# 25-Hydroxyvitamin $D_3$, the Prohormone of 1,25-Dihydroxyvitamin $D_3$, Inhibits the Proliferation of Primary Prostatic Epithelial Cells

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## Abstract

**The hormonal metabolite of vitamin D, 1$\alpha$,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] is known to inhibit the proliferation of prostatic epithelial cells. This has stimulated interest in vitamin D compounds as therapeutic agents for prostate cancer. However, the therapeutic use of 1,25$(OH)_2D_3$ is limited because elevations in serum 1,25$(OH)_2D_3$ can cause dangerous elevations in serum calcium levels. We wondered whether the prohormone of 1,25$(OH)_2D_3$, 25-hydroxyvitamin $D_3$ (25-OH-$D_3$), which is much less calcemic, could also achieve antiproliferative effects in prostatic cells. 25-OH-$D_3$ is converted to 1,25$(OH)_2D_3$ by the mitochondrial enzyme 1-$\alpha$-hydroxylase. We have recently shown that human prostatic cells also possess significant 1-$\alpha$-hydroxylase activity (Schwartz *et al.*, Cancer Epidemiol. Biomark. Prev., *7:* 391–395, 1998). We studied 1-$\alpha$-hydroxylase gene expression in four strains of primary human prostatic epithelial cells by reverse transcription PCR amplification (RT-PCR) of 1-$\alpha$-hydroxylase. Human prostatic stromal cells were negative for 1-$\alpha$-hydroxylase by RT-PCR. This led us to hypothesize that 25-OH-$D_3$ would inhibit the proliferation of prostatic epithelial cells because 25-OH-$D_3$ would be converted to 1,25$(OH)_2D_3$ intracellularly. We studied the effects of 25-OH-$D_3$ and 1,25$(OH)_2D_3$ on the proliferation of prostatic epithelial cells using high density growth and clonal growth assays on two different primary cell strains derived from normal human prostatic peripheral zone. 25-OH-$D_3$ and 1,25$(OH)_2D_3$ each inhibited growth in a dose- and time-dependent manner. Growth inhibition was evident at 1 nM, and maximal inhibition was observed at 100 nM within 10–12 days of exposure. The potencies of 25-OH-$D_3$ and 1,25$(OH)_2D_3$ were not significantly different. These data demonstrate that 25-OH-$D_3$, which previously was thought to have little biological activity, can become a potent antiproliferative hormone for prostatic cells that express 1-$\alpha$-hydroxylase. Because 25-OH-$D_3$ exhibits similar potency to 1,25$(OH)_2D_3$ but is less calcemic, 25-OH-$D_3$ may offer a safer option than 1,25$(OH)_2D_3$ for prostate cancer therapy. Moreover, because 25-OH-$D_3$ is produced endogenously from vitamin D, these findings support a potential role for vitamin D in the chemoprevention of prostate cancer.**

## Introduction

Prostate cancer is the most common non-skin cancer in United States males and the second leading cause of cancer death (1). Despite advances in early diagnosis, approximately 50% of the men diagnosed with prostate cancer each year have locally advanced or metastatic disease at the time of diagnosis and are not candidates for curative therapy (surgery or radiation; Ref. 2). Several therapeutic strategies have been devised to treat locally advanced or metastatic prostate cancer including chemotherapeutic, radiotherapeutic, and hormonal ablation therapy (2). However, these treatments are palliative, and new therapeutic approaches are urgently needed. Considerable recent data suggest that vitamin D compounds hold promise as therapeutic agents in prostate cancer (3–5).

"Vitamin" D is a hormone that is produced from 7-dehydrocholesterol by a series of reactions that culminates in the most active metabolite of vitamin D, 1,25$(OH)_2D_3$,[2] also known as calcitriol (for a recent review, see Ref. 6). When the skin is exposed to sunlight, UV rays convert 7-dehydrocholesterol in the skin to vitamin $D_3$ (cholecalciferol). Vitamin D can also be obtained from the diet, either as cholecalciferol or ergocalciferol (vitamin $D_2$). Cholecalciferol is transported to the liver, where the enzyme 25-hydroxylase converts it to 25-OH-$D_3$, also known as calcifidiol and calcidiol. This relatively inactive compound is the major circulating metabolite of vitamin D. From the liver, 25-OH-$D_3$ is transported to the kidneys, where 1-$\alpha$-hydroxylase converts 25-OH-$D_3$ to 1,25$(OH)_2D_3$, the active hormonal metabolite of vitamin D. In vitamin D-target tissues such as the intestine, 1,25$(OH)_2D_3$ exerts most of its biological activity through binding to the VDR. 1,25$(OH)_2D_3$ has approximately 500-1000 times the affinity for the nuclear VDR than does 25-OH-$D_3$ (7). 1,25$(OH)_2D_3$ plays a major role in mineral homeostasis by regulating serum calcium and phosphorus levels. In addition to its role in mineral homeostasis, it is now apparent that 1,25$(OH)_2D_3$ exhibits potent antiproliferative and differentiating properties in a variety of cell types, including prostatic cells (8–11).

