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Inhibition of Prostate Cancer Growth by Vitamin D: Regulation of Target Gene Expression

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Abstract Prostate cancer (PCa) cells express vitamin D receptors (VDR) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) inhibits the growth of epithelial cells derived from normal, benign prostate hyperplasia, and PCa as well as established PCa cell lines. The growth inhibitory effects of $1,25(OH)_2D_3$ in cell cultures are modulated tissue by the presence and activities of the enzymes 25-hydroxyvitamin D_3 24-hydroxylase which initiates the inactivation of $1,25(OH)_2D_3$ and 25-hydroxyvitamin D_3 1α-hydroxylase which catalyses its synthesis. In LNCaP human PCa cells $1,25(OH)_2D_3$ exerts antiproliferative activity predominantly by cell cycle arrest through the induction of IGF binding protein-3 (IGFBP-3) expression which in turn increases the levels of the cell cycle inhibitor p21 leading to growth arrest. cDNA microarray analyses of primary prostatic epithelial and PCa cells reveal that $1,25(OH)_2D_3$ regulates many target genes expanding the possible mechanisms of its anticancer activity and raising new potential therapeutic targets. Some of these target genes are involved in growth regulation, protection from oxidative stress, and cell–cell and cell–matrix interactions. A small clinical trial has shown that $1,25(OH)_2D_3$ can slow the rate of prostate specific antigen (PSA) rise in PCa patients demonstrating proof of concept that $1,25(OH)_2D_3$ exhibits therapeutic activity in men with PCa. Further investigation of the role of calcitriol and its analogs for the therapy or chemoprevention of PCa is currently being pursued. J. Cell. Biochem. 88: 363–371, 2003.

Key words: 1,25-dihydroxyvitamin D_3; vitamin D receptor; 24-hydroxylase; 1α-hydroxylase; cDNA microarray; IGFBP-3

Prostate cancer (PCa) is the most common malignancy in American men after skin cancer and is the second leading cause of male cancer deaths in the US [Hellerstedt and Pienta, 2002]. Circulating androgens promote PCa growth and androgen deprivation therapy remains the mainstay of PCa treatment [Hellerstedt and Pienta, 2002]. However, many patients eventually fail this therapy and develop androgen-independent PCa (AIPC) and metastatic disease that is not amenable to current therapies. There are several pathways by which AIPC can develop [Feldman and Feldman, 2001] understanding of which is essential for developing treatment strategies for this lethal form of PCa.

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One of the goals of current research on PCa and AIPC is the identification of new agents that would prevent and/or slow down the progression of this disease. In recent years, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D, has emerged as a promising therapeutic agent. $1,25(OH)_2D_3$ is an important regulator of calcium homeostasis and bone metabolism through its actions in intestine, bone, kidney, and the parathyroid glands [Feldman et al., 2001]. In addition to these classical actions, $1,25(OH)_2D_3$ also exerts antiproliferative and pro-differentiating effects in a number of tumors and malignant cells including PCa [Feldman et al., 1997; Blutt and Weigel, 1999; Konety et al., 1999; Miller, 1999; Feldman et al., 2000] raising the possibility of its use as an anti-cancer agent.

VITAMIN D AND PCa

Epidemiological and Genetic Studies

Epidemiological studies indicate that there are several risk factors for PCa including age,

race, and genetic influence [Gross et al., 1997; Blutt and Weigel, 1999; Konety et al., 1999; Miller, 1999; Feldman et al., 2000]. Schwartz et al. [Schwartz and Hulka, 1990] put forward the hypothesis that vitamin D deficiency increases the risk of PCa, based on the observations that mortality rates due to PCa in the US are inversely related to sunlight exposure and that UV light is essential for the synthesis of vitamin D in the skin. Although some studies suggest that lower serum $1,25(OH)_2D_3$ levels are a risk factor for PCa, others do not support this hypothesis [Gross et al., 1997; Blutt and Weigel, 1999; Konety et al., 1999; Miller, 1999; Feldman et al., 2000].

Several polymorphisms have been identified in the vitamin D receptor (VDR) gene [Uitterlinden et al., 2001]. Some of these polymorphisms may contribute to PCa risk, although not all studies could confirm this finding [Feldman, 1997; Ingles et al., 1998, review]. The role of VDR polymorphisms in diseases such as osteoporosis and PCa is being actively investigated and it is proposed that differences in the functional activity of the different VDR alleles might contribute to the risk of these diseases [Jurutka et al., 2000].

