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Advances in Brief

Reduced 1α-hydroxylase Activity in Human Prostate Cancer Cells Correlates with Decreased Susceptibility to 25-Hydroxyvitamin D_3-induced Growth Inhibition[1]

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Abstract

Evidence from epidemiological, molecular, and genetic studies suggests a role for vitamin D in the development and/or progression of prostate cancer. In experimental models and clinical trials, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] was shown to exert antiproliferative, prodifferentiating, and antimetastatic/invasive effects on prostatic epithelial cells. Because the direct clinical application of 1,25$(OH)_2D_3$ is limited by the major side effect of hypercalcemia, we investigated the potential therapeutic utility of its less calcemic precursor, 25-hydroxyvitamin D_3 [25$(OH)D_3$], which is converted locally within the prostate to 1,25$(OH)_2D_3$ by 1α-hydroxylase. Quantification of 1α-hydroxylase activity in human prostatic epithelial cells by enzyme-substrate reaction analyses revealed a significantly decreased activity in cells derived from adenocarcinomas compared with cells derived from normal tissues or benign prostatic hyperplasia (BPH). In growth assays, we found that 25$(OH)D_3$ inhibited growth of normal or BPH cells similarly to 1,25$(OH)_2D_3$. In contrast, in primary cultures of cancer cells and established cell lines, the antiproliferative action of 25$(OH)D_3$ was significantly less pronounced than that of 1,25$(OH)_2D_3$. Our results indicate that growth inhibition by 25$(OH)D_3$ depends on endogenous 1α-hydroxylase activity, and that this activity is deficient in prostate cancer cells. This finding has ramifications for both the prevention and therapy of prostate cancer with vitamin D compounds.

Introduction

Vitamin D is a member of the steroid hormone superfamily that is traditionally regarded as a major physiological modulator of mineral metabolism and bone homeostasis (1). Its active metabolite, 1,25$(OH)_2D_3$[3], is produced by a series of reactions involving several organs, beginning with the dietary absorption of vitamin D_2 or D_3 as well as cutaneous synthesis of vitamin D_3 after exposure to sunlight. Vitamin D_3 undergoes sequential hydroxylations, first in the liver to form the relatively inactive circulating prohormone, 25$(OH)D_3$. In the kidney, 25$(OH)D_3$ is activated by 1α-hydroxylase to 1,25$(OH)_2D_3$. The biological actions of 1,25$(OH)_2D_3$ are mediated through VDRs that act as ligand-dependent transcription factors (1). VDRs are present in a variety of tissues such as bone, parathyroid glands, skin, small intestine, colon, uterus, ovary, testes, and breast as well as prostate. The widespread distribution of VDRs has raised the possibility that vitamin D may be involved in cellular functions unrelated to bone and mineral metabolism (2).

The idea that vitamin D may be a protective factor in the development and/or progression of prostate CA was proposed by Schwartz and Hulka (3) based on epidemiological studies. Subsequently, considerable attention has focused on refining this hypothesis. The presence of VDRs has been demonstrated in prostatic epithelial cells (4). Moreover, we and others showed that 1,25$(OH)_2D_3$ inhibited the growth of established prostatic CA cell lines as well as primary cultures of prostatic epithelial cells (4). In addition to its antiproliferative effects, 1,25$(OH)_2D_3$ stimulated cellular differentiation by inducing expression of PSA (5) and inhibited the invasiveness of prostatic CA cells *in vitro* (6, 7). These antitumor activities of vitamin D have led to the investigation of 1,25$(OH)_2D_3$ as a therapeutic agent for prostate CA (5, 8). However, two clinical trials showed that the associated calcemic effect of 1,25$(OH)_2D_3$ (calcitriol) limits its clinical utility (9, 10), although the latter study did find a decrease in the rate of PSA increase in patients with recurrent CA after radiation therapy or prostatectomy. A number of analogues of 1,25$(OH)_2D_3$ with greater antiproliferative activity and less calcemic effects have been described (8, 11, 12). The analogues, like 1,25$(OH)_2D_3$, inhibited the proliferation of prostatic CA cells (13, 14) and are considered future therapeutic options.