In 1990, Schwartz and Hulka (3) proposed the vitamin D hypothesis for prostate cancer. On the basis of epidemiological

Received 7/19/99; revised 11/22/99; accepted 12/8/99.
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[2] The abbreviations used are: 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; VDR, vitamin D receptor; RT, reverse transcription; PSA, prostate-specific antigen; DPPD, 1,2 dianilinoethane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

data, they proposed that vitamin D maintains the normal phenotype of prostatic cells and that decreased vitamin D exposure increased the risk for clinical prostate cancer. Since the proposal of that hypothesis, several groups have demonstrated antiproliferative and prodifferentiatng effects of 1,25$(OH)_2D_3$ on human prostatic cells (8–11). These data have led to the investigation of 1,25$(OH)_2D_3$ as a therapeutic agent for prostate cancer. The first clinical trial with 1,25$(OH)_2D_3$ as a treatment for prostate cancer was performed by Osborn *et al.* (12). Osborn *et al.* assessed the effects of 1,25$(OH)_2D_3$ on patients with hormone-refractory (androgen-insensitive) metastatic prostate cancer. No partial responses (defined as a sustained 50% decrease in serum PSA) were observed, although two men showed substantial decreases in serum PSA (25% and 45% declines). Hypercalcemia was the major dose-limiting toxicity. More recently, Gross *et al.* (13) assessed the effects of 1,25$(OH)_2D_3$ in patients with biochemical recurrence of localized prostate cancer after definitive therapy. They demonstrated that 0.5–2.5 $\mu$g of 1,25$(OH)_2D_3$ given daily decreased the PSA doubling time by 50% every 3.3 month. However, several of these patients developed hypercalciuria, which resulted in a halt to the dose escalation during this trial. Thus, the calcemic effects associated with 1,25$(OH)_2D_3$ therapy limit the utility of this hormone for prostate cancer therapy.

The rate-limiting step in the synthesis of systemic 1,25$(OH)_2D_3$ is the conversion of 25-OH-$D_3$ to 1,25$(OH)_2D_3$ by renal 1-$\alpha$-hydroxylase. Local production of 1,25$(OH)_2D_3$ by nonrenal tissues that express 1-$\alpha$-hydroxylase has emerged as a new aspect of the vitamin D endocrine system. 1-$\alpha$-hydroxylase activity has been identified in a number of extrarenal sites in humans, including decidua, keratinocytes, macrophages, spleen cells, osteoblasts, and colon carcinoma cells (reviewed in Ref. 14). In keratinocytes, endogenously produced 1,25$(OH)_2D_3$ is involved in the control of keratinocyte growth and differentiation (15, 16). Recently, Schwartz *et al.* showed that prostate cancer cell lines and normal and benign prostatic hyperplasia primary cultures have 1-$\alpha$-hydroxylase activity and synthesize 1,25$(OH)_2D_3$ from 25-OH-$D_3$ (17). The 1-$\alpha$-hydroxylase activity of the primary human prostatic epithelial cells was comparable to that of primary renal proximal tubular cells.

The intracellular production of 1,25$(OH)_2D_3$ by the prostate suggested to us that 25-OH-$D_3$ might regulate the growth and differentiation of prostatic cells. To date, no study has reported on the antiproliferative effects of 25-OH-$D_3$ on human prostatic cells that express 1-$\alpha$-hydroxylase. In this report, we demonstrate that 25-OH-$D_3$ can inhibit primary prostatic epithelial cell growth in a time- and dose-dependent manner and that its effects are similar in magnitude to those of 1,25$(OH)_2D_3$.

## Materials and Methods

**Materials.** 1,25$(OH)_2D_3$ and 25-OH-$D_3$ were obtained from Biomol (Plymouth Meeting, PA). The purity of both 1,25$(OH)_2D_3$ and 25-OH-$D_3$ were 99% as reported by the manufacturer. DPPD was obtained from Aldrich Chemical Company (Milwaukee, WI). Concentrated stocks were prepared in 100% ethanol and stored at −20°C. The final concentration of ethanol in the media did not exceed 0.2%.

