

Androgen signaling is required for the vitamin D-mediated growth inhibition in human prostate cancer cells

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Epidemiological data on prostate cancer incidence has suggested that vitamin D deficiency may be a risk factor for prostate cancer. The antiproliferative activity of $1\alpha, 25$ -dihydroxyvitamin D₃ (1,25-VD) and its analogues has been demonstrated in many prostate cancer models, yet the detailed mechanisms underlying this protective effect of vitamin D remain to be determined. Here, we demonstrate that two androgen receptor (AR)-positive prostate cancer cell lines, LNCaP and CWR22R, are more sensitive to the growth inhibitory effects of 1,25-VD compared to the AR-negative prostate cancer cell lines, PC-3 and DU 145. 1,25-VD treatment inhibited cyclin-dependent kinase 2 (cdk2) activity and induced G0/G1 arrest. Interestingly, we also found that 1,25-VD treatment induced the expression of AR, and that the onset of the G0/G1 arrest in LNCaP and CWR22R cells is correlated with the onset of increasing expression of AR. This implies that the antiproliferative actions of 1,25-VD in AR-positive prostate cancer might be mediated through AR. Furthermore, a reduction in 1,25-VD-mediated growth inhibition was observed when AR signaling was blocked by antiandrogens, AR RNA interference, or targeted disruption of AR. Taken together, our data suggest that the androgen/AR signaling plays an important role in the antiproliferative effects of 1,25-VD and restoration of androgen responsiveness by 1,25-VD might be beneficial for the treatment of hormone-refractory prostate cancer patients.

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Introduction

Prostate cancer is the second leading cause of cancer deaths among North American men. Epidemiological evidence suggests that low exposure to sunlight and vitamin D deficiency may be risk factors for prostate cancer mortality (Schwartz and Hulka, 1990; Hanchette and Schwartz, 1992). Much research has focused on $1\alpha,$

25-dihydroxyvitamin D₃ (1,25-VD), the active metabolite of vitamin D, and its ability to induce either apoptosis or differentiation in many cell types, such as breast, colon, myeloid, and prostate (Pols *et al.*, 1990; Bikle, 1992; Peehl *et al.*, 1994; Jones *et al.*, 1998).

Biological responsiveness of target cells to 1,25-VD is mediated by an intracellular receptor, the vitamin D receptor (VDR). VDR is a member of the steroid/thyroid/retinoid receptor superfamily, and it functions as a ligand-activated transcription factor that binds to vitamin D response elements (VDREs) in the promoters of vitamin D-responsive genes (Darwish and DeLuca, 1993; MacDonald *et al.*, 1994; Haussler *et al.*, 1998). VDR binds with high affinity to VDREs by forming a heterodimeric complex with the retinoid X receptor (RXR), the nuclear receptor for 9-*cis*-retinoic acid (9-*cis*-RA). These VDR/RXR heterodimers function as transcription factors in regulating vitamin D-mediated gene expression (Liao *et al.*, 1990; Kliewer *et al.*, 1992; MacDonald *et al.*, 1993). Several reports have shown that 9-*cis*-RA and 1,25-VD act synergistically to inhibit the growth of prostate cancer cells via the VDR/RXR heterodimer (Blutt *et al.*, 1997; Zhao *et al.*, 1999).

Several established normal and malignant prostate cell lines express functional VDR and 1α -hydroxylase, which can convert 25-hydroxyvitamin D₃ to 1,25-VD, and are responsive to antiproliferative effects of 1,25-VD. Even though these cells express similar levels of functional VDRs, they respond to 1,25-VD-mediated inhibitory effects differently. For example, LNCaP, an androgen-dependent prostate cancer cell line, is significantly inhibited by 1,25-VD; however, the PC-3, DU 145, and ALVA-31 cell lines are less sensitive to the antiproliferative action mediated by 1,25-VD (Miller *et al.*, 1992; Schwartz *et al.*, 1994; Miller *et al.*, 1995; Blutt *et al.*, 1997). These results suggest that VDR expression is not solely responsible for the growth suppression induced by 1,25-VD (Skowronski *et al.*, 1993; Zhuang *et al.*, 1997; Zhuang and Burnstein, 1998). Understanding the molecular mechanisms of this growth inhibition and how cancer cells become resistant to 1,25-VD treatment will be useful for prostate cancer therapy.

