Pathways Mediating the Growth-Inhibitory Actions of Vitamin D in Prostate Cancer

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ABSTRACT Vitamin D is emerging as an important dietary factor that affects the incidence and progression of many malignancies including prostate cancer. The active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25(OH)₂D₃], inhibits the growth and stimulates the differentiation of prostate cancer cells. We have studied primary cultures of normal and cancer-derived prostatic epithelial cells as well as established human prostate cancer cell lines to elucidate the molecular pathways of 1,25(OH)₂D₃ actions. These pathways are varied and appear to be cell specific. In LNCaP cells, 1,25(OH)₂D₃ mainly causes growth arrest through the induction of insulin-like growth factor binding protein-3 and also stimulates apoptosis to a much smaller extent. We have used cDNA-microarray analyses to identify additional genes that are regulated by 1,25(OH)₂D₃ and to raise novel therapeutic targets for use in the chemoprevention or treatment of prostate cancer. Less calcemic analogs of 1,25(OH)₂D₃ that have more antiproliferative activity are being developed that will be more useful clinically. In target cells, 1,25(OH)₂D₃ induces 24-hydroxylase, the enzyme that catalyzes its self inactivation. Cotreatment with 24-hydroxylase inhibitors enhances the antiproliferative activity of 1,25(OH)₂D₃. The combination of other anticancer agents such as retinoids with vitamin D offers another promising therapeutic approach. A small clinical trial has shown that 1,25(OH)₂D₃ can slow the rate of prostate-specific antigen increase in prostate cancer patients, which demonstrates proof of the concept that vitamin D or its analogs are clinically effective. Our research is directed at understanding the mechanisms of vitamin D action in prostate cells with the goal of developing chemoprevention and treatment strategies to improve prostate cancer therapy. J. Nutr. 133: 2461S–2469S, 2003.

KEY WORDS: 1,25-dihydroxycholecalciferol • vitamin D analogs • vitamin D receptor • 24-hydroxylase • target genes

Background: hormonal actions of vitamin D in prostate

Vitamin D and its metabolites are candidates for prevention and therapy of prostate cancer. This concept is based on a large body of evidence from epidemiological, cell culture, animal and clinical studies that shows antitumor activity of vitamin D in prostate cells. The vitamin D axis involves a complex series of events, and the impact of vitamin D on the prostate occurs at several levels and is mediated by many different pathways.

Vitamin D metabolism and sunlight

The first step in the synthesis of the active form of vitamin D begins in the skin (1). Ultraviolet (UV) radiation converts the precursor 7-dehydrocholesterol to cholecalciferol (vitamin D-3). Epidemiological studies reveal a correlation between exposure to UV radiation and prostate cancer risk. Schwartz and Hulka (2) demonstrated that mortality rates from prostate cancer in the U.S.A. are inversely correlated with UV radiation, although this was an indirect link with vitamin D. Also, it is clear that age is the strongest risk factor for prostate cancer, and the elderly are frequently vitamin D deficient due to several factors that include less exposure to UV radiation (3). Race also is a risk factor for prostate cancer, with the clinical incidence in American blacks twice that of Caucasians. This also may be related to vitamin D deficiency in that high melanin levels in darkly pigmented skin block UV radiation and inhibit the formation of vitamin D-3 (4).

Diet. Dietary forms of vitamin D include calciferol in supplemented milk, ergocalciferol (vitamin D-2) in plants and vitamin D-3 in animal products. Diet is proposed to be a risk factor for prostate cancer, and the low risk of prostate cancer for indigenous Japanese is postulated to be related to their traditional diet. This diet, among other attributes, is rich in oily fish, which are an important dietary source of vitamin D-3.
**Metabolism of provitamin D to active vitamin D.** The active form of vitamin D, 1,25-dihydroxycholecalciferol \([1,25(\text{OH})_2\text{D}_3]\), is formed through two sequential hydroxylation steps (5,6). In the first step, hepatic hydroxylation of vitamin D-3 by vitamin D 25-hydroxylase generates 25-hydroxycholecalciferol \([25(\text{OH})\text{D}_3]\), which is then hydroxylated (mainly in the kidneys) to form 1,25(\text{OH})_2\text{D}_3 by the enzyme 25(\text{OH})\text{D}_3 1\alpha\text{-hydroxylase} (1\alpha\text{-hydroxylase}). Age has an impact at this point on the vitamin D axis as well, because activity of these enzymes declines with age (7). Thus, the elderly are frequently deficient in vitamin D not only due to less exposure to UV radiation, but also owing to a decline with increasing age in the ability to synthesize 1,25(\text{OH})_2\text{D}_3.

