter gene

Prostate cancer is the most commonly diagnosed and second most fatal cancer among U.S. men. Although the etiology of prostate cancer is incompletely understood, epidemiological and laboratory data increasingly support a protective role for vitamin D. For example, Schwartz and colleagues demonstrated that U.S. mortality rates from prostate cancer were correlated inversely ($P < 0.0001$) with the availability of ultraviolet radiation [Schwartz and Hulka, 1990]. The north-south gradient in prostate cancer mortality, and the greater risk for prostate cancer among Blacks, are reminiscent of the vitamin D deficiency disease, rickets. These findings suggested that one cause of prostate cancer might be vitamin D insufficiency [Schwartz and Hulka, 1990].

The vitamin D/prostate cancer hypothesis was supported by a 13-year follow-up of 19,000 middle-aged men in the Helsinki Heart Study [Ahonen et al., 2000]. In this study, 149 prostate cancer cases were identified and were matched to 566 sample controls. The study shows that (a) men with 25(OH)D level below the median concentration (40 nmol/L or 16 ng/ml) had an adjusted relative risk (OR) of 1.7 compared to men with 25(OH)D above the median, (b) the prostate cancer risk was highest among men younger than 52 years with low serum 25(OH)D with an OR of 3.5, (c) among men younger than 52 years, low serum 25(OH)D entailed a higher risk of non-localized cancer with an OR of 6.3. Thus, the study demonstrates that low levels of 25(OH)D are associated with an increased risk for subsequent earlier exposure and more aggressive development of prostate

Abbreviations: 25$(OH)D_3$, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α-OHase, 25-hydroxyvitamin D-1α-hydroxylase; 1α-OHase-GFP, 25-hydroxyvitamin D-1α-hydroxylase-green fluorescent protein; BDH, benign prostatic hyperplasia; VDR, vitamin D receptor.

Grant sponsor: The Commonwealth of Massachusetts; Grant numbers: 4118PP1017, 41211159016; Grant sponsor: US Army; Grant number: DAMD17-01-1-0025.

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Received 1 August 2002; Accepted 2 August 2002

DOI 10.1002/jcb.10342

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cancer, especially before the age of andropose (52 years old).

The biochemical evidence to support a role for vitamin D in prostate cancer includes the demonstration of specific vitamin D receptor (VDR) for $1\alpha,25(OH)_2D$ and the antiproliferative and pro-differentiation activities of $1\alpha,25(OH)_2D$ and its analogs in cultured prostate cancer cell lines, primary cultures of normal and cancerous prostate tissue, and the peripheral zone of the prostate in vitro and in vivo [Miller et al., 1992; Skowronski et al., 1993; Peehl et al., 1994; Schwartz et al., 1994; Chen et al., 2000].

Vitamin D_3 is formed in the skin after exposure to ultraviolet B portion (UVB) of the solar spectrum (290–315 nm). The amount of solar UVB radiation reaching the earth is a function of the solar zenith angle and depends on latitude, season, and time of day [Holick, 1995]. In addition, skin pigmentation competes and thus decreases the absorption of solar UVB radiation by epidermal 7-dehydrocholesterol (7-DHC), the precursor of vitamin D_3, and the epidermal 7-DHC stores decrease with age. Therefore, the production of vitamin D_3 is inversely related to latitude, degrees of skin pigmentation, and age [Holick, 1995]. To become biologically active, vitamin D_3 must be hydroxylated first in the liver at the 25 position to form 25-hydroxyvitamin D_3 [$25(OH)D_3$], the major circulating metabolite of vitamin D_3, and then in the kidney at the 1α-position catalyzed by 25(OH)D-1α-hydroxylase (1α-OHase), a mitochondria cytochrome P-450 enzyme, to form 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] [Jones et al., 1998]. The cDNAs encoding the mouse, rat, and human 1α-OHase have recently been cloned [Fu et al., 1997; St-Arnaud et al., 1997; Takeyama et al., 1997; Brenza et al., 1998].