Different mechanisms have been proposed for the antiproliferative effect of 1,25-VD. 1,25-VD treatment of LNCaP cells resulted in increased expression of cyclin-dependent kinase (cdk) inhibitors, p21^{waf1/cip1} and

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p27^{kip1}, as well as the protein level and hypophosphorylation of retinoblastoma (Rb) protein, therefore decreasing the transcriptional activity of E2F and causing the accumulation of cells in the G0/G1 phase of the cell cycle (Zhuang and Burnstein, 1998). The induction of p21^{waf1/cip1} by 1,25-VD is reported to be mediated by VDR/RXR binding to a VDRE identified in the p21^{waf1/cip1} promoter region (Liu *et al.*, 1996; Munker *et al.*, 1996). However, the effect of 1,25-VD on p21^{waf1/cip1} expression varies in different cell types. As reported, 1,25-VD increased p21^{waf1/cip1} protein level in LNCaP cells; however, there was no effect on mRNA expression or p21^{waf1/cip1} promoter regulation (Zhuang and Burnstein, 1998). The effects of 1,25-VD on p27^{kip1} expression seem more consistent in myeloid leukemic cell lines and LNCaP prostate cancer cells (Liu *et al.*, 1996; Munker *et al.*, 1996; Muto *et al.*, 1999). A recent study has also suggested that Sp1 and NF-Y cooperatively mediate 1,25-VD-induced p27^{kip1} gene promoter activity in myeloid leukemic cell lines that lack VDREs (Inoue *et al.*, 1999).

LNCaP cells express androgen receptor (AR), respond to androgen stimulation, and are highly sensitive to growth inhibition by 1,25-VD, whereas several other prostate cancer cell lines lacking AR, such as PC-3, DU 145, and ALVA-31, are much less sensitive to 1,25-VD (Hedlund *et al.*, 1996; Zhuang *et al.*, 1997). Furthermore, 1,25-VD inhibited LNCaP cell growth when the cells were cultured in FBS-containing medium, but no effect was observed in charcoal-stripped FBS-containing medium (Miller *et al.*, 1992; Zhao *et al.*, 1997). The growth inhibitory effect was restored by cotreating the cells with 1,25-VD and a low concentration of androgen in charcoal-stripped FBS medium. Meanwhile, the antiandrogen Casodex, which blocks AR function, can abolish the 1,25-VD-induced growth inhibitory effect. Based on these observations, it was hypothesized that the antiproliferative effect of 1,25-VD in LNCaP cells is androgen dependent (Zhao *et al.*, 1997).

In contrast, the growth inhibitory effects of 1,25-VD on two other AR-positive prostate cancer cell lines, MDA PCa 1a and MDA PCa 2b, were shown to be androgen independent (Zhao *et al.*, 2000b). More recently, it has been shown that the antiproliferative effects of 1,25-VD do not require androgen signaling (Yang *et al.*, 2002). Stable overexpression of AR in ALVA31 (AR-negative prostate cancer cell line) cells does not increase the sensitivity to 1,25-VD growth inhibition. Moreover, 1,25-VD inhibits the LNCaP-104R1 cell growth even in the absence of androgen, and the growth inhibition was not blocked by Casodex. Therefore, the role of androgen/AR in 1,25-VD-mediated cell growth inhibition remains controversial.