**Vitamin D and prostate cancer risk.** Evidence for vitamin D deficiency as a risk factor for prostate cancer first came from a prospective case-control study of vitamin D metabolite levels in sera collected in the San Francisco Bay area between 1964 and 1971 that were matched for age, race and day of storage. In this study (8), mean levels of serum 1,25(\text{OH})_2\text{D}_3 were 1.81 pg/mL lower in cases diagnosed with prostate cancer than in controls \((P = 0.002)\). This finding remains controversial, because other studies fail to confirm an association between serum levels of vitamin D metabolites and prostate cancer risk. Interestingly, another epidemiological study published recently (9) reports that high levels of dietary calcium are a significant risk factor for prostate cancer. This may be relevant to the relationship of vitamin D and prostate cancer, because high levels of serum calcium suppress parathyroid hormone and reduce the renal production of 1,25(\text{OH})_2\text{D}_3.

**Prostate as a target of 1,25(OH)_2D_3.** Classically, 1,25(\text{OH})_2\text{D}_3 is considered to interact exclusively with bone, intestine, kidney and parathyroid glands to regulate calcium and phosphate homeostasis (10). Now we recognize that 1,25(\text{OH})_2\text{D}_3 is a secosteroid hormone that regulates growth and differentiation in a wide variety of cells by binding to the vitamin D receptor (VDR), which is a member of the steroid hormone receptor gene superfamily (11,12). Evidence that VDR are present in human prostatic tissues dates back to 1988 (13), and epithelial cells are found (14) to be the primary target of 1,25(\text{OH})_2\text{D}_3 in the prostate.

Circulating 1,25(\text{OH})_2\text{D}_3 binds to VDR in prostatic epithelial cells and sets in motion a series of events that leads to several biological actions (11,12). VDR functions as a ligand-dependent transcription factor that binds to specific DNA sequences on target genes. Vitamin D response elements (VDRE) have been identified in several target genes. VDR bind to VDRE as heterodimers with the retinoid X receptor (RXR). Transcriptional activation by nuclear hormone receptors such as VDR requires their interaction with coactivators and corepressors.

Many factors influence the transcription program initiated by ligand binding to VDR and the end effect on cellular phenotype. Genetic polymorphisms may affect VDR function. Several molecular epidemiological studies link VDR polymorphisms with prostate cancer risk and/or progression (15). Many coactivators and corepressors modulate activity of VDR, and these also may have polymorphisms that affect individual response or may carry mutations in their genes in premalignant or cancer cells to make VDR more or less active. Also, cell-type specific responses to 1,25(\text{OH})_2\text{D}_3 are believed to some extent be due to cell-type specific expression of different sets of coactivators and/or corepressors (16). The number of VDR per cell also can be regulated in malignant cells by autocrine, paracrine or endocrine growth factors or hormones (17).

Strong evidence supports a role for 1,25(\text{OH})_2\text{D}_3 as a critical regulator of growth and differentiation in the prostate (14,18–21). Application of 1,25(\text{OH})_2\text{D}_3 to animal models of prostate cancer demonstrates efficacy as an antitumor agent in vivo. Finally, a pilot clinical study (22) shows that 1,25(\text{OH})_2\text{D}_3 can slow the rate of tumor doubling in patients with early recurrent prostate cancer after radical prostatectomy or radiation therapy. The combination of all of these observations at many different levels of investigation provides a strong rationale for additional studies on the development of vitamin D as a preventive and therapeutic agent in prostate cancer (14,18–21).

**FIGURE 1** Abrogation of 1,25-dihydroxycholecalciferol \([1,25(\text{OH})_2\text{D}_3]\)-mediated growth inhibition of LNCaP cells by insulin-like growth factor binding protein-3 (IGFBP-3) antisense oligonucleotides. Cells were seeded in 96-well plates and grown in serum-free growth medium for 4 d with 10 nmol/L of 1,25(\text{OH})_2\text{D}_3 or ethanol vehicle (+ and −, respectively) along with 8 \(\mu\text{g}\) of antisense or sense IGFBP-3 oligonucleotides/mL. No oligonucleotides were added to the control group. DNA concentrations were determined at the end of the experiment, and values in cells treated with vehicle for each group were defined as 100%. Values are shown as means ± SE of three experiments. [Reproduced with permission from Boyle et al. (2001) J. Urol. 165: 1319–1324.]
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inhibition in response to 1,25(OH)2D3. A notable exception is the DU 145 cell line, which expresses functional VDR but is resistant to growth inhibition. The LNCaP cells also are unique in comparison with other prostate cancer cell lines in that 1,25(OH)2D3 causes these cells to undergo apoptosis in addition to cell-cycle arrest. Blunt et al. (37) show evidence of apoptosis in LNCaP cells treated with 1,25(OH)2D3 for 6 d and also show the downregulation of the proapoptotic proteins Bcl-2 and Bcl-XL.