COMPARISON OF $1\alpha,25(OH)_2D_3$ AND ITS ANALOGS ON THE INHIBITION OF DIFFERENT CULTURED PROSTATE CELL LINES AND PRIMARY CULTURES DERIVED FROM CANCEROUS PROSTATE TISSUES

The antiproliferative activity of $1\alpha,25(OH)_2D_3$ in LNCaP, PC-3, and DU145 cancer cell lines and primary cultures of cells from a prostate cancer patient was evaluated by ^3H-thymidine incorporation into DNA using a protocol developed in our laboratory [Chen et al., 1995]. A dose-dependent inhibition in ^3H-thymidine incorporation was observed in the presence of $1\alpha,25(OH)_2D_3$ in the primary cultures of prostate cancer epithelial cells and cancer cell lines, LNCaP, PC-3, and DU145. However, the primary cultures were more responsive to $1\alpha,25(OH)_2D_3$ than the cell lines. For examples, we found that $1\alpha,25(OH)_2D_3$ caused $41 \pm 2\%$, $75 \pm 1\%$, and $88 \pm 1\%$ inhibition in primary cultured cells compared to 0%, $20 \pm 4\%$, and $53 \pm 3\%$ inhibition in LNCaP cells and 0%, $21 \pm 7\%$, and $50 \pm 3\%$ in PC-3 cells at 10^{-8}, 10^{-7}, and 10^{-6} M, respectively. No statistically significant differences were observed between PC-3 and LNCaP cells at the three concentrations of $1\alpha,25(OH)_2D_3$ tested. DU145 was not responsive to $1\alpha,25(OH)_2D_3$ at the concentrations between 10^{-8} and 10^{-6} M.

VITAMIN D AUTOCRINE SYSTEM

Until the mid 1980's, it was believed that, in normal individuals, the 1α-hydroxylation of 25(OH)D took place exclusively in the kidney. However, local synthesis of $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$ was first demonstrated in cultured keratinocytes [Bikle et al., 1986], where $1\alpha,25(OH)_2D_3$ acts locally to regulate cellular proliferation and differentiation [Chen et al., 1995]. Recently, it was demonstrated that human prostate cancer cell lines in culture, DU145 and PC-3, and cells derived from a normal prostate and a prostate with benign prostatic hyperplasia (BPH) also possess 1α-OHase activity and synthesize $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$ [Schwartz et al., 1998]. Interestingly, the primary culture of prostate cells from the BPH patient had lower 1α-OHase activity than that obtained from the normal donor, and DU145 and PC-3 cells possess even lower 1α-OHase activity than that found in BPH cells. Furthermore, no detectable 1α-OHase activities were found in the cancer cell line, LNCaP.

Since the primary cultures of prostate cells are capable of converting $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$, $25(OH)D_3$ added to the cultures should be converted to $1\alpha,25(OH)_2D_3$ and cause an inhibition in prostate cell proliferation. We found that both $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ caused a dose-dependent inhibition of the primary cultures derived from human prostate tissue although $25(OH)D_3$ was about 30-fold less active than $1\alpha,25(OH)_2D_3$ [Chen et al.,

2000]. Because $25(OH)D_3$ has only 1/1,000 of binding affinity for VDR as compared to $1\alpha,25(OH)_2D_3$, $25(OH)D_3$ usually has little biological activity on its own [Skowronski et al., 1995] such as in LNCaP cells which has little or no 1α-OHase activity [Schwartz et al., 1998]. The most likely explanation of the results is that portion of $25(OH)D_3$ was converted to $1\alpha,25(OH)_2D_3$ by 1α-OHase present in prostate cells during the 18-h incubation.

1α-OHase ACTIVITY IN PRIMARY CULTURES OF NORMAL AND PROSTATE CANCER CELLS AND CANCER CELL LINES

1α-OHase enzyme activity from the primary cultures of prostate epithelial cells derived from four prostate cancer patients (CaP), two BPH patients, three normal donors, and three prostate cancer cell lines (LNCaP, DU145, and PC-3) was determined by the conversion of ^3H-$25(OH)D_3$ to ^3H-$1\alpha,25(OH)_2D_3$ (Fig. 1). The normal cultures had an average activity of 3.0 ± 0.36 pmol/mg protein/h, whereas BPH and prostate cancer cultures had an average activity of 1.2 ± 0.28 and 0.46 ± 0.15 pmol/mg protein/h, respectively. Therefore, comparing to the primary cultured normal prostate cells, the enzyme activity was 60 and 85% lower in the primary cultured BPH and prostate cancer cells, respectively. DU145 and PC-3 had 0.2 ± 0.1 and 0.06 ± 0.01 pmol/mg protein/h, respectively [Whitlatch et al., 2002]. No activity was detected in LNCaP cells. Table I compares the 1α-OHase enzyme activity in a normal prostate cell line, PZHPV-7, to those of the three prostate cancer cell lines.