Hormone refractory prostate cancer (HRPC) remains a challenge in the management of prostate cancer patients. Although androgen ablation therapy is initially useful in controlling tumor progression, prostate cancer eventually will become hormone refractory. The lack of effective treatments has increased the need for more options to treat HRPC. In this study, we have

investigated the role of androgen/AR signaling in 1,25-VD-mediated growth inhibitory effects in two AR-positive prostate cancer cell lines, LNCaP and CWR22R, and two AR-negative prostate cancer cell lines, PC-3 and DU 145. We applied antiandrogen, AR RNA interference (RNAi), and gene targeted disruption of AR in AR-positive prostate cancer cells. The data strongly suggest that androgen/AR is an important regulator of 1,25-VD-mediated antiproliferative action in human prostate cancer cells. Our study provides valuable information in deciding which patients may be more responsive to vitamin D therapy, and may indicate an alternative vitamin D combination therapy to increase treatment effectiveness.

Results

Differential antiproliferative effects of 1,25-VD on human prostate cancer cell lines

We first explored the antiproliferative effects of 1,25-VD among the most commonly used human prostate cancer cell lines (LNCaP, CWR22R, PC-3, and DU 145). Cells were cultured in 10% FBS medium and treated with 100 nM 1,25-VD, and then harvested at indicated times for determination of cell proliferation. Cell growth was determined by MTT assay, which measures the mitochondrial dehydrogenase activity of living cells. As shown in Figure 1, 1,25-VD inhibited the growth of LNCaP and CWR22R cells to a similar degree, as the absorbance readings were reduced 40–50% at day 6 after 1,25-VD treatment, compared with vehicle control. However, 1,25-VD has much less inhibitory effect in PC-3 and DU 145 cells, with only 5–15% reduction of absorbance observed at day 6 after treatment.

Functional VDR is not sufficient for the antiproliferative effect of 1,25-VD

The actions of 1,25-VD are mainly mediated by its receptor, the VDR. Therefore, we first characterized the 1,25-VD actions in human prostate cancer cell lines. Using real-time PCR, mRNA expression levels of VDR were quantified and we found that the VDR mRNA expression levels are similar among these cell lines (Table 1A). Then, we evaluated the 1,25-VD sensitivity by examining the induction of the mRNA of 24-hydroxylase, a well-known 1,25-VD target gene, upon 1,25-VD treatment. As shown in Table 1B, LNCaP, CWR22R, and PC-3 cells have much higher 1,25-VD sensitivity than DU 145 cells (only 60% induction compared to LNCaP, CWR22R, and PC-3 cells). To our surprise, the expression level of VDR mRNA and the induction of 24-hydroxylase mRNA did not correlate well with the 1,25-VD antiproliferative effect in PC-3 cells. Therefore, these results indicate that there are factors other than VDR contributing to the growth inhibition induced by 1,25-VD. Our data show that AR-positive (LNCaP and CWR22R) human prostate cancer cell lines are more sensitive to growth inhibition by