Tumor-suppressive activity of 1,25(OH)2D3 or vitamin D analogs on prostate cancer cells also was shown in vivo. We reported that treatment of mice with 1,25(OH)2D3 reduces the size and weight of tumors in PC-3 and LNCaP xenograft models (14). Similar results were obtained by other investigators (38–40).

Vitamin D also modulates other phenotypic traits of prostate cancer cells in a manner that is consistent with tumor-suppressive activity. Schwartz et al. (41) found that 1,25(OH)2D3 inhibits the invasiveness of prostate cancer cells, which is presumably related to decreased expression of the matrix metalloproteinases MMP-2 and MMP-9 in 1,25(OH)2D3-treated cells. We showed (42) that 1,25(OH)2D3 decreases the expression of α6 and β4 integrins in prostate cancer cells, which results in reduced cellular invasion, adhesion and migration to the basement-membrane protein laminin. These effects of 1,25(OH)2D3 are considered to be related to the ability of 1,25(OH)2D3 to inhibit metastases, which was demonstrated in a rat model of prostate cancer (43).

Vitamin D activity in primary cultures of prostatic cells. In 1994, we were the first to show (44) that primary cultures of epithelial cells from normal, benign prostatic hyperplasia (BPH) and malignant human prostate tissues express VDR and that 1,25(OH)2D3 exhibits potent antiproliferative effects. In this initial study, we also noted that prostatic stromal cells have considerably fewer VDR than epithelial cells and are much less sensitive to growth suppression by 1,25(OH)2D3 than epithelial cells. Fresh, uncultured prostatic tissues also bind 1,25(OH)2D3, which demonstrates the presence of VDR in vivo. From these studies, we conclude that epithelial cells are the main target of vitamin D action in the prostate, and that primary cultures would provide a valuable model system to further investigate the role of vitamin D in the normal biology of the prostate as well as an alternative to established cancer cell lines for investigation of vitamin D activity. We noted that primary cultures differ in at least one significant way from cell lines in their response to 1,25(OH)2D3: the growth inhibition of cell lines is generally reversible even after rather lengthy 1,25(OH)2D3 exposure (45), whereas primary cultures (normal as well as malignant) become irreversibly growth suppressed after as little as 2 h of 1,25(OH)2D3 exposure (44).

Vitamin D activity in androgen-responsive prostate cells. Although the predominant effect of 1,25(OH)2D3 on prostatic epithelial cells is growth inhibition, Miller et al. reported (23) that growth of LNCaP cells is modestly stimulated by 1,25(OH)2D3 in medium that contains charcoal-stripped (androgen-depleted) serum instead of whole serum. We evaluated the response of primary cultures to 1,25(OH)2D3 in the presence of charcoal-stripped serum, but found that this did not make 1,25(OH)2D3 growth stimulatory (44). In subsequent studies from our laboratory and others, it was shown that this effect is uniquely related to the androgen-responsive nature of LNCaP cells. The actions of 1,25(OH)2D3 in LNCaP cells are in fact androgen dependent and are mediated in part by upregulation of the androgen receptor (AR) by 1,25(OH)2D3 (45–48). In another androgen-responsive pair of prostate cancer cell lines, MDA-PCa 2a and 2b (49) with unique mutations in the AR gene (50,51), we found that growth inhibition by 1,25(OH)2D3 occurs through androgen-dependent as well as androgen-independent mechanisms (52). Similar cross talk between the androgen-and vitamin D–signaling pathways is reported for ovarian (53) and breast cancer (54) cells in which VDR and AR are upregulated by their cognate ligands.