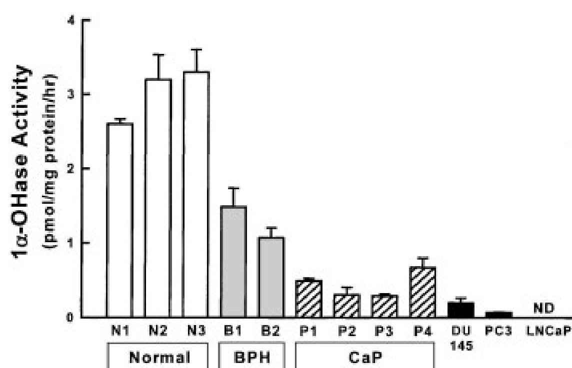

Fig. 1. Synthesis of $1\alpha,25(OH)_2D_3$ by primary cultures of normal, BPH, and prostate cancer cells (CaP), and by human prostate cancer cell lines, DU145, PC-3, and LNCaP cells. Bars shown are standard errors of three to six determinations.

1α-OHase GENE PROMOTER ACTIVITY IN PROSTATE CANCER CELL LINES

The finding that a marked decrease in 1α-OHase activity in cancer cells compared to normal cells led us to investigate the possibility whether the defect could be due to dysregulation of the enzyme at the promoter level. To test this hypothesis, a series of nucleotide base-pair deletion from the full length promoter (The 1.4 kb full length promoter is designated as AN1) was constructed and incorporated upstream into the luciferase reporter gene.

Among the six fragments, −1100 bp (AN2) and −463 bp (AN5) fragments which are upstream of ATG start site of the 1α-OHase gene were identified with high basal activity in transfected normal prostate PZHPV-7 cell line. When the reporter gene with either AN2 or AN5 was transfected into prostate cancer cells, we observed a substantially lower basal promoter activity in PC-3 cells and DU145 cells than that found in PZHPV-7 cells for both AN2 and AN5 constructs, and a complete loss of promoter activity in LNCaP (Table I).

TRANSFECTION OF LNCaP CELLS WITH 1α-OHase-GFP PLASMID

LNCaP cells are not growth inhibited in the presence of $25(OH)D_3$ [Skowronski et al., 1995]. To determine whether the lack of inhibition by $25(OH)D_3$ in LNCaP cells was due to the absence of 1α-OHase enzyme activity, we transfected LNCaP cells with 1α-OHase cDNA plasmid and then evaluated the catalytic activity of the expressed 1α-OHase and the antiproliferative activity of $25(OH)D_3$. A human 1α-OHase-green fluorescence protein (GFP) fusion construct was generated to examine the expression and the appearance of cellular distribution of the 1α-OHase-GFP fusion protein by scanning laser confocal microscopy (600×). Using a 530 nm filter, the green fluorescence of the 1α-OHase-GFP protein in live transfected LNCaP cells showed that the expressed 1α-OHase-GFP protein was perinuclear and punctuate in appearance, consistent with cytochrome P_{450}-1α-OHase localization in the mitochondria [Jones, 1998]. The same live transfected LNCaP cells were also stained with a mitochondria-specific red fluorescent indicator MitoTracker using a 580 nm filter. When both images were superimposed, the resulting image showed co-localization of the 1α-OHase-

TABLE I. Comparison of the Enzymatic Activity and Gene Promoter Activity of 25-Hydroxyvitamin D-1α-Hydroxylase in Prostate Cell Lines

	Enzyme activity (% conversion)	Promoter luciferase activity	
		AN2	AN5
LNCaP	ND*	95 ± 18	ND
PC-3	1.5 ± 0.8	1,633 ± 118	5,364 ± 290
DU145	3.7 ± 0.6	8,581 ± 223	14,432 ± 863
PZHPV-7	37 ± 3.0	30,076 ± 857	46,347 ± 735

Results are means ± SD of three to four determinations.
*Non-detectable.

GFP green fluorescence with the mitochondria red fluorescence that appears yellowish-green, confirming that the 1α-OHase-GFP protein was expressed in the mitochondria. This in contrast to a live LNCaP cell that was transfected with the control GFP plasmid. There was uniform fluorescence throughout the cytoplasm, consistent with cytoplasmic GFP protein expression [Whitlatch et al., 2002].