**Cell Culture.** Primary human epithelial cell cultures from histologically normal prostate peripheral zones were obtained from radical prostatectomies performed at Wake Forest University School of Medicine and were maintained as described previously (18, 19). Briefly, a small piece of tissue from each specimen was removed and minced. The tissue was digested with collagenase overnight. To remove the collagenase and the majority of the stromal cells, the tissue was rinsed and centrifuged. The tissue was inoculated into a 60-mm tissue culture dish coated with collagen type I (Collagen Corporation, Palo Alto, CA) and grown in medium PFMR-4A supplemented with growth factors and hormones (19). Previous studies have demonstrated that prostatic stromal cells do not grow in the serum-free conditions used in this study, yet these conditions maintain the growth and differentiation of prostatic epithelial cells (19). Morphological characteristics of the cultures used in the present study were consistent with the growth of epithelial cells. The cells that grew out from the tissue were aliquoted and stored in liquid nitrogen. The histology of each specimen was verified by inking and fixing the prostate after dissection and serially sectioning the marked area. The histology of sections immediately adjacent to the area of the dissection was reviewed. The frozen aliquots were thawed to produce secondary cultures, which were grown in medium MCDB 105 (Sigma, St. Louis, MO) supplemented with growth factors and hormones (19). Nomenclature for epithelial cell strains is "E" followed by the histology of origin (peripheral zone) and then the strain number (*e.g.,* E-PZ-1).

Stromal cell cultures were established by inoculating collagenase-digested tissues into medium MCDB 105 with 10% fetal bovine serum and 100 $\mu$g/ml gentamicin in 60-mm tissue culture dishes (20). Although epithelial cells attach in this medium, they do not grow well and are lost after the first passage. Passaged cultures were aliquoted and stored frozen in liquid nitrogen until use. Stromal cell cultures do not express keratin but express vimentin and fibronectin (20). Nomenclature for stromal cell strains is "F" followed by the histology of origin (peripheral zone) and then the strain number (*e.g.,* F-PZ-1). Strain numbers for stromal cells do not necessarily correlate with strain numbers for epithelial cells.

**Experimental Media.** Complete medium for the primary prostatic epithelial cells is defined as MCDB 105 supplemented with cholera toxin (10 ng/ml), epidermal growth factor (10 ng/ml), bovine pituitary extract (10 $\mu$g/ml), phosphoethanolamine (0.1 mM), hydrocortisone (1 $\mu$g/ml), selenium ($3 \times 10^{-8}$ M), gentamicin (100 $\mu$g/ml), retinoic acid (0.01 ng/ml), insulin (4 $\mu$g/ml), and vitamin E ($2.3 \times 10^{-6}$ M). Experimental medium is defined as MCDB-105 with the aforementioned supplements, 20 $\mu$M DPPD [an antioxidant used to prevent the generic conversion of 25-OH-D3 to 1,25$(OH)_2D_3$], and increasing dosages of either 25-OH-D3 or 1,25$(OH)_2D_3$.

**High-Density Dose Response and Time Course.** Prostatic epithelial cells ($5 \times 10^3$) from thawed secondary cultures were inoculated into 35-mm tissue culture dishes coated with type I collagen and containing complete MCDB 105 medium. After 48 h of growth at 37°C in a humidified incubator with 5% $CO_2$, the media were replaced and cells were treated with vehicle (0.1% ethanol and 20 $\mu$M DPPD) or with media containing 0.1–100 nM 25-OH-$D_3$ or 1,25$(OH)_2D_3$. The media were changed every 3 days. For the dose response, cells were grown for 11 more days and then trypsinized and counted with a hemocytometer. Each dose was tested on triplicate plates. For the time course, three plates were trypsinized and counted as $T_0$ on the first day the experimental medium was added. Cell counts were done in triplicate on days 1, 2, 4, 6, 8, and 10 for each experimental medium. Results are expressed as the mean cell number per plate ± SE.

**Clonal Growth Assay.** Clonal growth assays were performed as described previously (10, 18). Six hundred cells from thawed primary cultures were inoculated into 60-mm tissue culture