Activity of vitamin D in patients with prostate cancer

In 1995, Osborn et al. (55) published the results of a phase II clinical trial in which patients with hormone-refractory prostate cancer were treated with 1,25(OH)2D3. Although no objective responses were seen, serum levels of PSA declined in several of the patients. In 1998, we reported the benefit of 1,25(OH)2D3 in a pilot clinical trial of early recurrent prostate cancer after radical prostatectomy or radiation therapy (22). As shown in Table 1, all of the seven subjects showed a statistically significant prolongation of the serum PSA doubling time, thereby establishing proof of principle that 1,25(OH)2D3 has beneficial effects when used to treat men with prostate cancer. Recently, we developed a rationale for combining ketoconazole and 1,25(OH)2D3 or vitamin D analogs to treat prostate cancer (36). Ketoconazole inhibits cytochrome P450 enzymes such as those that synthesize androgens (57) and is used as a second-line androgen-aborlition therapy to treat advanced prostate cancer. However, ketoconazole also inhibits the enzyme 1α-hydroxylase that synthesizes 1,25(OH)2D3, and therefore, men on ketoconazole therapy are likely to be vitamin D deficient. Van Veldhuizen et al. (58) showed that treating vitamin D deficiency in patients with metastatic prostate cancer may improve bone pain and muscle strength. We reasoned that combination therapy with ketoconazole and 1,25(OH)2D3 might alleviate the side effects of vitamin D deficiency caused by ketoconazole as well as provide additional therapeutic activity through antitumor effects of 1,25(OH)2D3. Indeed, our studies on primary prostatic epithelial cultures reveal enhanced antiproliferative activity of the combined drugs (59).

Unfortunately, the hypercalcemic effect of pharmacological doses of 1,25(OH)2D3 limits its clinical application as a therapy.

### Table 1

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<th>Patient no.</th>
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<td>9.4</td>
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1. Patient no. 1 had a decrease in serum prostate-specific antigen (PSA) so that his doubling time was negative, which indicates a halving of PSA. During this study, therapy was discontinued in patients no. 1, 2 and 3, and their doubling times returned to approximately pretreatment values. This resumption in PSA rise indicates that 1,25-dihydroxycholecalciferol treatment inhibited tumor-cell growth and PSA secretion but did not kill the cancer cells. [Reproduced with permission from Gross et al. (1998) J. Urol. 159: 2035–2039.]
a therapeutic agent. A number of analogs with less calcemic activity and equal or greater antiproliferative activity than 1,25(OH)\textsubscript{2}D\textsubscript{3} have been developed (60). We compared the biological actions of 1,25(OH)\textsubscript{2}D\textsubscript{3} on LNCaP cells to synthetic analogs of vitamin D (25). Several analogs exhibit up to fourfold greater inhibitory activity than 1,25(OH)\textsubscript{2}D\textsubscript{3}. Interestingly, we noted that the potency of antiproliferative activity does not directly correlate with affinity of the analogs for VDR, which indicates that other factors are involved (61).

Factors that modulate activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} on prostatic epithelial cells

Combination therapy. In addition to searching for less-calcemic vitamin D analogs that could be used more efficaciously for therapy than 1,25(OH)\textsubscript{2}D\textsubscript{3}, investigators have tested a variety of agents for additive or synergistic antiproliferative activity for use in combination with 1,25(OH)\textsubscript{2}D\textsubscript{3}. Clinically relevant agents that enhance the antiproliferative activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} on prostatic epithelial cells include platinum drugs (62) and paclitaxel (63). Using primary cultures of prostate cancer cells, we found enhanced activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} when combined with suramin (64). Suramin is a polysulfonated napthylurea compound that is used with some efficacy to treat patients with advanced prostate cancer. For the most part, the mechanism of additive or synergistic activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} with these drugs is not understood.

Interactions between vitamin D and retinoids have been recognized for some time and are believed to occur through the RXR, with which both VDR and the retinoic acid (RA) receptor dimerize (65). When we tested a variety of factors for the ability to enhance the response of primary cultures of normal and malignant prostatic epithelial cells to vitamin D, we found synergistic activity between all-trans RA and 1,25(OH)\textsubscript{2}D\textsubscript{3} (64). We found similar synergistic growth-inhibitory activity of 9-cis RA and 1,25(OH)\textsubscript{2}D\textsubscript{3} on LNCaP cells (48) as noted by others (45,66). Various other retinoids sensitize prostate cancer cell lines to 1,25(OH)\textsubscript{2}D\textsubscript{3} or analogs (67-69). Retinoids have been tested in clinical trials for prostate cancer, but as with 1,25(OH)\textsubscript{2}D\textsubscript{3}, therapeutic efficacy is limited by toxicity.