CONVERSION OF $25(OH)D_3$ TO $1\alpha,25(OH)_2D_3$ BY LNCaP CELLS TRANSFECTED WITH 1α-OHase CDNA

To determine whether the expressed 1α-OHase-GFP was functional, we first evaluated the efficiency of ^3H-$25(OH)D_3$ conversion to ^3H-$1\alpha,25(OH)_2D_3$ in LNCaP cells, which were transiently transfected with 1α-OHase cDNA plasmid. As noted above, LNCaP cells have little or no 1α-OHase activity (Fig. 1) and $25(OH)D_3$ had no antiproliferative activity in them [Skowronski et al., 1995]. HPLC analysis revealed that a significant increase in the conversion of ^3H-$25(OH)D_3$ to ^3H-$1\alpha,25(OH)_2D_3$ in cells transfected with 1α-OHase-cDNA plasmid compared to an undetectable amount of conversion by cells that were mock-transfected with pCR 3.1 vector alone. In LNCaP cells transfected with 1α-OHase-cDNA plasmid, 36 ± 5% of ^3H-$25(OH)D_3$ was converted to ^3H-$1\alpha,25(OH)_2D_3$, compared to no conversion by LNCaP cells which were mock-transfected with pCR 3.1 vector alone. The HPLC chromatogram illustrated that $1\alpha,25(OH)_2D_3$ fraction was well-separated from $25(OH)D_3$ and $24,25(OH)_2D_3$ fractions. When the enzyme activity was expressed as pmol $1\alpha,25(OH)_2D_3$ produced/mg protein/h, LNCaP cells transfected with 1α-OHase-cDNA plasmid had 1α-OHase activity of 4.95 ± 0.69 pmol/mg protein/h compared to 0.07 ± 0.07pmol/mg protein/h of $1\alpha,25(OH)_2D_3$ produced in LNCaP cells which were mock-transfected with pCR 3.1 vector alone (Fig. 2). Similarly, LNCaP cells stably transfected with 1α-OHase-cDNA plasmid had 1α-OHase activity of 5.8 ± 0.7 pmol/mg protein/h vs. 0.3 ± 0.3 pmol/mg protein/h of $1\alpha,25(OH)_2D_3$ produced in LNCaP cells, which were transfected with empty pCR 3.1 vector ($P < 0.01$).

EFFECT OF $25(OH)D_3$ ON ^3H-THYMIDINE INCORPORATION INTO DNA IN 1α-OHase TRANSFECTED LNCaP CELLS

After we established that LNCaP cells transfected with 1α-OHase cDNA either transiently or stably had restored 1α-OHase activity, we next evaluated whether the conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ would result in the appearance of the antiproliferative effects of $25(OH)D_3$ in these cells. Transient transfection with sense 1α-OHase cDNA plasmid into LNCaP cells caused a significant decrease in ^3H-thymidine incorporation (37 ± 6%), vs. no

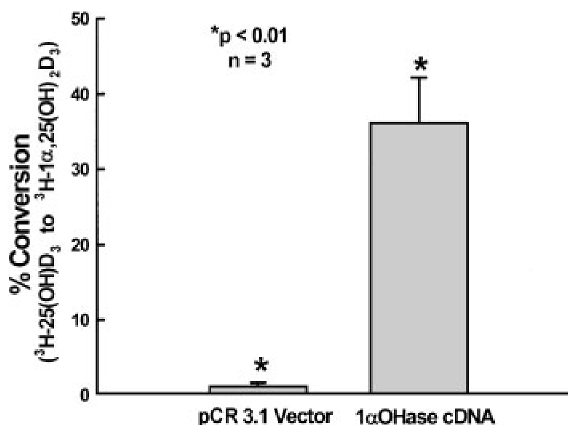

Fig. 2. Effect of transfection of 1α-OHase cDNA on LNCaP conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$. Bars represent the standard deviation of three determinations. *$P < 0.01$.

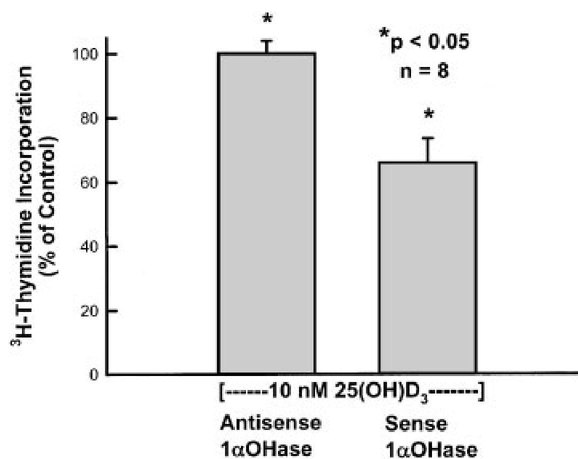

Fig. 3. Effect of $25(OH)D_3$ on ^3H-thymidine incorporation into DNA in LNCaP cells transfected with sense and anti-sense 1α-OHase cDNA. Bars indicate the standard deviation of eight determinations. $*P < 0.05$.