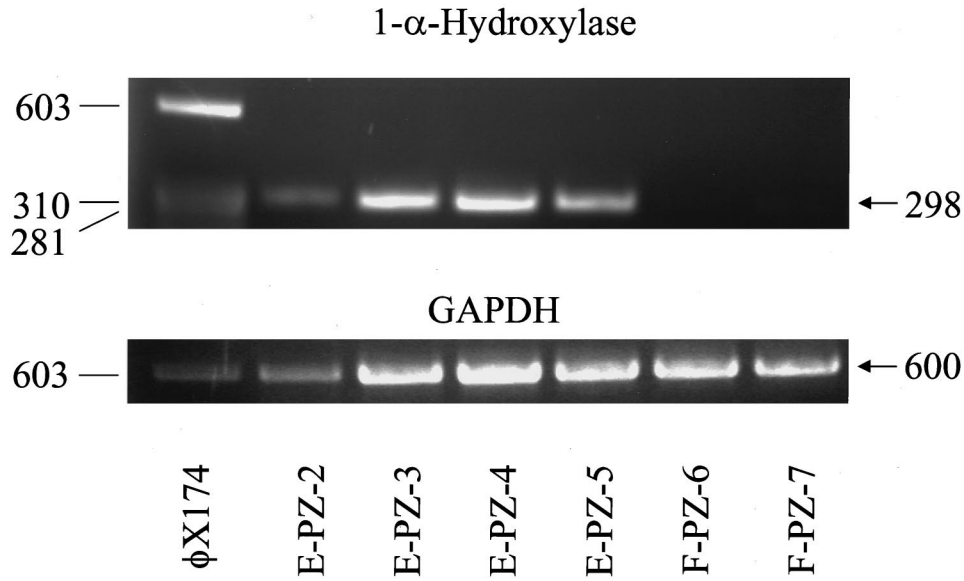


*Fig. 1.* 1-α-hydroxylase expression in primary cultures of human prostatic cells. Twenty μl of each RT-PCR of 1-α-hydroxylase and GAPDH were fractionated on a 2% agarose gel and photographed as described in "Materials and Methods." *Top panel*, amplification of 1-α-hydroxylase. *Bottom panel*, amplification of GAPDH from the same RT reaction used for the corresponding 1-α-hydroxylase amplification. *Numbers to the left*, the marker sizes in bp.; *numbers to the right*, the predicted size of the RT-PCR product in bp. φX174 = 500 ng of φX174 DNA digested with *Hae*III restriction enzyme. *Bottom of each Lane*, the strains from which RNA was isolated (see "Materials and Methods" for nomenclature details).

dishes coated with type I collagen containing experimental medium. Cells were grown at 37°C in a humidified incubator with 5% $CO_2$ for 13–14 days, at the end of which, cells were fixed with 10% formalin and stained with 0.1% crystal violet. The number and size of stained colonies were then determined on each plate.

**1-α-Hydroxylase mRNA Expression.** Total RNA was isolated from cultured cells by the guanidinium-phenol-chloroform extraction method (21). Oligo dT-primed RT with 1 μg of total RNA was performed using the GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's protocol. The RT reaction [6 μl (⅓)] was used for subsequent PCR amplification of the 3′ untranslated region of the 1-α-hydroxylase cDNA from base 1853 to 2150 of the sequence reported by Monkawa *et al.* (22).[3] The reaction was performed with 150 nM 5′ primer (5′-TGGTCTCTCTGCTTGCTTGG-3′), 150 nM 3′ primer (5′-GACACCTAGTCAGAGACAGG-3′), 1× PCR buffer (Promega, Madison, WI), 150 μM each dNTP, and 2 mM $MgCl_2$ in a final reaction volume of 100 μl. All of the thermal reactions were performed in a Perkin Elmer Applied Biosystems GenAmp 2400 (South San Francisco, CA). Samples were incubated for 5 min at 94°C followed by 5 min at 80°C, during which 2.5 units of Taq DNA polymerase (Promega) were added. Samples were subjected to 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, followed by a 7-min final extension at 72°C. RT-PCR reactions were fractionated on 2% agarose gels and visualized by ethidium bromide staining using an IS-500 digital imaging system (Alpha Innotech, San Leandro, CA) and processed with the software provided by the manufacturer. φX174 genomic DNA, digested with *Hae*III restriction enzyme (Promega), was fractionated in a parallel lane as a size marker. For a positive control of RT, the GAPDH mRNA was amplified using human GAPDH primers purchased from Stratagene (La Jolla, CA) according to the manufacturer's protocol.