After the recent cloning of renal 1α-hydroxylase (15) and the discovery of extra-renal 1α-hydroxylase in various tissues (16–19), Schwartz *et al.* (20) demonstrated expression of 1α-hydroxylase in human prostatic epithelial cells. These authors raised the possibility that treatment with 25$(OH)D_3$ could potentially inhibit the growth of prostate CA attributable to intraprostatic production of 1,25$(OH)_2D_3$ without the systemic side effect of hypercalcemia (20, 21). Thus, treatment with the prohormone and local conversion would serve as a new mechanism through which an anti-CA effect is locally achieved within the prostate without systemic side effects (20). The ability of 25$(OH)D_3$ to cause hypercalcemia is reduced because of its low affinity for the VDR, which requires 200- to 500-fold higher concentrations than does 1,25$(OH)_2D_3$ for equivalent activation of the VDR (22).

To further explore the feasibility of using 25$(OH)D_3$ therapeutically, we quantitated the levels of endogenous 1α-hydroxylase activity in a series of primary cultures of human prostatic epithelial cells derived from normal tissues, BPH, and adenocarcinomas. Several prostatic CA cell lines were also evaluated. We also examined the antiproliferative activity of 25$(OH)D_3$ compared with 1,25$(OH)_2D_3$ on prostatic cells and correlated the antiproliferative potency with levels of 1α-hydroxylase activity. We found that CA cells had approximately 10- to 20-fold lower levels of 1α-hydroxylase activity compared with cells from normal tissues. Cells from BPH had lower levels of 1α-hydroxylase activity than normal cells, but still significantly higher than CA-derived cells. Furthermore, the reduced levels of 1α-hydroxylase in cells from adenocarcinomas correlated with a diminished antiproliferative response to 25$(OH)D_3$. Our findings indicate that growth-inhibitory activity of 25$(OH)D_3$ is dependent upon levels of endogenous 1α-hydroxylase and suggest that prostate CA

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[3] The abbreviations used are: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25$(OH)D_3$, 25-hydroxyvitamin D_3; BPH, benign prostatic hyperplasia; CA, cancer; CZ, central zone; PSA, prostate-specific antigen; PZ, peripheral zone; TLC, thin layer chromatography; VDR, vitamin D receptor.

therapy with $25(OH)D_3$ might not be feasible because of the reduced levels of 1α-hydroxylase activity in CA cells.

Materials and Methods

Cell Culture. Primary cultures of prostatic epithelial cells were isolated from tissues obtained at radical prostatectomy and grown according to previously described protocols (23). With one exception (described in "Results"), only specimens from individuals untreated prior to surgery were used. Histological diagnosis established the tissues as normal prostate (PZ or CZ), BPH, or CA. CAs were graded according to the Gleason system. Nomenclature for epithelial cell strains is "E" followed by the histology of origin and then the strain number (*i.e.*, E-CZ-1). Cells strains used in this study were at similar passage number (~10–20 population doublings). Prostatic CA cell lines LNCaP, PC-3, and DU 145 were purchased from the American Type Culture Collection (Rockville, MD), and were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin. The prostatic CA cell line MDA-PCa 2b was obtained from Dr. Nora Navone (M. D. Anderson CA Center, Houston, TX; Ref. 24).

Cell Proliferation Assays. Cells were seeded at 5×10^5 cells/dish into 60-mm dishes coated with type-I collagen and containing the serum-free medium Complete 105 (23) with concentrations of $1,25(OH)_2D_3$ and $25(OH)D_3$ (generous gifts of Dr. M. Uskokovic, Hoffmann-LaRoche, Inc., Nutley, NJ) ranging from 0.01 to 10 nM. Cells treated with diluent (0.1% ethanol) were included as controls. After 3 days, the medium was replaced with the serum-free medium Complete PFMR-4A (23) containing fresh vitamin D compounds. Cells were then harvested on day 6 for determination of DNA content as a measure of accumulated cell mass using the diphenylamine-colorimetric assay of Burton (25). All of the reagents were obtained from Sigma (St. Louis, MO). The percentage of growth inhibition was calculated as follows: (total DNA content of treated cells/DNA content of diluent-treated cells) × 100%. Statistical analyses were performed using ANOVA. Differences were considered statistically significant when $P < 0.05$ or $P < 0.005$.