Inhibitory Effect of Vitamin D on Prostate Cell Growth—In Vitro Studies

The presence of VDR has been demonstrated in human PCa cell lines [Miller et al., 1992; Skowronski et al., 1993], primary cultures of epithelial cells from normal prostate, benign prostatic hyperplasia (BPH), and adenocarcinoma [Peehl et al., 1994] as well as in the epithelial cells of the normal prostate gland [Kivineva et al., 1998]. Additional experiments have shown that the prostate is a vitamin D target organ. $1,25(OH)_2D_3$ inhibits the growth of primary prostatic epithelial cells from normal tissue, BPH, and cancer [Peehl et al., 1994]. The well-known human PCa cell lines such as LNCaP, PC-3, and DU 145 [Skowronski et al., 1993] as well as other human PCa cell lines such as ALVA 31, PPC-1 [Miller et al., 1995], and MDA PCa 2a and 2b [Zhao et al., 2000] also respond to $1,25(OH)_2D_3$ with growth inhibition. Interestingly, the vitamin D mediated growth inhibition in the primary prostatic epithelial cells appears to be irreversible [Peehl et al., 1994]. Among the well-studied PCa cell lines, $1,25(OH)_2D_3$ exerts a substantial inhibitory effect on LNCaP cell growth, has an intermediate inhibitory effect on PC-3 cells, and only minimally inhibits DU 145 cells [Skowronski et al., 1993].

Vitamin D Analogs

The concentrations of $1,25(OH)_2D_3$ required to produce a significant anti-proliferative effect in vivo (see section on clinical trials below) cause hypercalcemia and/or hypercalciuria and renal stone formation which limits its therapeutic use [Gross et al., 1998]. Consequently, structural analogs of $1,25(OH)_2D_3$ have been developed which exhibit significant antiproliferative activity but are less calcemic in vivo [Feldman et al., 1997; Hisatake et al., 1999]. Several studies have shown that a number of these analogs are very effective in inhibiting the growth of PCa cells [Gross et al., 1997; Blutt and Weigel, 1999; Konety et al., 1999; Miller, 1999; Feldman et al., 2000]. The mechanisms underlying differential activity of the analogs have been extensively studied [Feldman et al., 1997] and include differences in pharmacokinetics, metabolism, structural interactions with the VDR and recruitment of co-modulators to the ligand–VDR complex. It is hoped that vitamin D analogs with enhanced antiproliferative activity and with reduced calcemic side effects would emerge as clinically useful anti-cancer agents.

Cellular Responsiveness to $1,25(OH)_2D_3$—Role of Vitamin D Metabolism

25-Hydroxyvitamin D_3 24-hydroxylase. $1,25(OH)_2D_3$ induces the expression of the enzyme 25-hydroxyvitamin D_3 24-hydroxylase (24-hydroxylase) in target cells which catalyzes the initial step in the conversion of the active molecule $1,25(OH)_2D_3$ into less active metabolites. In prostate cells, the degree of growth inhibition by vitamin D appears to be inversely proportional to the 24-hydroxylase activity in the cells. Among the human PCa cell lines DU 145, PC-3, and LNCaP, DU 145 cells exhibit a high level of 24-hydoxylase induction and are least responsive to $1,25(OH)_2D_3$ in terms of growth inhibition. On the other hand, the basal and induced expression of 24-hydroxylase is very low in LNCaP cells and growth inhibition by $1,25(OH)_2D_3$ is substantial. Ly et al. [1999] have shown that in DU 145 cells, liarozole (an imidazole derivative which inhibits P450

hydroxylases) causes significant inhibition of 24-hydroxylase activity leading to increased $1,25(OH)_2D_3$ half-life in these cells. They further demonstrated that in the presence of liarozole, $1,25(OH)_2D_3$ could elicit a significant growth inhibitory response in DU 145 cells. Miller et al. [1995] have also demonstrated that the differences in $1,25(OH)_2D_3$ mediated growth inhibition between various PCa cell lines correlate inversely to 24-hydroxylase expression in these cells. A recent study by Peehl et al. [2002] has shown that in primary human PCa cells, the use of the P450 inhibitor ketoconazole potentiates the growth inhibitory effects of $1,25(OH)_2D_3$ or its structural analog EB 1089 by inhibiting the 24-hydroxylase activity in these cells. Thus, combinations of $1,25(OH)_2D_3$ with inhibitors of 24-hydroxylase such as ketoconazole or liarozole may enhance its antitumor effects in PCa therapy. The combination approach may also allow the use of $1,25(OH)_2D_3$ at lower concentrations thereby reducing the hypercalcemic side effects.