For sequence analysis of RT-PCR product, a sample was fractionated on a 2% agarose gel, and the 298-bp fragment was sliced out of the gel and extracted from the agarose using the GeneClean DNA purification kit (Bio 101, Vista, CA). The nucleotide sequence of the purified PCR product was determined by automated fluorescent nucleotide sequencing with an ABI Prism 377 DNA Sequencer by the DNA sequencing facility of the Comprehensive Cancer Center of Wake Forest University. Sequence comparison with the published 1-α-hydroxylase was determined using the Blast software at the National Center for Biotechnology Information Web site.[4]

**Statistics.** Statistical analyses were performed using the SAS statistical package release 6.12 TS Level 0020 Windows version 4.0.950, (SAS Institute Inc., Cary, NC). Multiple ANOVA controlling for dose or time and vitamin D compound [ethanol (the control), 25-OH-$D_3$, or 1,25$(OH)_2D_3$] was used to detect differences between vitamin D treatment groups. ANOVA followed by Tukey-Kramer Multiple Comparison Test was used to evaluate significant differences within each treatment group (*i.e.*, different doses of 25-OH-$D_3$).

## Results

We used RT-PCR to validate the expression of 1-α-hydroxylase in epithelial cells grown under the conditions used in this study. Four epithelial strains and two stromal strains derived from human prostatectomy specimens were evaluated for 1-α-hydroxylase gene expression. Fig. 1 shows that all of the four epithelial cell strains expressed detectable 1-α-hydroxylase transcripts. Sequence analysis of the PCR product verified the 1-α-hydroxylase sequence. Neither of the stromal cell strains expressed any detectable 1-α-hydroxylase transcript.

To determine whether exposure to 25-OH-$D_3$ inhibits the proliferation of primary prostatic epithelial cells, we performed high-density growth assays. Two different strains of normal primary human prostatic epithelial cells were treated with various concentrations of 25-OH-$D_3$ and 1,25$(OH)_2D_3$ in the presence of DPPD to prevent spontaneous oxidation of 25-OH-$D_3$ (23). Cell number was determined after 11 days. Fig. 2 shows

[3] GenBank Accession No. AB005038.

[4] Internet address: http://www.ncbi.nlm.nih.gov/BLAST/

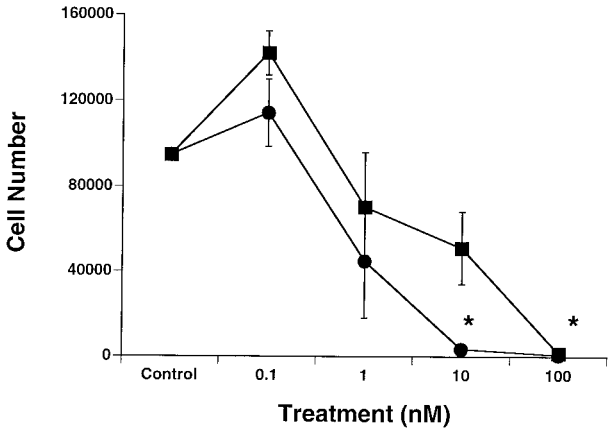


*Fig. 2.* 25-OH-$D_3$ inhibits primary prostatic epithelial cell high-density growth in a dose-dependent manner. Cells (5000) were inoculated into 35-mm, collagen-coated dishes containing serum-free media. After 48 h, experimental media with vehicle, or 0.1–100 nM 25-OH-$D_3$, or 1,25$(OH)_2D_3$ were added. After 11 days of growth, dishes were trypsinized and counted with hemocytometer. Each point represents the mean ± SE of duplicate or triplicate dishes. ■, 25-OH-$D_3$-treated cultures; ●, 1,25$(OH)_2D_3$-treated cultures; ∗, significantly different from control, $P = 0.05$.

the results of a representative experiment showing high-density growth inhibition by 25-OH-$D_3$ and 1,25$(OH)_2D_3$ of strain E-PZ-1. Both 25-OH-$D_3$ and 1,25$(OH)_2D_3$ inhibited growth in a dose-dependent manner. The dose-response curves for the two compounds were parallel, with the effects of both compounds apparent at 10 nM. The two curves were not statistically different. However, we observed a trend for a higher potency of 1,25$(OH)_2D_3$ *versus* 25-OH-$D_3$, with 1,25$(OH)_2D_3$ approximately 10-fold more potent than 25-OH-$D_3$. ANOVA performed on the individual dose-response curves demonstrated that significant growth inhibition occurred at 10 nM 1,25$(OH)_2D_3$, but that 100 nM 25-OH-$D_3$ was required for this effect. These results were repeated once with this strain (E-PZ-1) and twice with an additional strain (E-PZ-2; data not shown).