Another clinically relevant observation of ours is that hydrocortisone at 3 \textmu M/L dramatically reduces the growth-inhibitory action of 1,25(OH)\textsubscript{2}D\textsubscript{3} on primary cultures of prostate cancer cells. In the absence of hydrocortisone, 1,25(OH)\textsubscript{2}D\textsubscript{3} is at least 10-fold more potent as an antiproliferative agent (64). Our observation that hydrocortisone mutes the antiproliferative activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} is noteworthy, because a clinical trial combining 1,25(OH)\textsubscript{2}D\textsubscript{3} and glucocorticoid therapy is in progress at the University of Pittsburgh. The rationale for this trial is based on enhanced activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} with glucocorticoids in other experimental systems (70) and the expectation that glucocorticoid action on the intestine will reduce the tendency toward hypercalcemia. However, the outcome of combining vitamin D and glucocorticoids is cell-type dependent (71,72), and our finding suggests that this approach may be contraindicated for prostate cancer. Glucocorticoids are shown to regulate VDR levels in a number of experimental systems (17,71). However, we did not find changes in VDR levels in primary cultures of prostatic cancer cells in response to hydrocortisone (64), which suggests that the downregulation of VDR is apparently not the mechanism by which hydrocortisone blocks the antiproliferative activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} in these cells.

Vitamin D and genistein. Vitamin D and its analogs are believed to have the potential to prevent as well as treat prostate cancer (73). The antiproliferative and differentiating effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on normal epithelial cells in the rat prostate (74) in conjunction with in vitro studies of cultured cells support this concept. The vitamin D analog Ro24-5531 shows chemopreventive activity in the androgen-promoted carcinaoma model of the Lobund-Wistar rat seminal vesicle and prostate (75). Therefore, in addition to considering combinations of vitamin D and other agents that might have additive or synergistic therapeutic activity, investigators have begun to search for combinations that could have enhanced chemopreventive properties. One such factor that is emerging is genistein. This compound is the most-abundant isoflavone in soy, and the association between decreased risk of certain cancers, including prostate cancer, and soy consumption is attributed to genistein. A recent study demonstrates that genistein potentiates the antiproliferative effect of vitamin D analogs on HL-60 leukemia cells (76), and a similar enhancement of activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} by genistein is found in prostate cells (77). The mechanism is unclear, but several recent abstracts (78,79) suggest that genistein may potentiate activity of vitamin D by decreasing enzymatic activity of 24-hydroxylase, which metabolizes 1,25(OH)\textsubscript{2}D\textsubscript{3} to less-active products.

Resistance of some prostate cancer cells to growth suppression by vitamin D. Although many of the established prostate cancer cell lines or primary cultures of adenocarcinoma-derived cells respond to vitamin D, there is abundant evidence that prostate cancer cells may become resistant to antiproliferative activity of 1,25(OH)\textsubscript{2}D\textsubscript{3}. This resistance can develop at several levels such as through the loss or decreased expression of VDR or RXR, through VDR polymorphisms that diminish its function, through elevated expression of VDR corepressors, by increased expression of enzymes that metabolize 1,25(OH)\textsubscript{2}D\textsubscript{3} or by other means. An example of loss of response to vitamin D through loss of VDR is shown by the cancer cell line JCA-1 (formerly believed to be prostatic but now of questionable origin) (80). VDR is not detectable in these cells, and they do not respond to 1,25(OH)\textsubscript{2}D\textsubscript{3}. Stable transfection of the cells with VDR cDNA makes these cells sensitive to growth inhibition by vitamin D (81). We (24) and others noted that the growth of DU 145 cells is not inhibited by 1,25(OH)\textsubscript{2}D\textsubscript{3} despite the presence of a functional VDR with apparently normal binding affinity for 1,25(OH)\textsubscript{2}D\textsubscript{3} in these cells. It seems that several factors can explain the insensitivity of DU 145 cells to growth inhibition by 1,25(OH)\textsubscript{2}D\textsubscript{3}. VDR abundance to some degree determines the magnitude of response to 1,25(OH)\textsubscript{2}D\textsubscript{3}. Like JCA-1 and PC-3 cells, DU 145 cells have relatively low numbers of VDR. Stable transfection of VDR into these cells results in increased levels of receptors and increased growth inhibition by 1,25(OH)\textsubscript{2}D\textsubscript{3} (28). Therefore, increased levels of VDR are one mechanism by which cells may become more responsive to vitamin D.