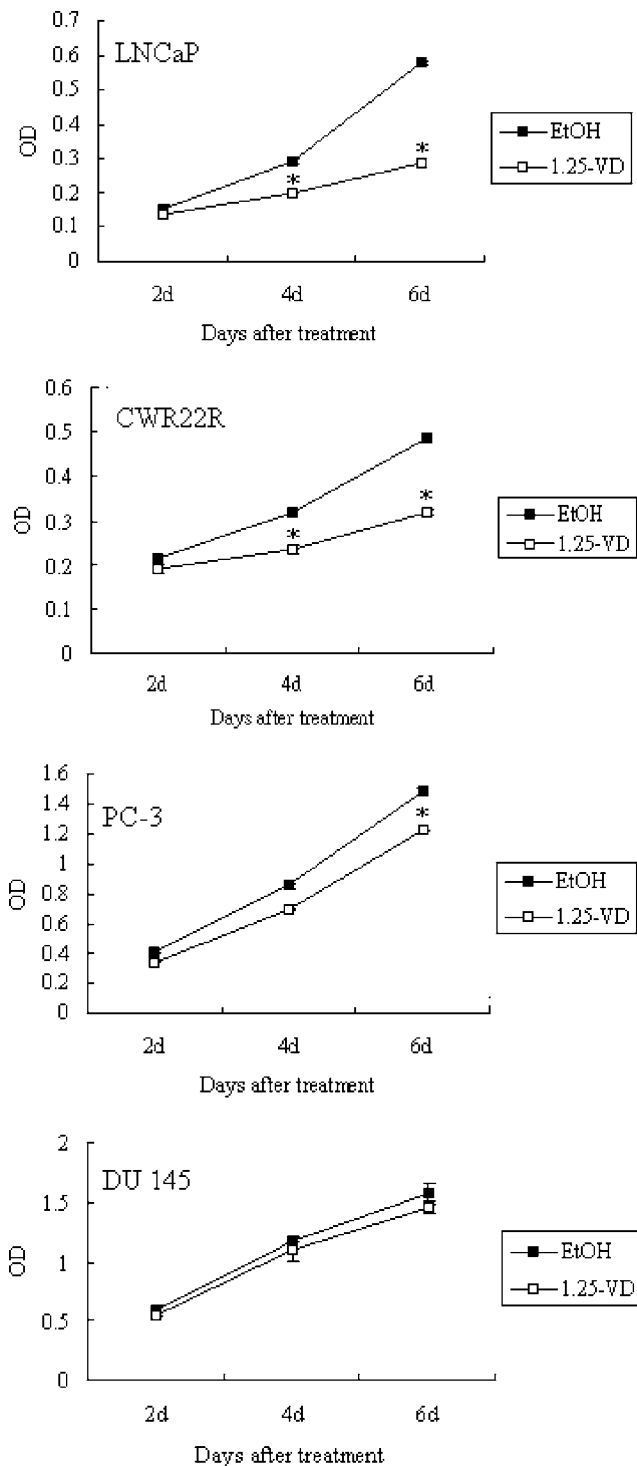


Figure 1 The antiproliferative effects of 1,25-VD on human prostate cancer cell lines. Cells were plated at a density of 5600 cells/cm², and 24 h later treated with 100 nM 1,25-VD or vehicle (ethanol, 0.1%). At the indicated time points, the media was changed and levels of cell proliferation were determined by MTT assay. Data are expressed as the mean \pm s.d. of triplicate samples. *Significant ($P < 0.05$) differences between control and 1,25-VD-treated groups

1,25-VD than AR-negative (PC-3 and DU 145) cell lines (Figure 1), suggesting androgen actions might be correlated with the antiproliferative effects of 1,25-VD.

1,25-VD causes G0/G1 accumulation of AR-positive human prostate cancer cell lines

To understand the mechanism underlying the antiproliferative effects of 1,25-VD on AR-positive prostate cancer cell lines, we examined the cell cycle profile of 1,25-VD-treated cells using flow cytometric analysis after staining with propidium iodide. As shown in Figure 2, we found that 1,25-VD caused androgen-dependent human prostate cancer cell lines (LNCaP and CWR22R) to accumulate in the G0/G1 phase of the cell cycle with different latencies. In LNCaP cells, accumulation in the G0/G1 phase (from 56 ± 2.5 to $70 \pm 0.6\%$) begins within 1 day of 1,25-VD treatment, and is more obvious (from 53 ± 2.3 to $82 \pm 0.4\%$) after 2 days of treatment. In CWR22R cells, G0/G1 accumulation (from 44 ± 0.5 to $54 \pm 1.8\%$) occurred 3 days after 1,25-VD treatment, indicating a much slower response compared with that of LNCaP cells. However, 1,25-VD does not have significant effects on the cell cycle profile of PC-3 and DU 145 cells. These results suggest that 1,25-VD might regulate some G0/G1 regulatory proteins to trigger G0/G1 arrest in LNCaP and CWR22R cells, but not in PC-3 and DU 145 cells.