25-Hydroxyvitamin D_3 1α-hydroxylase. Endogenously synthesized and dietary vitamin D are transported to the liver where they are hydroxylated at the C-25 position to form the prohormone, 25-hydroxyvitamin D_3 [$25(OH)D_3$] [Feldman et al., 2001]. The active hormone $1,25(OH)_2D_3$ is then formed in the kidney by the hydroxylation of $25(OH)D_3$ at the C-1 position by the enzyme 1α-hydroxylase. In recent years, the presence of extra-renal 1α-hydroxylase has been demonstrated and Schwartz et al. [1998] have shown that human prostatic epithelial cells express 1α-hydroxylase. They raised the possibility that treatment with $25(OH)D_3$ could potentially inhibit the growth of PCa due to production of $1,25(OH)_2D_3$ within the prostate, without the systemic side effect of hypercalcemia. The ability of $25(OH)D_3$ to cause hypercalcemia is much reduced because of its lower affinity for the VDR. A recent study from our laboratory [Hsu et al., 2001] quantitated the levels of 1α-hydroxylase in primary prostatic epithelial cells derived from normal tissue, BPH, or cancer as well as in established PCa cell lines. This study shows that epithelial cells from normal prostate have more 1α-hydroxylase activity than those derived from BPH or cancer. The activity in primary cancer cells is lower than BPH and the PCa cell lines express the lowest 1α-hydroxylase activity. The antiproliferative effect of $25(OH)D_3$ correlates with the endogenous 1α-hydroxylase activity in these cells. As illustrated in Figure 1, the growth of primary epithelial cells from normal tissue or BPH is inhibited by $25(OH)D_3$ to an extent similar to $1,25(OH)_2D_3$ as it could be converted to $1,25(OH)_2D_3$ by endogenous 1α-hydroxylase activity. In contrast, in primary epithelial cells from cancer or in the PCa cell line LNCaP, with very low endogenous 1α-hydroxylase activity, the antiproliferative action of $25(OH)D_3$ is much less pronounced in comparison with $1,25(OH)_2D_3$. We conclude that a decrease in 1α-hydroxylase activity may represent an important mechanism in PCa development and/or progression and suggest that the administration of $25(OH)D_3$ might be an effective chemopreventive approach while 1α-hydroxylase is initially still high within the prostate.

Vitamin D and Androgen Interactions

Androgens acting through the androgen receptor (AR) regulate prostate growth and play an important role in the development and progression of PCa [Hellerstedt and Pienta, 2002]. Studies from our laboratory [Zhao et al., 1997] have shown that there is cross talk between $1,25(OH)_2D_3$ and androgen signaling in the androgen-responsive PCa cell line LNCaP. $1,25(OH)_2D_3$ up-regulates AR gene expression at both mRNA and protein levels in LNCaP cells [Zhao et al., 1999] and consequently the secretion of prostate specific antigen (PSA) by these cells is synergistically enhanced when the cells are exposed to a combination of androgens and $1,25(OH)_2D_3$ [Zhao et al., 1999]. The antiproliferative action of $1,25(OH)_2D_3$ in LNCaP cells appears to be androgen-dependent as it could be blocked by the AR antagonist casodex [Zhao et al., 1997]. This androgen-dependent mechanism of $1,25(OH)_2D_3$ action may be specific to LNCaP cells because $1,25(OH)_2D_3$ also inhibits the growth of other PCa cells which do not express the AR [Skowronski et al., 1993; Peehl et al., 1994]. Zhao et al. [2000] have shown that $1,25(OH)_2D_3$ inhibits the growth and up-regulates AR expression in MDA PCa 2a and 2b cells that were recently established from the bone metastasis of a patient who exhibited advanced AIPC. In contrast to LNCaP, the growth inhibitory action of $1,25(OH)_2D_3$ in the MDA PCa cells appears to be androgen-independent. Importantly these findings support the potential therapeutic role of vitamin D in the treatment of AIPC.