Quantitation of 1α-hydroxylase Activity. 1α-hydroxylase activity was determined using methods previously described with modifications. Cells were seeded in 6-well plates at 10^5 cells/well. At 24 h, $[^3H]25(OH)D_3$ (5 nM) and $25(OH)D_3$ (1 μM) were added as substrate. *N,N′*-diphenyl-*p*-phenylenediamine was included to inhibit auto-oxidation of $25(OH)D_3$ to $1,25(OH)_2D_3$ (20). After 4 h of incubation at 37°C, media and cells were collected for extraction of vitamin D metabolites with methanol/chloroform (2:1). The extract was then dried and redissolved in hexane/isopropanol (9:1) and subjected to TLC on silica gel TLC sheets (EM Science, Gibbstown, NJ). Mobility of $1,25(OH)_2D_3$ and $24,25(OH)D_3$ were determined by comigration of authentic standards. The production of $[^3H]1,25(OH)_2D_3$ was quantitated by scintillation counting. The corresponding protein concentration was determined by the method of Bradford (26). Enzymatic activity was expressed as picomoles of $1,25(OH)_2D_3$/mg protein/h. Data are expressed as mean ± SD obtained from triplicate wells of at least three independent experiments.

Results

Levels of 1α-hydroxylase Activity in Prostatic Epithelial Cells. Because the intracellular conversion of $25(OH)D_3$ to the biologically active $1,25(OH)_2D_3$ is dependent on 1α-hydroxylase, we measured endogenous enzymatic activities in prostatic epithelial cells cultured from prostatectomy specimens. Table 1 summarizes the activities of 1α-hydroxylase measured by enzyme-substrate reaction in 18 strains of normal prostatic epithelial cells, 8 strains of BPH cells, 15 cell strains derived from adenocarcinomas, and 4 established prostatic CA cell lines. Normal epithelial cells displayed the highest levels of 1α-hydroxylase activity, ranging from 1.19 to 3.1 pmol/mg protein/h. BPH cells tended to have lower levels of activity (1.21 to 1.71 pmol/mg protein/h), and the difference between BPH and normal cells reached statistical significance (Fig. 1). Primary cultures of CA cells and prostatic CA cell lines in general possessed significantly reduced 1α-hydroxylase activities (0.006–0.72 pmol/mg protein/h). Two exceptional prostatic CA cell strains, E-CA-14 and E-CA-15, exhibited relatively higher levels of 1α-hydroxylase activity (1.17 and 1.26 pmol/mg protein/h, respectively) than other CA cells. Interestingly, E-CA-14 was derived from an intraductal carcinoma and E-CA-15 was isolated from a patient who underwent antiandrogen therapy prior to radical prostatectomy. Overall, our data demonstrated a substantially reduced level of 1α-hydroxylase activity in prostatic CA cells (Table 1; Fig. 1).

Differential Antiproliferative Effects of $25(OH)D_3$ on Normal and CA Cells. The effect of $25(OH)D_3$ on cell proliferation was investigated in several strains of normal (E-CZ-2, E-PZ-8, and E-PZ-12) and CA-derived epithelial cells (E-CA-6, E-CA-10, and E-CA-12), as well as in the LNCaP prostate CA cell line. As shown in Fig. 2*A*, $25(OH)D_3$ displayed dose-dependent growth inhibition of normal prostatic epithelial cells that was statistically indistinguishable from that displayed by $1,25(OH)_2D_3$. Although primary cultures derived from prostatic adenocarcinomas did respond slightly to $25(OH)D_3$, the potency of this compound was significantly reduced compared with that of $1,25(OH)_2D_3$ (Fig. 2*B*). Moreover, treatment of LNCaP cells

Table 1 *Synthesis of $1,25(OH)_2D_3$ by human prostatic cells and cancer cell lines*[a]