inhibition in LNCaP cells transfected with antisense 1α-OHase cDNA (Fig. 3). There was no significant difference in ^3H-thymidine incorporation in cells either non-transfected or transfected with vector alone or antisense 1α-OHase cDNA [Whitlatch et al., 2002]. Similar increase in cellular sensitivity to the antiproliferative activity of the pro-hormone, $25(OH)D_3$ was also observed in cells stably transfected with 1α-OHase-cDNA [Whitlatch et al., 2002].

DISCUSSION

Our observations indicate that primary cultures of prostate cancer cells and prostate cancer cell lines demonstrate a marked decline in 1α-OHase activity compared to the primary cultures of normal prostate cells (Fig. 1). These findings have important implications and suggest that the loss of 1α-OHase activity, an enzyme that synthesizes the growth-inhibitory hormone $1\alpha,25(OH)_2D$, may be associated with prostate cancer. One approach to investigate this association is to transfect prostate cells with 1α-OHase cDNA plasmid to restore or enhance the antiproliferative activity of 25(OH)D in the transfected cells. The ideal cells for this study would be a cell type that has very low or no 1α-OHase activity and whose growth is not inhibited in the presence of 25(OH)D but can be inhibited by $1\alpha,25(OH)_2D$. Therefore, we re-evaluated the antiproliferative effect of $1\alpha,25(OH)_2D_3$ in the primary cultures of prostate cancer cells and three cancer cell lines, LNCaP, PC-3, and DU145. Our results confirm the previously published observations showing inhibition in LNCaP and PC-3 cells, but not in DU145 cells [Miller et al., 1992; Skowronski et al., 1993; Peehl et al., 1994; Schwartz et al., 1994]. However, the present study did not show statistically significant differences in ^3H-thymidine incorporation in the presence of $1\alpha,25(OH)_2D_3$ between LNCaP and PC-3 cells. Although the reasons for this discrepancy are not clear, it may be due to different assay procedures or culture conditions employed between our laboratory and others. Since LNCaP cells responded to $1\alpha,25(OH)_2D_3$ but not to $25(OH)D_3$ [Skowronski et al., 1995], and these cells had no 1α-OHase activity (Fig. 1), we were able to successfully transfect these cells with 1α-OHase cDNA plasmid either transiently or stably, and furthermore, we demonstrated that this restored the 1α-OHase enzyme activity and thereby conferred inhibition of cell growth by $25(OH)D_3$ [Whitlatch et al., 2002]. Thus, our results strongly suggest that the lack of $25(OH)D_3$'s antiproliferative activity in LNCaP cells is due to the loss of the 1α-OHase enzyme activity in those cells. Although we cannot determine from the present data whether loss of 1α-OHase is a cause or a consequence of prostate carcinogenesis, our findings that growth inhibition by $25(OH)D_3$ was restored in cells transfected with 1α-OHase cDNA plasmid (Fig. 3) supports our interpretation that the loss of 1α-OHase may be causally associated with prostate cancer. Additionally, because the expression of 1α-OHase was approximately threefold higher in normal than in cancerous cells, the relative absence of 1α-OHase may be useful as a functional marker by which cancerous prostatic cells could be separated from noncancerous ones.

There is an important functional difference between the renal 1α-OHase and the prostatic 1α-OHase; the renal 1α-OHase is tightly regulated by parathyroid hormone [Chen et al., 1989; Jones et al., 1998], whereas the prostatic enzyme, like other extra renal 1α-OHases, is not [Dusso et al., 1994; Chen et al., 1998]. This suggests that the increased synthesis of $1\alpha,25(OH)_2D$ within the prostate could be influenced by the increases in the systemic levels of 25(OH)D. The lack of regulation by parathyroid hormone, together with the finding of decreased 1α-OHase activity in cancer cells, suggest that the apparent autocrine role of

1α-OHase in the prostate may be operative in vivo. This interpretation is consistent with the results of some (but not all) epidemiological studies, which show that lower serum levels of 25(OH)D are significantly associated with a higher risk of subsequent prostate cancer [Ma et al., 1998; Ahonen et al., 2000].