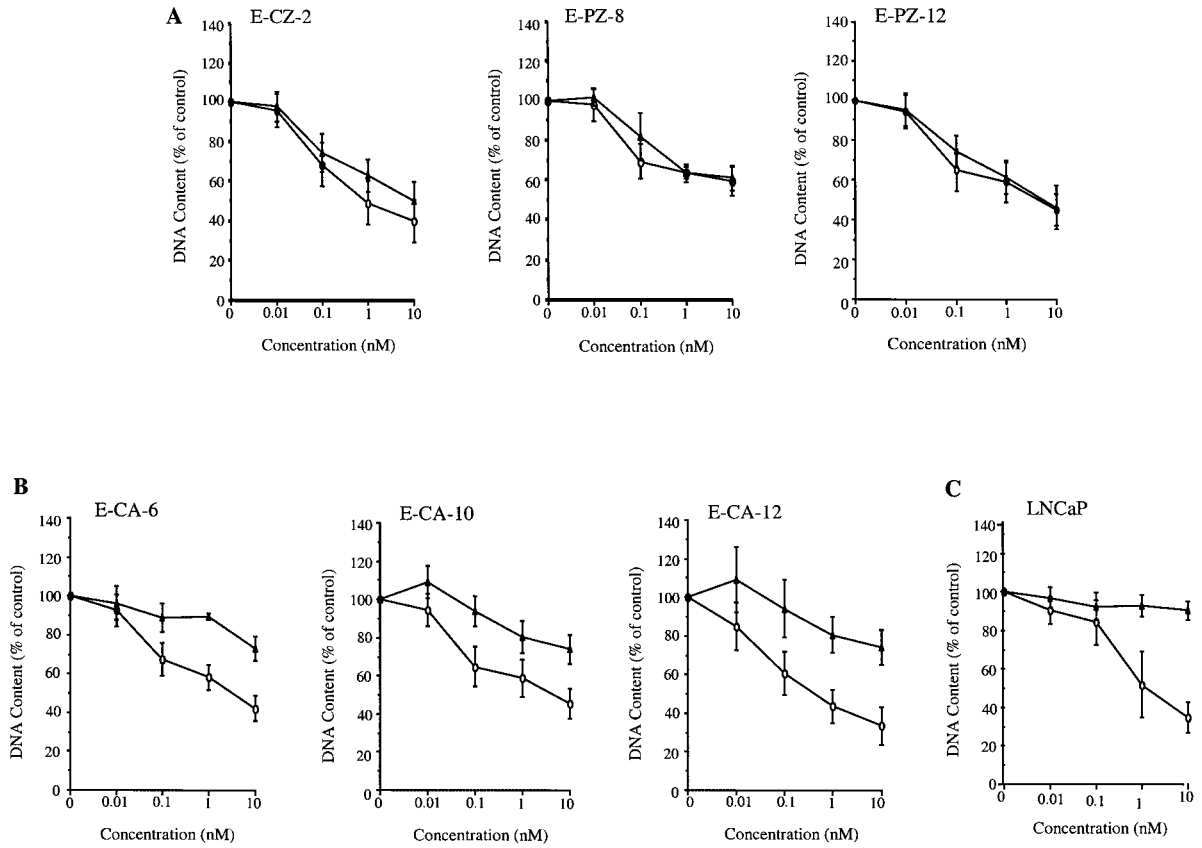

Fig. 1. Growth inhibition by 25(OH)D_3 (*triangles*) in comparison with 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) (*circles*) in prostate epithelial cells. **A**: In normal prostatic epithelial cells (E-CZ-2, E-PZ-8, and E-PZ-12), 25(OH)D_3, and 1,25$(OH)_2D_3$ were equally growth inhibitory at concentrations between 0.01 and 10 nM. **B**: In primary cultures of cancer cells (E-CA-6, E-CA-10, E-CA-12), 1,25$(OH)_2D_3$ induced ~20–40% more growth inhibition than 25(OH)D_3. **C**: The prostate cancer (PCa) cell line LNCaP, although fully responsive to 1,25$(OH)_2D_3$, was resistant to treatment with 25(OH)D_3. [Reproduced with permission from Hsu et al. [2001]].

In Vivo Studies in Rodents

Although several mouse and rat models of PCa have been developed [Lucia et al., 1998], there is still a lack of a perfect model for human PCa. Many in vivo studies have investigated the effects of 1,25$(OH)_2D_3$ or its analogs on the establishment and growth of human PCa cells as xenografts in immune-compromised mice [Gross et al., 1997; Blutt and Weigel, 1999] and showed that 1,25$(OH)_2D_3$ or its analogs inhibit the growth of PCa xenografts causing significant reductions in tumor size and volume. Although imperfect, xenograft models provide an in vivo system to validate the anti-tumor effects of 1,25$(OH)_2D_3$ or its analogs and monitor their ability to elevate serum calcium levels. Clinical studies on the effects of 1,25$(OH)_2D_3$ in PCa patients will be discussed at the conclusion of this review.