The three cell lines mentioned as well as another, TSU-Pr1 (also of questionable origin), have the highest levels of 1,25(OH)\textsubscript{2}D\textsubscript{3}-inducible 24-hydroxylase of all of the prostate cancer cell lines studied so far. This enzyme initiates the catabolism of 1,25(OH)\textsubscript{2}D\textsubscript{3} to inactive metabolites. Studies from our laboratory (82) show that inhibition of 24-hydroxylase by liarozole, a compound that blocks P450 enzymes including 24-hydroxylase, permits growth inhibition of DU 145 cells by physiological concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} (Fig. 2). Clinical application of drugs such as liarozole or ketoconazole with similar anti-P450 activity (83) may improve the efficacy of vitamin D therapy. The greater response of DU 145 cells to certain vitamin D analogs than to 1,25(OH)\textsubscript{2}D\textsubscript{3} also may relate to lesser metabolism of the analogs by 24-hydroxylase or to...
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other interactions of the analogs with receptors or vitamin D-binding protein (31,84). Thus, overexpression of 24-hydroxylase is a mechanism that can limit growth-suppressive activity of 1,25(OH)_{2}D_{3} on prostate cancer cells.

We also recognized the loss of sensitivity to 1,25(OH)_{2}D_{3}-mediated growth inhibition by simian virus 40 (SV40), but not human papillomavirus (HPV) transformation of prostatic epithelial cells (85,86). Other types of cells transformed by the SV40 large T antigen also become resistant to 1,25(OH)_{2}D_{3} (87), which shows the generality of this finding. This resistance was overcome in breast cells by the overexpression of VDR (87), but the mechanism by which large T antigen blocks activity of vitamin D is still not completely understood.

One important concept to emerge from these studies is that different signaling pathways are used by VDR to regulate genes involved in growth control versus genes involved in other functions. This is apparent in the ability of cells resistant to growth inhibition by 1,25(OH)_{2}D_{3} to still exhibit VDR upregulation and/or 24-hydroxylase induction in response to 1,25(OH)_{2}D_{3} (24). The presence of analogs with less-calcemic but equal or enhanced growth-inhibitory effects also suggests that different signaling pathways may be involved in these responses.

1α-hydroxylase activity in prostatic cells. Schwartz et al. (88) initially reported that normal human prostatic epithelial cells have 1α-hydroxylase activity and synthesize 1,25(OH)_{2}D_{3}, whereas tumor cells derived from cancer (89). Whereas normal cells indeed had significant 1α-hydroxylase activity, 14 of 15 cancer-derived cell strains had significantly reduced activity (5- to 10-fold lower than normal cells). The biological significance of this observation is evident from the data shown in Figure 3. Because of the very low activity of 1α-hydroxylase in primary cancer strains, 25(OH)_{2}D_{3} is unable to inhibit the growth of these cancer cells due to lack of conversion to the active hormone 1,25(OH)_{2}D_{3}. Normal cells, on the other hand, are inhibited by 25(OH)_{2}D_{3} through conversion to 1,25(OH)_{2}D_{3} by 1α-hydroxylase activity (89). We did not detect 1α-hydroxylase mRNA in prostatic stromal cells, which indicates that expression of this enzyme is specific to epithelial cells of the prostate. The prostate joins the list of extrarenal sites of 1α-hydroxylase production, and the significance of local production of 1,25(OH)_{2}D_{3} is a subject of considerable interest. If reduced activity of 1α-hydroxylase is confirmed in prostate cancer tissues, then loss of local production of 1,25(OH)_{2}D_{3} may be an important mechanism by which cancer escapes the antitumor effects of dietary vitamin D or circulating 1,25(OH)_{2}D_{3}.