Normal cells	$1,25(OH)_2D_3$ production[b]	BPH	$1,25(OH)_2D_3$ production[b]	Primary cancer	Gleason grade	$1,25(OH)_2D_3$ production[b]	Cancer cell lines	$1,25(OH)_2D_3$ production[b]
E-CZ-1	2.04	BPH-1	1.37	E-CA-1	3/3	0.17	LNCaP	0.006
E-CZ-2	2.25	BPH-2	1.46	E-CA-2	3/4	0.40	PC-3	0.071
E-CZ-3	1.27	BPH-3	1.71	E-CA-3	3/4	0.24	DU 145	0.279
E-CZ-4	2.10	BPH-4	1.65	E-CA-4	3/3	0.41	MDA-PCa 2b	0.34
E-PZ-1	2.14	BPH-5	1.21	E-CA-5	3/3	0.21		
E-PZ-2	2.60	BPH-6	1.57	E-CA-6	3/4	0.23		
E-PZ-3	2.10	BPH-7	1.61	E-CA-7	3/3	0.27		
E-PZ-4	1.23	BPH-8	1.28	E-CA-8	3/3	0.31		
E-PZ-5	1.19			E-CA-9	3/3	0.46		
E-PZ-6	2.34			E-CA-10	3/3	0.72		
E-PZ-7	3.10			E-CA-11	30% IDC[c]/70% 4	0.31		
E-PZ-8	2.63			E-CA-12	3/3	0.27		
E-PZ-9	2.43			E-CA-13	4/3	0.17		
E-PZ-10	2.31			E-CA-14	IDC	1.17		
E-PZ-11	2.70			E-CA-15	Antiandrogen therapy	1.26		
E-PZ-12	3.01							
E-PZ-13	1.30							
E-PZ-14	2.92							

[a] Values represent means of assays performed in triplicate. Strains E-CZ-2, E-PZ-8, E-CA-9, E-CA-14, E-CA15, and all four cancer cell lines were evaluated two and/or three times with values differing by <10%.

[b] pmol/mg protein/h.

[c] IDC, intraductal carcinoma.

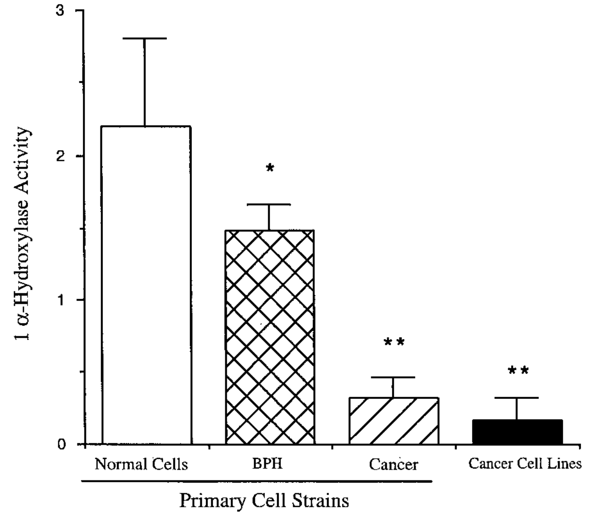

Fig. 1. 1α-hydroxylase activity in prostatic epithelial cells *in vitro*. Quantitative measurement of 1α-hydroxylase activity was performed in 18 strains of normal prostatic epithelial cells (☐), 8 strains of BPH cells (▩), 13 strains of CA-derived cells (▨) (E-CA-14 and E-CA-15 were excluded), and four prostatic CA cell lines (■). The mean ± SD for each group is shown. The 1α-hydroxylase activity in BPH cells was lower than in normal cells, and the difference reached statistical significance (*, $P < 0.05$). Furthermore, both prostatic CA cell strains and cell lines displayed a substantial reduction in 1α-hydroxylase activity compared with normal cells and BPH (**, $P < 0.005$). E-CA-14 and E-CA-15 were excluded.

with $25(OH)D_3$ failed to confer any antiproliferative effect, whereas $1,25(OH)_2D_3$ was quite inhibitory (Fig. 2*C*).

Responsiveness to $25(OH)D_3$ Correlates with Endogenous 1α-hydroxylase Activity. To correlate the antiproliferative activity of $25(OH)D_3$ with 1α-hydroxylase activity, we plotted the ratio of $25(OH)D_3$:$1,25(OH)_2D_3$-mediated growth inhibition against the level of 1α-hydroxylase activity for each cell strain or cell line (Fig. 3). Our data demonstrate a highly significant correlation (*r*, 0.93), indicating that the level of 1α-hydroxylase activity in prostatic epithelial cells