Mechanisms of Vitamin D Mediated Growth Inhibition

Several studies have investigated the molecular mechanisms by which 1,25$(OH)_2D_3$ mediates growth inhibitory effects on prostate cells. As described below, 1,25$(OH)_2D_3$ seems to have multiple and diverse actions [Freedman, 1999], often cell-specific, including effects on cell cycle arrest, apoptosis, inhibition of metastasis, and angiogenesis. Research from our laboratory and others have also attempted to identify novel 1,25$(OH)_2D_3$ target genes mediating its various actions especially regulation of cell growth.

Growth arrest. In many cancer cells, 1,25$(OH)_2D_3$ treatment has been shown to result in the accumulation of cells in the G_1 phase of the cell cycle and this has been observed in the case of LNCaP cells as well [Blutt et al., 1997]. The retinoblastoma protein (Rb) is a key

regulator of G_1 to S phase transition. Hyperphosphorylation of the Rb protein by G_1 cyclins and their cyclin-dependent protein kinase (CDK) partners inactivates the Rb protein and releases the repression on E2F transcriptional activity allowing cells to progress from G_1 to S phase. Zhuang and Burnstein [1998] have shown that in LNCaP cells $1,25(OH)_2D_3$ exerts its effects on some of these key steps. $1,25(OH)_2D_3$ treatment of LNCaP cells causes an increase in the expression of the CDK inhibitor p21, a decrease in CDK2 activity leading to a decrease in the phosphorylation of Rb and repression of E2F transcriptional activity resulting in G_1 arrest of the cells. Liu et al. [1996] have shown that $1,25(OH)_2D_3$ directly up-regulates *p21* gene expression in U937 leukemia cells, by binding to the VDR and acting through a putative vitamin D response element (VDRE) in the promoter of the *p21* gene. However, in LNCaP cells, the regulation of *p21* gene expression appears to be indirect [Campbell et al., 1997; Zhuang and Burnstein, 1998]. Studies from our laboratory [Boyle et al., 2001] have shown that the induction of IGF binding protein-3 (IGFBP-3) gene expression by $1,25(OH)_2D_3$ results in increased p21 protein levels. The role of IGFBP-3 in mediating the growth inhibitory effect of $1,25(OH)_2D_3$ is discussed in detail in a following section on growth factor actions. Although a functional Rb plays a key role in cell cycle control, lack of a functional Rb gene in DU 145 cells does not appear to be the critical reason for their reduced sensitivity to growth inhibition by $1,25(OH)_2D_3$. As detailed earlier in the section on 24-hydroxylase, a combination of $1,25(OH)_2D_3$ and the 24-hydroxylase inhibitor, liarozole causes appreciable growth inhibition in these cells. Also, transfection of a functional Rb into DU 145 cells could not render the cells more sensitive to growth inhibition by $1,25(OH)_2D_3$ [Gross et al., 1997] even though the Rb transfected DU 145 cells exhibited reduced tumorigenicity as xenografts in nude mice [Bookstein et al., 1990]. $1,25(OH)_2D_3$ does not increase p21 expression in PC-3 cells which is consistent with the lack of G1 accumulation of these cells following $1,25(OH)_2D_3$ treatment. Thus, the regulation of cell cycle distribution by $1,25(OH)_2D_3$ appears to be cell specific and may involve multiple pathways of action.

Apoptosis. Induction of apoptosis or programmed cell death by $1,25(OH)_2D_3$ is not uniformly seen in all cancer cells [Gross et al., 1997; Blutt and Weigel, 1999; Konety et al., 1999; Miller, 1999; Feldman et al., 2000]. In the case of PCa, investigators have mostly focused on LNCaP cells and the findings have been variable [Blutt and Weigel, 1999; Feldman et al., 2000]. Recently, Blutt et al., [2000] showed evidence of apoptosis in LNCaP cells treated with $1,25(OH)_2D_3$ for 6 days and also showed the down-regulation of the pro-apoptotic proteins Bcl-2 and Bcl-X_L. They went on to demonstrate the involvement of Bcl-2 in $1,25(OH)_2D_3$ mediated apoptosis by stably transfecting the *Bcl-2* gene in LNCaP cells and showing the lack of an apoptosis response to $1,25(OH)_2D_3$ in the Bcl-2 transfected LNCaP cells. In LNCaP cells, therefore, $1,25(OH)_2D_3$ stimulates both growth arrest and to a lesser extent, apoptosis.