VDR genotype and prostate cancer risk. Several polymorphisms are identified in the VDR gene (90), and some studies report that these polymorphisms are associated with prostate cancer risk (15,91). We are particularly interested in the FokI polymorphism, because this polymorphism occurs in the first of two potential translational start codons (ATG) in the gene sequence (92). The F allele lacks the first ATG, and translation starts at the second ATG with the resulting VDR being shorter by three amino acids than that of the f allele that starts at the first ATG. Therefore, unlike the three polymorphisms in the VDR gene that do not alter the VDR coding sequence, the FokI polymorphism alters the structure of the VDR by causing the deletion of the first three amino acids. We examined the association of the VDR FokI genotype with histopathological characteristics and prognosis of prostate cancer among 191 subjects who had undergone radical prostatectomy between 1984 and 1992 at Stanford (93). Subjects with the ff genotype (longer VDR) had a lower mean percent of Gleason grade 4/5 cancer than subjects with the FF or Ff genotypes. These data suggest that the presence of an F allele (shorter VDR) increases the risk of being diagnosed with more aggressive cancer, because a higher percent of Gleason grade 4/5 is associated with a worse prognosis (94). However, functional studies that assess the activity of the FokI alleles have had variable results (95,96), although recent data suggest that the F or short allele is more active in mediating vitamin D action in fibroblasts than the f or long allele (97,98).

Identification of new target genes by cDNA microarray

Transactivation of target genes by 1,25(OH)_{2}D_{3}. Molecular mechanisms of action of 1,25(OH)_{2}D_{3} are beginning to be resolved (11). It is known that 1,25(OH)_{2}D_{3} elicits biological responses through the regulation of transcription of various target genes that are involved in cell growth, apoptosis and cell differentiation (14,18–21). Genes regulated by 1,25(OH)_{2}D_{3} range from those involved in bone mineralization and immune modulation to those implicated in signaling pathways that regulate cell cycle, proliferation and expression of structural proteins. However, it is clear that the effects of 1,25(OH)_{2}D_{3} on cellular phenotype are cell and tissue specific, and data show that these specific effects have their basis in differential gene expression profiles induced by 1,25(OH)_{2}D_{3} in different tissues.

Gene expression profiles. To understand the basis of the differential phenotypic responses to 1,25(OH)_{2}D_{3} among primary cultures and various cell lines and to begin to identify its molecular targets, we performed cDNA-microarray analyses of primary cell cultures (one strain of normal cells and one strain derived from an adenocarcinoma of Gleason grade 3/3) and LNCaP cells. Cells were exposed to fresh medium 2 d before vehicle or 50 nmol of 1,25(OH)_{2}D_{3}/L were added, and mRNA was isolated at 6 and 24 h after treatment. Microarray analyses were conducted with chips carrying ~20,000 genes. When comparing our results with the published profiles of genes that are regulated by 1,25(OH)_{2}D_{3} in squamous carcinoma cells (99), we found no commonly regulated genes. We found a very limited number of genes commonly regulated between prostate...
cells and breast cells after microarray analyses of 1,25(OH)2D3-treated breast cancer cell lines MCF-7 and MDA-MB 231 (unpublished data). As additional findings from genetic profiling become available, it appears that the spectrum of genes induced by any given factor differs markedly among different cell types (100). Prostate-specific effects of 1,25(OH)2D3 are therefore expected and can be used to develop molecular markers that reflect activity of vitamin D in the prostate.

Several noteworthy observations emerged from microarray analyses. First, we found the highest fold increase in the expression of 24-hydroxylase in both normal and cancer-derived primary cell cultures. We had previously seen upregulation of this gene by 1,25(OH)2D3 at both the RNA and enzymatic level in primary cultures, which confirms the microarray technique. The promoter of this gene contains VDRE, and its regulation by 1,25(OH)2D3 in the kidney is very important for maintaining vitamin D homeostasis in the body. In contrast, we did not detect any increase in 24-hydroxylase RNA in our microarray analysis of LNCaP cells, which confirms our previously reported results that were based on Northern blot analyses (24).

There was overlap in the profiles of genes regulated by 1,25(OH)2D3 in normal and cancer-derived primary cultures, but there also were striking differences. Of the early-response genes (i.e., upregulated twofold or more at 6 h), dual-specificity phosphatase 10 was the most highly upregulated gene in both normal and cancer-derived cultures. Dual phosphatase 10 inactivates mitogen-activated protein kinase (MAPK), which suggests that an important feature of the growth-inhibitory activity of 1,25(OH)2D3 may be the inhibition of the growth-promoting activity of MAPK. Early upregulation of protein kinase A anchor protein (gravin) 12 by 1,25(OH)2D3 in both normal and cancer cells also is of interest and is perhaps related to the recently reported 1,25(OH)2D3-regulated packaging of protein kinase C in chondrocytes (101).