Effect of 1,25-VD on cell cycle regulatory molecules in AR-positive human prostate cancer cell lines

Cell cycle progression is largely governed by a series of cdk, which target critical substrates such as Rb. Cdk activity is positively regulated by cyclins and negatively regulated by cdk inhibitors (cdki). Hyperphosphorylation of Rb results in the release of E2F, with the consequent activation of E2F target genes, and then progression of cells from the G1 to S phase. We examined the effects of 1,25-VD on the phosphorylation status of Rb in LNCaP. As shown in Figure 3A, we found that the levels of hypophosphorylated Rb were 20% increased 6 h after 1,25-VD treatment in LNCaP cells. However, there was no obvious change in the phosphorylation status of Rb in CWR22R cells (data not shown). In LNCaP cells, 1,25-VD did not change the total protein levels of p21 or p27 (Figure 3A), cyclin D1, D2, E, or cdk5 (data not shown) during 2 days of treatment. Then, we examined cdk activity and found that the activity of cdk2 was inhibited after 48 h treatment in LNCaP cells, but not in PC-3 cells (Figure 3B). The cdk2-associated complexes were then immunoprecipitated and examined by Western blotting. As shown in Figure 3B, we found that the amount of cdk2-associated p27 increased in LNCaP cells, yet there was no significant change in PC-3 cells. These data suggest that 1,25-VD increases the association between cdk2 and p27, decreases cdk2 activity, and therefore causes the cells to undergo G0/G1 arrest.

Table 1 (A) Relative VDR mRNA expression levels and (B) time-dependent effect of 1,25-VD treatment on the expression of 24-hydroxylase mRNA in LNCaP, CWR22R, PC-3, and DU 145 cells

Cell lines	ΔCT	Relative expression level ^b		
(A)				
LNCaP	8.15 ± 0.07	1.00		
CWR22R	8.05 ± 0.07	0.99		
PC-3	7.75 ± 0.07	0.95		
DU 145	7.95 ± 0.21	0.98		
Cell lines	Treatment	ΔCT	$\Delta\Delta CT$	Relative induction fold ^b
(B)				
LNCaP	EtOH	19.00		1.00
	1,25-VD	4.10	-14.90 ± 0.21	
CWR22R	EtOH	18.85		0.99
	1,25-VD	4.15	-14.70 ± 0.01	
PC-3	EtOH	17.60		0.96
	1,25-VD	3.30	-14.30 ± 0.14	
DU 145	EtOH	12.70		*0.60
	1,25-VD	3.75	-8.95 ± 0.21	

Cells were cultured and gene expression was analysed by real-time PCR (as in A). Cells were cultured and treated with either ethanol vehicle or 100 nM 1,25-VD for 24 h and gene expression was analysed by real-time PCR (as in B). ^aValues represent the fold differences in gene expression relative to LNCaP cells set at 1.00. ^bValues represent the relative fold differences compared to LNCaP induction fold set at 1.00. * $P < 0.05$

1,25-VD upregulates cellular AR, and the timing of expression correlates with the onset of G0/G1 arrest

Since there are differential onset times of the G0/G1 arrest between LNCaP and CWR22R human prostate cancer cell lines, we were interested to know whether the expression levels of AR and VDR changed upon 1,25-VD treatment. As shown in Figure 4A, an increase of AR expression in LNCaP cells was observed 12–24 h after 1,25-VD treatment, concurrent with the accumulation of cells in the G0/G1 phase of the cell cycle. Similarly, in CWR22R cells, AR protein expression increased 60–72 h after 1,25-VD treatment (Figure 4B), which also correlates with the time required for cell accumulation in the G0/G1 phase of the cell cycle. VDR protein expression was slightly increased after 1,25-VD treatment in both cell lines (Figure 4). Again, these data suggest that androgen actions might be correlated with the antiproliferative effects of 1,25-VD.

Blockade of antiproliferative effects of 1,25-VD by the antiandrogen, Casodex

Next, we applied an antiandrogen to evaluate the possible role of androgen/AR in the antiproliferative effects of 1,25-VD. The antagonistic effects of Casodex were tested first by examining its ability to inhibit androgen/AR-mediated transcriptional activity in LNCaP cells. As shown in Figure 5A, Casodex has no androgenic activity (lane 2) and completely suppressed androgen-induced AR transcriptional activity (lane 4 vs 3).