Differentiation. $1,25(OH)_2D_3$ has been shown to induce the differentiation of a number of normal and malignant cells [Feldman et al., 1997]. However, there is no clear evidence supporting a role for vitamin D as a differentiation-promoting agent in PCa. Peehl et al. [1994] did not find any changes in cell morphology or in the expression of various keratins as markers of epithelial cell differentiation when primary human prostatic epithelial cells were exposed to $1,25(OH)_2D_3$. Konety et al. [1996] harvested prostate tissue from castrated rats treated with vehicle, testosterone (T), $1,25(OH)_2D_3$, or a combination of T and $1,25(OH)_2D_3$. Histological examination of the prostate tissue revealed a greater degree of epithelial cellular differentiation in rats treated with T and $1,25(OH)_2D_3$ compared to rats treated with T alone. In the PCa cells, LNCaP and MDA PCa 2a and 2b, $1,25(OH)_2D_3$ increases the expression of PSA [Zhao et al., 1999; Zhao et al., 2000], which is regarded as a differentiation marker for prostatic epithelial cells. However, the effect appears to be much smaller compared to the induction of PSA expression by androgens and the synergistic increase in PSA in cells treated with both $1,25(OH)_2D_3$ and androgens is in part due to the up-regulation of AR levels by $1,25(OH)_2D_3$ [Zhao et al., 2000].

Growth factor actions. Growth factors play an important role in the regulation of prostate epithelial cell growth by autocrine and paracrine mechanisms. Expression of autocrine growth factors by the epithelium may contribute to progression of PCa through the development of independence from epithelial–stromal interactions that modulate the growth

and development of the normal prostate gland. Some important growth factors that regulate prostate epithelial growth include epidermal growth factor, keratinocyte growth factor, basic fibroblast growth factor, transforming growth factors (TGFs), and insulin-like growth factors (IGFs). In PC-3 and ALVA 31 cells, $1,25(OH)_2D_3$ decreases the availability of IGF by increasing the expression of its binding proteins IGFBP-3 and IGFBP-5 [Drivdahl et al., 1995; Huynh et al., 1998]. Studies from our laboratory [Boyle et al., 2001] have provided evidence that the up-regulation of IGFBP-3 expression by $1,25(OH)_2D_3$ is a necessary component of $1,25(OH)_2D_3$ mediated inhibition of LNCaP cell growth. $1,25(OH)_2D_3$ treatment increases IGFBP-3 mRNA levels. Importantly, addition of IGFBP-3 anti-sense oligonucleotides abrogates $1,25(OH)_2D_3$ mediated growth inhibition (Fig. 2), suggesting that in LNCaP cells the growth inhibitory action of $1,25(OH)_2D_3$ depends on IGFBP-3 up-regulation. Immunoneutralization of the IGFBP-3 protein similarly abrogates growth inhibition by $1,25(OH)_2D_3$. This study also demonstrates that the increase in the levels of the CDK inhibitor protein p21 elicited by $1,25(OH)_2D_3$ could be blocked by anti-IGFBP-3 antibodies showing that IGFBP-3 induction is necessary for up-regulation of p21 expression by $1,25(OH)_2D_3$.

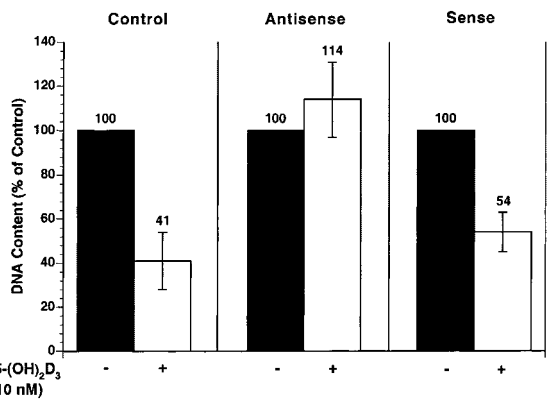

Fig. 2. Abrogation of $1,25(OH)_2D_3$ mediated growth inhibition of LNCaP cells by IGF binding protein-3 (IGFBP-3) anti-sense oligonucleotides. Cells were seeded in 96-well plates and grown in serum-free growth medium for 4 days with 10 nM $1,25(OH)_2D_3$ (+) or ethanol vehicle (−), along with 8 μg/ml of anti-sense or sense IGFBP-3 oligonucleotides. 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 was defined as 100%. Values are shown as mean ± SEM of three experiments. [Reproduced with permission from Boyle et al. [2001]].