Our observation that 1α-OHase expression is greater in non-cancerous primary cultures and non-cancerous PZHPV-7 cell line than in cancerous primary cultures and cancer cell lines suggests that loss of 1α-OHase activity may be part of the natural history of malignancy in the prostate gland. Because the donor prostates were from younger men, it is conceivable that age may contribute to the differences we observed in 1α-OHase activity. However, in prostate cultures of comparable ages, the cancerous or BPH status appears to be the determinative factor. For example, one culture was prepared from a 42-year old African-American organ donor and another culture was derived from a 50-year old African-American patient with prostate cancer. The ages of these two subjects are comparable, but they had a tenfold difference in 1α-OHase activity. Although these findings are consistent with our initial observations, larger series of patients with cancer and BPH will be required to confirm these observations. A report with a larger sample size [Hsu et al., 2001] also demonstrated a marked decline in 1α-OHase activity in BPH and prostate cancer cells compared to the normal prostate cells in specimens from men of similar ages.

Unlike the primary cultures, the level of 1α-OHase expression within the prostate cancer cell lines is not well correlated with the degree of differentiation of the cell lines. For example, although LNCaP cells are widely recognized as the most well-differentiated cell line, these cells possess little or no 1α-OHase activity. Conversely, DU 145 cells are the most poorly differentiated cell line, yet they express the most 1α-OHase of the three well-characterized cell lines. However, it is important to note that the magnitude of the difference between normal ($\sim$3 pmol/mg protein/h) and cancerous primary cultures (about 0.5 pmol/mg protein h) is far greater than the differences between cell lines ($\sim$0.3 pmol/mg protein/h in DU 145 cells vs. undetectable in LNCaP cells). Thus, although differences in 1α-OHase expression appear to differentiate cancerous from non-cancerous primary cultures, factors other than 1α-OHase expression must account for the differences among cancerous cells themselves.

The presence of 1α-OHase activity is clearly necessary for the antiproliferative effects of the pro-hormone 25(OH)D, but it is not sufficient. For example, DU145 cells express 1α-OHase, but are not growth inhibited by $25(OH)D_3$ or by $1\alpha,25(OH)_2D_3$. It has been suggested that high levels of 24R-OHase in DU145 cells may contribute to the faster catabolism of $1\alpha,25(OH)_2D_3$ and consequently, the ineffectiveness of 1α, $25(OH)_2D_3$'s antiproliferative action in this cell line [Zhao et al., 1999]. The addition of a specific inhibitor of the 24R-OHase P-450, Liarozole, caused a significant increase in the half-life of $1\alpha,25(OH)_2D_3$ from 11 to 31 h in DU145 cells. In support of this interpretation, the combination of $1\alpha,25(OH)_2D_3$ and Liarozole retarded cell growth more effectively than did $1\alpha,25(OH)_2D_3$ alone. Therefore, it is possible that the 24R-OHase may play a role in the progression of prostate cancer either indirectly or directly, by regulating the levels of $1\alpha,25(OH)_2D_3$. Thus, both enhancement of the vitamin D catabolic pathway (via up-regulation of 24R-OHase) and loss of the synthetic pathway (via down-regulation of 1α-OHase) appear to be involved in the pathophysiology of prostate cancer.

We examined the antiproliferative activity of $1\alpha,25(OH)_2D_3$,19-nor-$1\alpha,25(OH)_2D_2$ and hexa-fluoro-$1\alpha,25(OH)_2D_3$ in LNCaP cells and in primary cultures of prostate cancer cells by ^{3}H-thymidine incorporation assay [Chen et al., 2000]. All three compounds had a greater effect in the primary cultures of prostate cancer cells than in the LNCaP prostate cancer cell line, suggesting that primary cultures may be a more sensitive system to differentiate the effectiveness of different vitamin D compounds in vitro. 1α, $25(OH)_2D_3$ was previously shown to decrease cyclin-dependent kinase2 activity, resulting in decreased retinoblastoma (Rb) protein phosphorylation and accumulation of LNCaP cells in G1 phase of the cell cycle [Zhuang and Burnstein, 1998]. Since a functional Rb pathway appears to be required for the maximal antiproliferative effects of $1\alpha,25$-$(OH)_2D_3$, primary prostatic cultures may exhibit increased growth inhibition by $1\alpha,25$-$(OH)_2D_3$, hexa-fluoro-$1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ because, compared to the cell lines, these cultures are less likely to have mutations in this pathway.