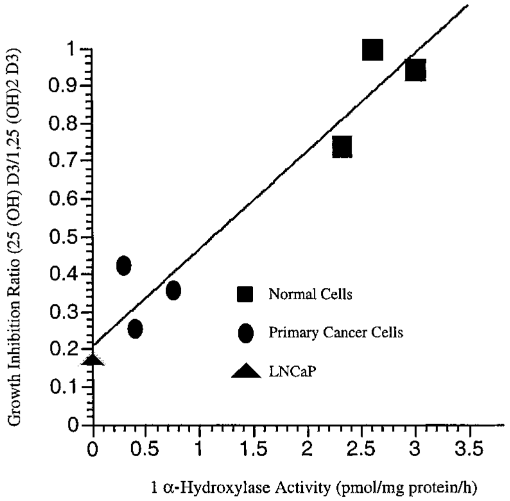

Fig. 3. Correlation of growth-inhibitory activity and 1α-hydroxylase activity. The ratio of growth inhibition induced by $25(OH)D_3/1,25(OH)_2D_3$ (concentration at 1 nm) correlated with endogenous 1α-hydroxylase activity in normal prostatic epithelial cells (■), primary cultures of CA cells (●), and the LNCaP CA cell line (▲). The *X* axis represents the mean enzymatic activity, expressed as pmol of $1,25(OH)_2D_3$ production/mg total protein/h from triplicate wells (refer to Fig. 1). The *Y* axis depicts the mean percentage of growth inhibition after treatment with $25(OH)D_3$ compared with $1,25(OH)_2D_3$ (see Fig. 2). At concentrations of $25(OH)D_3$ from 0.1 to 10 nm, the efficacy of antiproliferative effect correlates with the level of 1α-hydroxylase activity (data not shown). The computer-determined regression line was plotted according to the equation $f(X) = 2.579617\ E\text{-}1 * X + 2.112030\ E\text{-}1$. *R*, 0.93.