The time-dependent effects of high-density growth inhibition were evaluated in both of the prostatic strains. Fig. 3 shows a time course of the effects of both 25-OH-$D_3$ and 1,25$(OH)_2D_3$ on the primary prostatic epithelial cell strain E-PZ-1. Three different concentrations of each of the compounds were studied [1 nM (Fig. 3*A*), 10 nM (Fig. 3*B*), and 100 nM (Fig. 3*C*)]. Both 25-OH-$D_3$ and 1,25$(OH)_2D_3$ inhibited growth in a time-dependent manner at all of the three concentrations tested, although this effect was not statistically significant at 1 nM. The difference in growth between control- and vitamin D-treated cultures is significant by 10 days with 100 nM, whereas it took 12 days for this effect to be significant with 10 nM. As with the dose response, the effects of 25-OH-$D_3$ and 1,25$(OH)_2D_3$ were not statistically different. These results were repeated once with this strain and twice with strain E-PZ-2 (data not shown).

Clonogenic growth assays measure the effects of compounds on cell growth under conditions of very low cell density. Under these conditions, the effects of secreted factors on paracrine growth are limited because of the diluting effects of the large volume of medium in which the cells are grown (18). We next evaluated the effects of 25-OH-$D_3$ and 1,25$(OH)_2D_3$ on the clonogenic growth of the two strains of prostatic epithelial cells that we tested for high-density

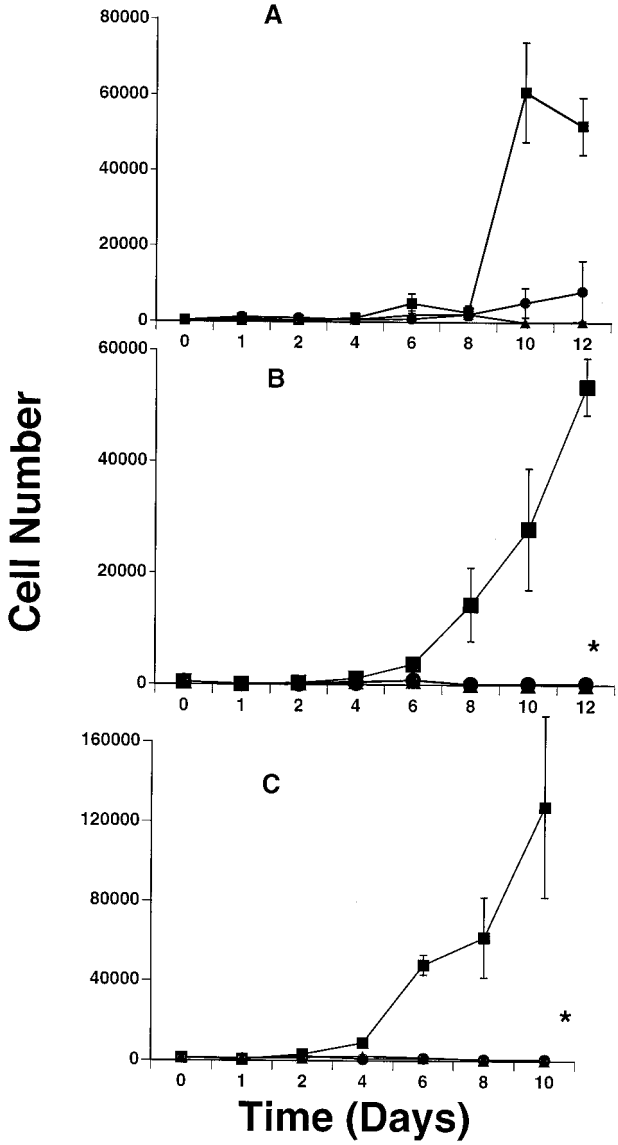


*Fig. 3.* 25-OH-$D_3$ inhibits primary prostatic epithelial cell high-density growth in a time-dependent manner. Cells (5000) were inoculated into 35-mm, collagen-coated dishes containing serum-free media. After 48 h, three dishes were trypsinized and counted with a hemocytometer ($T_0$), and experimental media were added to remaining dishes. Three dishes from each treatment were trypsinized and counted on days 1, 2, 4, 6, 8, 10, and 12. Treatments were as follows: *A*, 1 nM of 25-OH-$D_3$ or 1,25$(OH)_2D_3$; *B*, 10 nM of 25-OH-$D_3$ or 1,25$(OH)_2D_3$; *C*, 100 nM of 25-OH-$D_3$ or 1,25$(OH)_2D_3$. ■, vehicle control-treated cultures; ●, 25-OH-$D_3$-treated cultures; ▲, 1,25$(OH)_2D_3$-treated cultures. *Each point*, the mean ± SE of triplicate dishes; ∗, significantly different from control, $P = 0.05$.

growth. Both 25-OH-$D_3$ and 1,25$(OH)_2D_3$ inhibited growth in a dose-dependent manner. Fig. 4 shows representative clonogenic growth plates from strain E-PZ-1 treated with various concentrations of 25-OH-$D_3$ or 1,25$(OH)_2D_3$ and stained with crystal violet after 14 days of growth. The results were essentially identical to the results from high-density growth with 1,25$(OH)_2D_3$. The assay was performed three times with strain E-PZ-1 and three times with strain E-PZ-2. All of the experiments gave similar results.