The human 1α-OHase contains nine exons and eight introns spanning 6.5 kb and a 1.4-kb 5′-flanking promoter region [Kong et al., 1999]. The promoter region contains consensus or highly conserved sequences for TATA, Pu, and CCAAT boxes, four cAMP response elements, two activator protein-1 (AP-1) response elements, two AP-2 response elements, three specific protein-1 (Sp1) response elements, and four NF-kB binding sites, but no vitamin D response element (VDRE). By using luciferase reporter gene constructs of the full length promoter (AN1) and various truncated forms (AN2-AN6) of the 1α-OHase promoter transfected into normal prostate cell line, PZHPV-7, we observed high basal transcriptional activities associated with AN2 and AN5 promoter fragments. Further determination of the AN2 and AN5 transcriptional activity in DU145, PC-3, and LNCaP prostate cancer cells revealed that the order of transcriptional activity among those four prostate cells was identical to that of the enzyme activities in the four prostate cell lines (Table I). Thus, the results suggest that the lower enzyme activity found in prostate cancer cell lines is likely due to the decreased promoter activity in those cancer cells. Identifying the transcription factor(s) involved in the regulation of 1α-OHase gene promoter activity could very well provide critical information regarding the etiology of prostate cancer.

In summary, the presence of variable 1α-OHase activities found in primary cultured cells derived from normal, BPH and prostate cancer tissue, normal prostate cell line and prostate cancer cell lines suggest that a defect in 1α-OHase activity and/or expression may be involved in the etiology of some human prostate cancers. The defect in enzyme activity may result from the decreased promoter activity in prostate cancer cells. Transfection of 1α-OHase cDNA plasmid into LNCaP cells confers antiproliferative activity on the pro-hormonal form of vitamin D. Because increases in systemic levels of 25(OH)D could result in increased local production of 1α,25$(OH)_2$D in the prostatic cells without inducing systemic hypercalcemia [Osborn et al., 1995; Gross et al., 1998], the introduction of the 1α-OHase gene into the prostate cancer cells together with the systemic administration of 25(OH)D could offer a novel and less toxic approach for treating prostate cancer than treatment with 1α,25$(OH)_2$D.

ACKNOWLEDGMENTS

The authors thank Kelly Persons for technical assistance and California Tan for generous gift of the confocal microscope. This work was supported in part by grants 4118PP1017 and 41211159016 from The Commonwealth of Massachusetts, and US Army DAMD17-01-1-0025.

REFERENCES

Ahonen MH, Tenkanen L, Teppo L, Hakama M, Tuohimaa P. 2000. Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes and Control 11:847–852.

Bikle DD, Nemanic MK, Whitney JO, Elias PW. 1986. Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D_3. Biochemistry 25:1545–1548.

Brenza HL, Kimmel-Jehan C, Jehan F, Shinki T, Wakino S, Anazawa H, Suda T, DeLuca HF. 1998. Parathyroid hormone activation of the 25-hydroxyvitamin D_3-1α-hydroxylase gene promoter. Proc Natl Acad Sci USA 95: 1387–1391.

Chen TC, Curthoys NP, Lagenaur CF, Puschett JB. 1989. Characterization of primary cell cultures derived from rat renal proximal tubules. In Vitro 25:714–722.

Chen TC, Persons K, Liu W-W, Chen ML, Holick MF. 1995. The antiproliferative and differentiative activities of 1,25-dihydroxyvitamin D_3 are potentiated by epidermal growth factor and attenuated by insulin in cultured human keratinocytes. J Invest Dermatol 104:113–117.

Chen TC, Young MV, Whitlatch LW, Zhu XH, Kong XF, Schwartz GG, Lokeshwar BL, Holick MF. 1998. Expression and regulation of 25-hydroxyvitamin D_3-1-alpha-hydroxylase in cultured human prostatic cells. Bone 23(Suppl 5):S262.

Chen TC, Schwartz GG, Burnstein KL, Lokeshwar BL, Holick MF. 2000. The in vitro evaluation of 25-hydroxyvitamin D_3 and 19-nor-1α,25-dihydroxyvitamin D_2 for prostate cancer therapy. Clin Cancer Res 6:901–908.

Dusso A, Brown A, Slatopolsky E. 1994. Extrarenal production of calcitriol. Semin Nephrol 14:144–155.