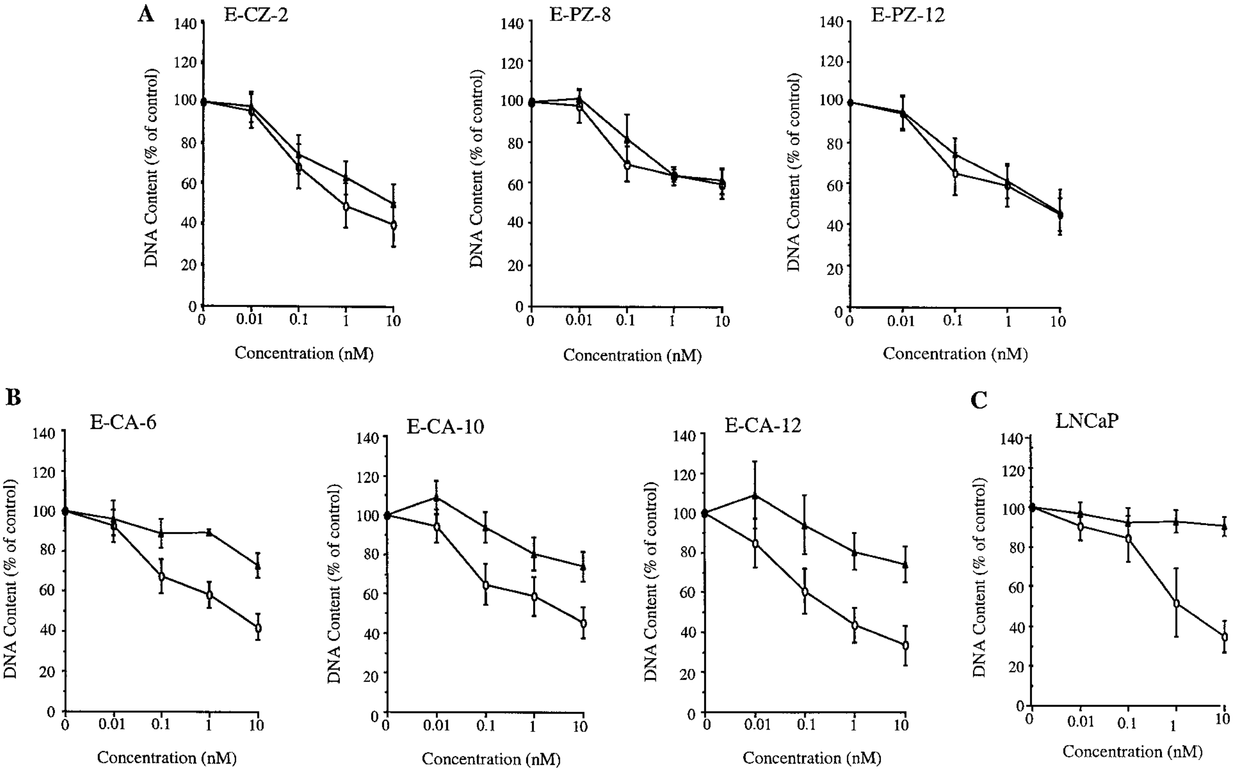

Fig. 2. Growth inhibition by $25(OH)D_3$ (*triangles*) in comparison with $1,25(OH)_2D_3$ (*circles*) in prostatic epithelial cells. *A*, in normal prostatic epithelial cells (E-PZ-8, E-PZ-12, and E-CZ-2), $25(OH)D_3$ and $1,25(OH)_2D_3$ were equally growth inhibitory at concentrations between 0.01 and 10 nm. *B*, in primary cultures of CA cells (E-CA-6, E-CA-10, and E-CA-12), $1,25(OH)_2D_3$ induced ~20–40% more growth inhibition than $25(OH)D_3$. *C*, a prostate CA cell line, LNCaP, although fully responsive to $1,25(OH)_2D_3$, was resistant to treatment with $25(OH)D_3$.

determines the degree of growth inhibition by $25(OH)D_3$. Normal epithelial cells, with high endogenous 1α-hydroxylase activities, were as responsive to $25(OH)D_3$ as to $1,25(OH)_2D_3$. Growth inhibition of 40–50% was induced by both $25(OH)D_3$ and $1,25(OH)_2D_3$ in these cells at a concentration of 1 nM. The same dose of $25(OH)D_3$ only suppressed proliferation of primary cultures of prostatic CA cells by ~10–20%, whereas $1,25(OH)D_3$ inhibited growth by 40–50%. Interestingly, LNCaP cells, which exhibited the lowest level of 1α-hydroxylase activity (0.006 pmol/mg protein/h; Table 1), were totally resistant to $25(OH)D_3$ (Fig. 2*C*).

Discussion

Because the mortality of prostate CA continues unabated, the development of new therapeutic agents has become an urgent need. Vitamin D has emerged as a therapeutic option from a wealth of epidemiological, experimental, and clinical studies (4). It is now clear that the prostate is a source (20) as well as a target organ of $1,25(OH)_2D_3$, and that the hormone stimulates prostatic cellular differentiation and regulates growth. However, the therapeutic potential of vitamin D cannot be fully exploited because of the dose-dependent hypercalcemia associated with the administration of active hormone, $1,25(OH)_2D_3$ (9, 10). To circumvent the toxicity, relatively noncalcemic vitamin D analogues have been synthesized and tested for antineoplastic activity (8, 11–14).

As an alternative approach, we evaluated the possible use of the less calcemic $25(OH)D_3$ as a prodrug for prostate CA treatment. This compound is the natural precursor of $1,25(OH)_2D_3$, and its reduced calcemic activity would allow substantial increases in dosage compared with the active metabolite. Systemic conversion to $1,25(OH)_2D_3$ would be limited by suppression of parathyroid hormone, a requirement of 1α-hydroxylase in kidney but not in prostate. Recently, Barreto *et al.* (21) reported that $25(OH)D_3$ exhibited an antiproliferative effect on normal prostatic epithelial cells through intracellular conversion to $1,25(OH)_2D_3$. Although we confirmed the ability of $25(OH)D_3$ to inhibit normal prostatic epithelial cells, the important conclusion from our study is that primary cultures of CA cells have significantly reduced levels of 1α-hydroxylase and are not inhibited by $25(OH)D_3$.