Inhibition of invasion and metastasis. In addition to the inhibition of proliferation in malignant cells, $1,25(OH)_2D_3$ is also believed to play a role in tumor invasion and metastasis. $1,25(OH)_2D_3$ can inhibit the invasiveness of breast and lung carcinoma cells in vitro [Hansen et al., 1994; Young et al., 1995]. $1,25(OH)_2D_3$ decreases the tumor size and lung metastasis of the highly metastatic Mat-Ly-Lu and R 3327-AT-2 Dunning PCa cells in vivo [Schwartz et al., 1995]. A recent study from our laboratory [Sung and Feldman, 2000] shows that in DU 145 and PC-3 PCa cells, $1,25(OH)_2D_3$ inhibits invasiveness, cell adhesion and migration to the basement membrane matrix protein laminin due in part to decreasing the expression of α6 and β4 integrins. In LNCaP and PC-3 cells, $1,25(OH)_2D_3$ and its analogs have also been shown to increase the expression of E-cadherin, a tumor suppressor gene whose expression is inversely correlated to the metastatic potential of the cells [Campbell et al., 1997].

Angiogenesis. Angiogenesis or the process of new blood vessel formation is critical for tumor progression and metastasis. $1,25(OH)_2D_3$ has been shown to inhibit tumor cell-induced angiogenesis in mice [Majewski et al., 1996; Mantell et al., 2000] and, therefore, may have a therapeutic application in advanced metastatic cancer.

Novel 1,25D target genes. Acting through the VDR, a classical nuclear receptor, $1,25(OH)_2D_3$ initiates its effects on cell growth and differentiation by the direct activation or repression of target genes. The identification of such genes is one of the goals of the current research on vitamin D. Freedman et al. used a differential screening technique to isolate putative $1,25(OH)_2D_3$-inducible target genes during myeloid cell differentiation and identified genes encoding the CDK inhibitor p21 and the homeobox protein HoxA10 as vitamin D targets [Freedman, 1999]. We have recently performed cDNA microarray analyses in primary human prostatic epithelial cells as well as LNCaP cells to identify molecular targets of $1,25(OH)_2D_3$ involved in the regulation of prostate epithelial cell growth. We used arrays carrying 20,000 genes to study the gene expression patterns in primary epithelial cells derived from normal prostate or adenocarcinoma of Gleason grade 3/3 treated with vehicle or 50 nM $1,25(OH)_2D_3$.

Several interesting and noteworthy observations have emerged from our study. We find that

24-hydroxylase, the classical $1,25(OH)_2D_3$ inducible target gene, is maximally up-regulated in both normal and cancer-derived primary cell cultures. Earlier studies on primary cells in our laboratory have shown a significant induction of 24-hydroxylase mRNA and a substantial increase in enzyme activity following $1,25(OH)_2D_3$ treatment, confirming the microarray data. In contrast, in LNCaP cells we find the basal and induced levels of 24-hydroxylase to be extremely low by Northern blot analysis [Skowronski et al., 1993] and we do not detect 24-hydroxylase up-regulation by microarray analyses. In LNCaP cells, the expression of IGFBP-3 gene shows the highest fold-increase after $1,25(OH)_2D_3$ treatment, which is in agreement with our study showing that the up-regulation of IGFBP-3 expression is essential for $1,25(OH)_2D_3$ mediated growth inhibition in these cells. We could not detect any regulation of IGFBP-3 in our analysis of primary cells consistent with the fact that primary cells do not express IGFBP-3