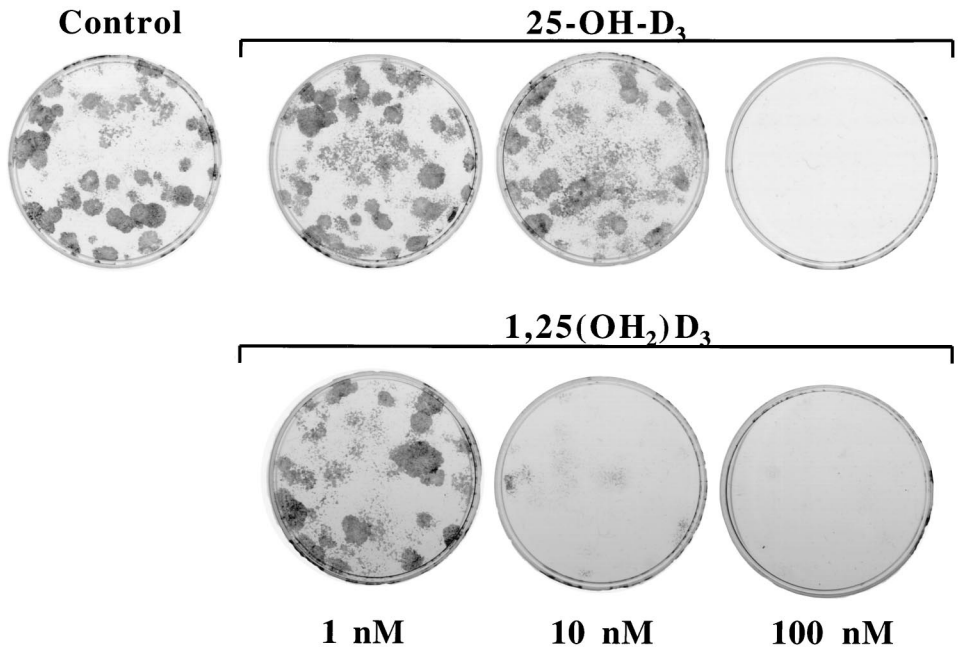


*Fig. 4.* Clonal growth of prostatic epithelial cells in response to 25-OH-$D_3$ and 1,25$(OH)_2D_3$. Six hundred cells were inoculated into 60-mm, collagen-coated dishes containing serum-free media with vehicle or the indicated concentrations of 25-OH-$D_3$ or 1,25$(OH)_2D_3$. After 14 days of incubation, the cells were fixed and stained. Each dose was tested on triplicate dishes. Representative dishes from each treatment are shown.

## Discussion

The recent identification of 1-$\alpha$-hydroxylase in human prostatic cells by Schwartz *et al.* (17) suggested that intracellular production of 1,25$(OH)_2D_3$ may be an important modulator of prostatic growth. In this report we describe the growth inhibitory properties of 25-OH-$D_3$ on primary human prostatic epithelial cells. Maximal growth inhibition (essentially no growth) was observed at 100 nM 25-OH-$D_3$ by 6–8 days. The concentrations of 25-OH-$D_3$ used in this study are within the normal physiological range in humans (35–100 nM; Ref. 7) and may play a role in the normal modulation of prostatic growth and differentiation. However, *in vivo* studies will be required to validate this hypothesis. Interestingly, the concentrations of 1,25$(OH)_2D_3$ needed to inhibit prostatic growth range from 1 to 100 nM, but the physiological range of serum 1,25$(OH)_2D_3$ in normal healthy adults is in the pM range. Therefore, systemic 1,25$(OH)_2D_3$ levels are less likely to play a normal role in prostatic growth and differentiation. Concentrations of 1,25$(OH)_2D_3$ above the normal physiological range, which are needed to inhibit prostatic growth, are known to cause hypercalciuria and/or hypercalcemia (12, 13). A noncalcemic form of vitamin D that inhibits prostatic growth, therefore, would be very useful for the treatment of prostate cancer.