Our finding differs from that of Chen *et al.* (27), who reported that $25(OH)D_3$ and $1,25(OH)_2D_3$ were equipotent inhibitors of the growth of primary cultures of human prostatic CA cells. The details were not provided regarding the protocols used by these investigators to obtain samples of adenocarcinomas. Because this is a complex procedure, we suggest that perhaps the cells used by Chen *et al.* were inadvertently derived from normal tissues rather than from CA. The histopathological descriptions of the CAs of origin were not provided, and the number of different cell strains tested was not clear. If indeed the cultures were actually derived from malignant tissues, then it is possible that those CAs had features in common with the exceptional CAs that in our study gave rise to cell strains with relatively normal levels of 1α-hydroxylase. One of these two cell strains was derived from a tumor with an unusual pathology of intraductal carcinoma (E-CA-14) and the other from a patient treated by androgen-ablation prior to surgery (E-CA-15). In contrast to all of the other cell strains, these two particular cell strains grew very poorly, rendering assays for growth inhibition not feasible.

The discrepancy between our results and those of Chen *et al.* emphasizes the necessity of precise histopathological characterization of prostatic tissues from which primary cultures are isolated. This is essential because no markers have been available to definitely identify prostatic CA cells in culture. In many aspects, primary cultures of human prostatic CA cells resemble those derived from normal tissues or BPH. Morphologies are similar, responses to growth factors are the same, and all are mortal and nontumorigenic in host animals (23). Tang *et al.* (28) found a difference in growth rate between primary cultures of normal *versus* CA cells, but in our culture system, growth rates between the two types of cultures are generally very similar. The only consistent difference that we have found between normal and CA-derived primary cultures is that normal cell strains are diploid, whereas CA-derived primary cultures have cytogenetic abnormalities (29). Therefore, the consistent reduction in activity of 1α-hydroxylase found in almost all of the CA-derived cultures is quite remarkable and may provide a novel *in vitro* marker to distinguish normal from CA cells. It is worth noting that both normal and CA-derived cell cultures in this study were isolated and grown under identical conditions, and, in fact, one set of normal and malignant cell cultures (E-CZ-3, E-PZ-13, and E-CA-11) were derived from the same individual.

The activity of 1α-hydroxylase was significantly different between normal and BPH. In a previous report by Schwartz *et al.* (20), the one cell strain from BPH that was tested also had lower 1α-hydroxylase activity than the cell strain derived from normal tissue. Whereas this was attributed to age differences between the donors of the two specimens, our results suggest that the difference may instead reflect biological differences between normal and BPH cells, because the donors of our cell strains were all within a similar age range. Although 1α-hydroxylase levels in BPH cells were intermediate between normal and CA-derived cells, BPH is not considered to be a precursor of prostate CA; therefore, reduced activity of 1α-hydroxylase in BPH does not represent a step toward development of prostate CA.

The levels of 1α-hydroxylase activity that we found in the established CA cell lines were very similar to those reported previously (20), with DU 145 cells having the highest activity and LNCaP cells the least. The MDA PCa 2b cell line, derived from a bony metastasis of CA of the prostate (24), had a level of 1α-hydroxylase activity similar to that of DU 145. Overall, activity of 1α-hydroxylase was low in the established prostate CA cell lines as well as in primary cultures of CA cells.

Our findings of reduced 1α-hydroxylase activity in CA-derived prostatic cells raises the possibility that this difference may endow the malignant cells with an intrinsic growth advantage because of the resultant decrease in production of local growth inhibitory $1,25(OH)_2D_3$. In addition, local deficiency of $1,25(OH)_2D_3$ may allow cellular de-differentiation and invasion, hallmarks of malignancy. We conclude that decreased activity of 1α-hydroxylase may represent an important mechanism in prostate CA development and/or progression. Because most of the malignant cell strains that we investigated originated from adenocarcinomas of Gleason grades 3/3 to 4/3, it appears that reduction of 1α-hydroxylase activity occurs at an early stage of development of prostate CA. Given the potential of 1α-hydroxylase as a diagnostic and/or prognostic marker and as a future therapeutic target, it will be important to examine the protein expression of 1α-hydroxylase *in situ*. When the appropriate reagents become available, we will evaluate tissue samples of various CA stages including the premalignant lesion, prostatic intraepithelial neoplasia. It is also hoped that understanding how the activity of 1α-hydroxylase is regulated at a molecular level may shed light on the pathogenesis of prostate CA. Finally, although use of $25(OH)D_3$ may not represent a feasible therapeutic approach for established prostate CA, administering $25(OH)D_3$ might be an effective approach to prevent or slow the development of prostate CA.

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