Metallothionein genes are particularly noteworthy for their preferential induction by 1,25(OH)2D3 in normal but not cancer-derived primary epithelial cells. In fact, several metallothionein genes are significantly downregulated by 1,25(OH)2D3 in cancer cells. Metallothioneins constitute the majority of intracellular protein thiols and as such are considered to act as cell-survival factors. Upregulation of metallothioneins in normal prostatic epithelial cells is consistent with the lack of apoptotic activity of 1,25(OH)2D3 in primary prostatic epithelial cells. Certain metallothioneins are reportedly overexpressed in prostate cancer (102), and the downregulation of metallothioneins in cancer cells by 1,25(OH)2D3, as we noted in this study, might be beneficial for therapy. Vitamin D is believed to participate in the antioxidant machinery (103), and our microarray results attest to this type of activity in prostate cells. Thioredoxin reductase 1, which is involved in maintaining redox balance, is an early-response gene in both normal and cancer-derived primary cultures. Glutathione-S-transferase-1-like-1 and superoxide dismutase 2, late-response genes induced by 1,25(OH)2D3 in normal and cancer-derived primary cultures, respectively, also are linked to protection from oxidative damage. These genes are unlikely to be directly involved in growth suppression but undoubtedly are key elements in mediating the chemopreventive activity of 1,25(OH)2D3 by preventing DNA damage caused by reactive oxygen species.

Our results also are noteworthy for the absence of regulation of certain genes by 1,25(OH)2D3. More than 50 genes are described as being responsive to vitamin D including at least 26 genes that contain promoters in which VDRE are identified (104,105). Calbindin, osteocalcin and osteopontin are not regulated in primary cultures of prostatic cells. The human prostate cancer cell line PC-3 expresses osteocalcin, and an osteocalcin promoter–luciferase construct exhibits strong vitamin D–induced activity in these cells (106). The cell-cycle inhibitor p21 also is not induced by 1,25(OH)2D3 in our primary prostate cell strains. Reports regarding the role of p21 in mediating vitamin D signaling are variable. Although the promoter of p21 has a VDRE (107), p21 is not consistently regulated by vitamin D in different types of cells. The p27 gene, which is also implicated in growth inhibition by vitamin D, is not regulated in primary cultures of prostatic cells. However,
vitamin D is believed to upregulate p27 by increasing the rate of mRNA translation and extending the half-life of the p27 protein rather than by altering the level of p27 mRNA (108).

In our microarray study using LNCaP cells, the expression of the IGFBP-3 gene shows the highest fold increase after 1,25(OH)2D3 treatment (33-fold induction at 24 h), yet this gene does not appear to be regulated by 1,25(OH)2D3 in our analysis of primary cultures. We previously implicated IGFBP-3 (26) as a key mediator of vitamin D activity in LNCaP cells (see Fig. 1). In contrast, we do not see regulation of IGFBP-3 by 1,25(OH)2D3 in primary cultures, and in fact, primary cultures do not express IGFBP-3. Clearly, IGFBP-3 is not mandatory for growth inhibition of all prostastic epithelial cells. IGFBP-3 may be linked to apoptosis induced by 1,25(OH)2D3 (109).

In LNCaP cells, however, 1,25(OH)2D3 induces IGFBP-3, which in turn increases p21 expression and results in growth arrest (26). Vitamin D does not induce apoptosis in primary cultures, and the lack of regulation of IGFBP-3 as well as Bax or Bcl-2 is consistent with this response. In contrast, 1,25(OH)2D3 alters the ratio of expression of Bcl-2/Bax in LNCaP cells, which undergo apoptosis in response to vitamin D (37).

In conclusion, the microarray studies reveal describe studied reveal many biologically relevant molecular targets of 1,25(OH)2D3 in human prostatic epithelial cells and provide a starting point for additional investigations to more fully elucidate the mechanism of vitamin D action in prostate. The identification of genes primarily responsible for the anticancer effects of 1,25(OH)2D3 could lead to a tissue or serum marker that shows the status of vitamin D-mediated tumor-suppressor activity in the prostate. Such markers could be used to identify individuals who need supplemental vitamin D treatment for prostate cancer prevention or therapy. Less-calcemic analogs of 1,25(OH)2D3 with more potent antiproliferative activity will be more useful clinically. Cotreatment with 24-hydroxylase inhibitors enhances the antiproliferative activity of 1,25(OH)2D3. The combination of other anticancer agents such as retinoids with vitamin D is another promising therapeutic approach. In the future, new therapeutic targets may become apparent when the mechanism for vitamin D-induced anticancer activity is more completely understood.

LITERATURE CITED


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