Fu GK, Lin D, Zhang MYH, Bikle DD, Shackleton CHL, Miller WL, Portale AA. 1997. Cloning of human 25-hydroxyvitamin D-1α-hydroxylase and mutations causing vitamin D-dependent rickets type 1. Mol Endocrinol 11:1961–1970.

Gross C, Stamey T, Hancock S, Feldman D. 1998. Treatment of early recurrent prostate cancer with 1,25-Dihydroxyvitamin D_3 (calcitriol). J Urol 159:2035–2040.

Holick MF. 1995. Vitamin D: Photobiology, metabolism, and clinical applications. In: DeGroot L, Besser H, Burger HG, editors. Endocrinology, 3rd Ed. Philadelphia: W.B. Saunders. pp 990–1013.

Hsu JY, Feldman D, McNeal JE, Peehl DM. 2001. Reduced 1α-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D_3-induced growth inhibition. Cancer Res 61: 2852–2856.

Jones G. Metabolism and catabolism of vitamin D, its metabolites, and clinically relevant analogs. 1998. Holick M.F, editor. Vitamin D: Physiology, molecular biology,

and clinical applications, Humana Press, Totowa, New Jersey. pp 57–84.

Jones G, Strugnell SA, DeLuca HF. 1998. Current understanding of the molecular actions of vitamin D. Physiol Rev 78:1193–1231.

Kong XF, Zhu XH, Pei YL, Jackson DM, Holick MF. 1999. Molecular cloning, characterization, and promoter analysis of the human 25-hydroxhvitamin D_3-1α-hydroxylase gene. Proc Natl Acad Sci 96:6988–6993.

Ma J, Satmpfer MJ, Gann PH, Hough HL, Giovannucci E, Kelsey KT, Hennekens CH, Hunter DJ. 1998. Vitamin D receptor polymorphisms, circulating vitamin D metabolites, and risk of prostate cancer in United States physicians. Cancer Epidem Biomar Prev 7:385–390.

Miller GJ, Stapleton GE, Ferrara JA, Lucia MS, Pfister S, Hedlund TE, Upadhya P. 1992. The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1,25-dihydroxyvitamin D_3. Cancer Res 52:515–520.

Osborn JL, Schwartz GG, Bahnson R, Smith DC, Trump DL. 1995. Phase II trial of oral 1,25-Dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer. Urol Oncol 1:195–198.

Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D. 1994. Antiproliferative effects of 1,25-dihydroxyvitamin D_3 on primary cultures of human prostatic cells. Cancer Res 54:805–810.

Schwartz GG, Hulka BS. 1990. Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). Anticancer Res 10:1307–1311.

Schwartz GG, Oeler TA, Uskokovic MR, Bahnson RR. 1994. Human prostate cancer cells: Inhibition of proliferation by vitamin D analogs. Anticancer Res 14:1077–1081.

Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL, Holick MF. 1998. Human prostate cells synthesize 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3. Cancer Epidemiol, Biomark & Prev 7:391–395.

Skowronski RJ, Peehl DM, Feldman D. 1993. Vitamin D and prostate cancer: 1,25-Dihydroxyvitamin D_3 receptors and actions in human prostate cancer cell lines. Endocrinology 132:1952–1960.

Skowronski RJ, Peehl DM, Feldman D. 1995. Actions of vitamin D_3 analogs on human prostate cancer cell lines: Comparison with 1,25-dihydroxyvitamin D_3. Endocrinology 136:20–26.

St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH. 1997. The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. J Bone Miner Res 12:1552–1559.

Takeyama KI, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S. 1997. 25-Hydroxyvitamin D_3 1α-hydroxylase and vitamin D synthesis. Science 277: 1827–1830.

Whitlatch LW, Young MV, Schwartz GG, Flanagan JN, Burnstein KL, Lokeshwar BL, Rich ES, Holick MF, Chen TC. 2002. 25-Hydroxyvitamin D-1α-hydroxylase activity is diminished in human prostate cancer cells and is enhanced by gene transfer. J Steroid Biochem Mol Biol 81:135–140.

Zhao XY, Ly LH, Holloway L, Feldman D. 1999. Liarozole acts synergistically with 1,25-dihydroxyvitamin D3 to inhibit DU-145 human prostate cancer cell growth by blocking 24-hydroxylase activity. Endocrinology 140: 2071–2076.

Zhuang S-H, Burnstein KL. 1998. Antiproliferative effect of 1α,25-dihydroxyvitamin D_3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. Endocrinology 139:1197–1207.