Our analyses have revealed several novel putative vitamin D target genes in primary epithelial cells. In general, there is an appreciable overlap in the profiles of $1,25(OH)_2D_3$ regulated genes in normal and cancer derived primary cells. In both of these cultured cells, the expression of dual specificity phosphatase 10 shows maximal up-regulation. Dual specificity phosphatase 10 inactivates mitogen activated protein kinase (MAPK), suggesting that an important feature of the growth inhibitory activity of $1,25(OH)_2D_3$ in these cells may be an inhibition of the growth promoting effect of MAPK. Early up-regulation of the kinase anchoring protein gravin that is known to coordinate the localization of protein kinase C (PKC) and protein kinase A (PKA) is of interest and may be related the recently reported effect of vitamin D on the packaging of PKC in chondrocytes [Schwartz et al., 2002]. Our data are supportive of the role of vitamin D as an antioxidant in primary prostate cells. Thioredoxin reductase 1, involved in redox balance, is an early response gene in both normal and cancer cells. Up-regulation of superoxide dismutase 2 is also indicative of protection from oxidative damage. The regulation of the expression of metallothionein genes by $1,25(OH)_2D_3$ is different between the normal and cancer derived primary cells, the former showing an up-regulation and the latter a significant down-regulation. Metallothioneins constitute the majority of intracellular protein thiols and as such are considered to act as cell survival factors. Up-regulation of metallothioneins in normal prostatic epithelial cells is consistent with the anti-apoptotic effect of $1,25(OH)_2D_3$ in these cells. It is also note worthy that $1,25(OH)_2D_3$ up-regulates the expression of the anti-apoptotic protein surviving in these cells. Certain metallothioneins have been reported to be over-expressed in PCa [Zhang et al., 1996] and hence a down-regulation of their expression in the cancer-derived cells may be therapeutically beneficial.

In summary, cDNA microarray analysis is a powerful tool that has revealed biologically important targets of $1,25(OH)_2D_3$ in prostate cells and has provided a starting point for additional investigations into the molecular mechanisms underlying the anti-cancer effect of $1,25(OH)_2D_3$ and its analogs.

Clinical Studies

A few investigators have undertaken clinical trials in PCa patients to evaluate the safety and efficacy of treatment with vitamin D or its analogs. Osborn et al. [1995] reported a small phase II trial of $1,25(OH)_2D_3$ in 13 patients with hormone refractory metastatic PCa. No objective responses ($> 50\%$ reduction in serum PSA levels or $> 30\%$ reduction in measurable tumor mass) could be seen and the median time to progression was 10.6 weeks. A pilot study from our laboratory [Gross et al., 1998] used increasing concentrations of $1,25(OH)_2D_3$ to treat seven patients with early recurrent PCa following radiation or surgery. At the beginning of the trial, the patients showed no evidence of metastasis and the only sign of recurrent disease was rising levels of serum PSA. We compared the doubling time of serum PSA before and after $1,25(OH)_2D_3$ treatment in the same patient. As shown in Table I, in all seven patients the rate of PSA rise was substantially decreased by $1,25(OH)_2D_3$ and in the case of patient 1, the serum PSA levels actually decreased registering a negative doubling time. Due to hypercalciuria, vitamin D therapy was discontinued in three of the seven patients. Withdrawal from therapy resulted in increases in PSA doubling time, with the values reaching those of pretreatment levels in these patients (Table I). This study provides evidence that vitamin D could be effective in slowing the progression of PCa. Both

TABLE I. Prostate Specific Antigen (PSA) Doubling Time in Months Before and After 1,25D Treatment

Patient No.	Before therapy	During therapy	*P* value	Therapy discontinued
1	17.1	−3.3	0.005	10.8
2	5.5	12.5	< 0.0001	2.1
3	12.0	37.6	0.009	13.7
4	10.8	15.8	0.002	
5	23.2	43.0	0.029	
6	12.5	16.7	0.453	
7	4.1	9.4	0.0009	

[Reproduced with permission from Gross et al. [1998]].

these clinical trials found incidences of hypercalciuria or hypercalcemia and the development of renal stones in some patients. This finding underscores the fact that development of hypercalcemia would preclude the use of very high doses of $1,25(OH)_2D_3$ and, therefore, limits its therapeutic benefit that may only be realized at high doses. As discussed before, several structural analogs of $1,25(OH)_2D_3$ which are more potent as antiproliferative agents but with less calcemic effects are being developed and characterized.

CONCLUSIONS

A number of studies have established the role of $1,25(OH)_2D_3$ as an antiproliferative agent in normal and malignant prostate cells. The mechanisms underlying the anti-cancer effects of vitamin D in PCa cells are varied and cell specific and include growth arrest, apoptosis, pro-differentiation effects, modification of growth factor activity, inhibition of tumor cell invasiveness, and interactions with androgen signaling. Investigators are making progress in identifying $1,25(OH)_2D_3$ regulated genes and understanding their role in the mediation of the above mentioned effects. Such studies would unveil novel $1,25(OH)_2D_3$ regulated genes and provide new therapeutic targets. Another challenging area of research involves defining the mechanisms by which various vitamin D analogs maintain potent growth inhibitory effects and yet are less calcemic. We hope that progress in these areas of research will result in rational drug design and lead to the development of more potent and safer vitamin D compounds to be employed in the treatment of PCa.

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