One way to circumvent the problem of hypercalcemia is via the use of synthetic, relatively noncalcemic forms of 1,25$(OH)_2D_3$, such as EB 1089 (24), which is presently in human clinical trials for prostate cancer. We propose that the prohormone 25-OH-$D_3$ may be another way to circumvent this problem. 25-OH-$D_3$ appears to be an ideal candidate for a therapeutic vitamin D compound for several reasons: (*a*) it is effective in inhibiting prostatic growth at concentrations within its normal physiological range; (*b*) it is safe at levels far exceeding its physiological range (7); (*c*) oral dosing of 50 $\mu$g per day has been used in long-term studies for other diseases with no adverse side-effects (25); and (*d*) oral formulations of 25-OH-$D_3$ have long been available (*e.g.*, for the treatment of vitamin D insufficiency), and their safety has long been established.

The relative affinity of 25-OH-$D_3$ for the VDR is approximately 500- to 1000-fold lower than the affinity of 1,25$(OH)_2D_3$ for the VDR (7). On the basis of these affinities, one would expect a 500- to 1000-fold difference in biological activities between 25-OH-$D_3$ and 1,25$(OH)_2D_3$ if both compounds were acting by directly binding to the VDR. We found no significant difference between 25-OH-$D_3$ and 1,25$(OH)_2D_3$ in their ability to inhibit prostatic growth. Our data (and those of others) strongly suggest that the growth inhibition by 25-OH-$D_3$ is due to prostatic conversion of 25-OH-$D_3$ to 1,25$(OH)_2D_3$. In support of this interpretation, the human prostatic cell line LNCaP expresses VDR but not 1-$\alpha$-hydroxylase. One hundred nM 1,25$(OH)_2D_3$ will cause maximal growth inhibition of LNCaP cells; however, 100 nM 25-OH-$D_3$ has no effect on the proliferation of these cells (26). To further validate this interpretation, we examined the expression of 1-$\alpha$-hydroxylase in our primary culture system. All of the four epithelial strains were positive for 1$\alpha$-hydroxylase, including the strain E-PZ-2 used in this study. Although some variation in 1-$\alpha$-hydroxylase expression was evident, the assay that we used was not quantitative and should not be used to interpret differences in level of expression. It is interesting to note that the two prostatic stromal cultures were essentially negative for 1-$\alpha$-hydroxylase expression, indicating little or no 1-$\alpha$-hydroxylase expression in prostatic stromal cultures. To our knowledge, this is the first evidence that 1-$\alpha$-hydroxylase expression is specific to the epithelial cells of the prostate.

In addition to the potential use of 25-OH-$D_3$ for prostate cancer therapy, our findings have implications for the molecular epidemiology of prostate cancer. For example, little is known about the relative expression of prostatic 1-$\alpha$-hydroxylase between individuals. Variations of 1-$\alpha$-hydroxylase have been observed between primary prostatic epithelial cells and three commonly used prostatic cell lines (PC3, DU-145, and LNCaP; Ref. 17). Expression of 1-$\alpha$-hydroxylase in the primary prostatic cultures is approximately 10 to 40 times that in the cell lines. Because 1-$\alpha$-hydroxylase governs the synthesis of a key autocrine hormone in the prostate, heterogeneity in the expres-

sion of this enzyme may have important physiological consequences. Our data with primary prostatic cultures that show complete growth inhibition with 100 nM 25-OH-$D_3$, in conjunction with data showing no growth inhibition of LNCaP cells with 100 nM 25-OH-$D_3$, further reinforce the significance of 1-$\alpha$-hydroxylase expression in the prostate-vitamin D endocrine system.

In summary, we report that primary human prostatic epithelial cells are growth-inhibited by physiological concentrations of 25-OH-$D_3$ in a dose- and time-dependent manner that did not differ significantly from the effects of 1,25$(OH)_2D_3$. These data imply that the normal prostate-vitamin D endocrine system involves the conversion of 25-OH-$D_3$ to 1,25$(OH)_2D_3$ by prostatic 1-$\alpha$-hydroxylase. These findings support the use of 25-OH-$D_3$ as a chemotherapeutic agent in the treatment of prostate cancer. A preclinical trial of 25-OH-$D_3$ in mice xenografted with human prostatic tumors is presently underway in our laboratory. Finally, because 25-OH-$D_3$ is produced endogenously from exposure to vitamin D, our data raise the exciting possibility that vitamin D (*i.e.*, cholecalciferol or ergocalciferol), which is inexpensive and relatively nontoxic, may be useful as a chemopreventive agent to reduce the risk of prostate cancer (3).

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# 25-Hydroxyvitamin $D_3$, the Prohormone of 1,25-Dihydroxyvitamin $D_3$, Inhibits the Proliferation of Primary Prostatic Epithelial Cells

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*Cancer Epidemiol Biomarkers Prev* 2000;9:265-270.