We then applied Casodex to examine the role of androgen/AR in the 1,25-VD-mediated cell cycle arrest in the cell growth assay. As shown in Figure 5B, in the presence of 1 μM Casodex, the addition of 1,25-VD did

not further inhibit cell growth in LNCaP cells. Intriguingly, Casodex slightly reversed 1,25-VD-induced AR, VDR expression, and hypophosphorylation of Rb (Figure 5C, lane 4 vs 3), as well as the G0/G1 accumulation caused by 1,25-VD (Figure 5D). These data further support our hypothesis that intact androgen/AR signaling may be involved in the 1,25-VD-mediated antiproliferative pathway. However, Casodex also inhibits cell growth and increases the doubling time of LNCaP cells. Therefore, whether 1,25-VD loses its antiproliferative ability in the presence of Casodex needs to be further investigated.

Blockade of 1,25-VD antiproliferative effects by AR RNAi

In order to further test our hypothesis, we used the vector-based RNAi technique to silence the expression of AR in LNCaP cells and evaluate the consequences of the loss of androgen/AR function relative to 1,25-VD-mediated antiproliferative effects. Several different AR RNAi molecules were constructed into the BS/U6 vector (Sui et al., 2002) and tested for their ability to inhibit androgen/AR-mediated transcriptional activation. Among these, one AR RNAi was found to inhibit androgen/AR transcriptional activity in LNCaP cells by approximately 80% (Figure 6A), and was used for further testing.

We applied immunofluorescence analysis to test the AR RNAi specificity. In order to monitor the transfection efficiency and distinguish transfected cells from untransfected cells, fourfold amounts of BS/U6 or AR RNAi were cotransfected with green fluorescent protein (GFP) expression plasmid. We then examined the AR and VDR expression in GFP-positive cells. As shown in

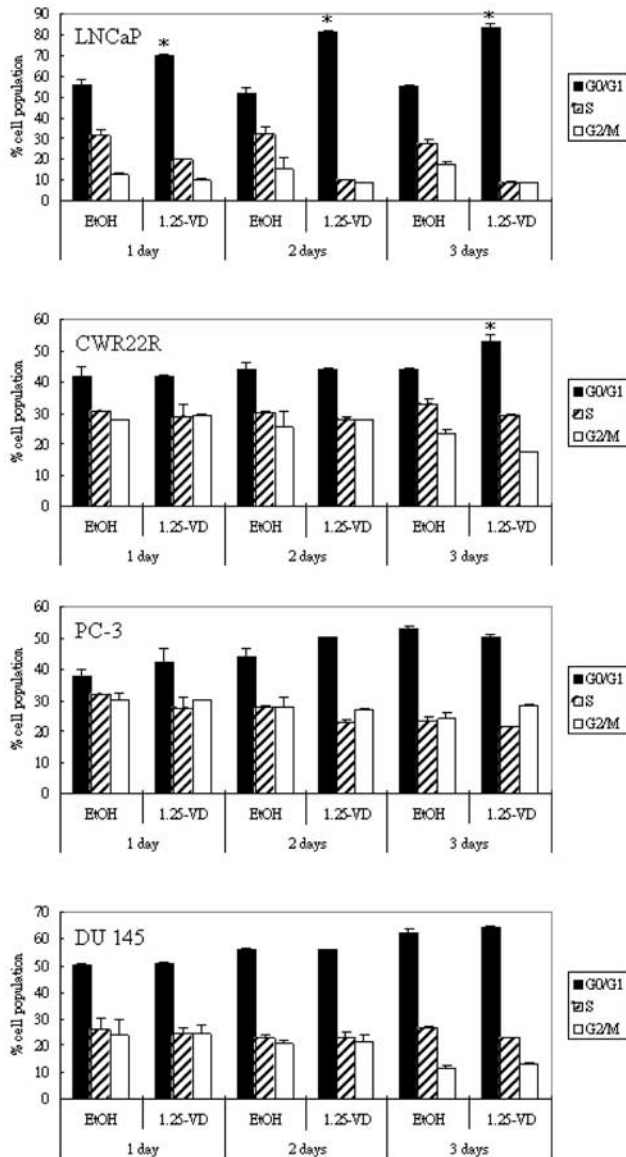


Figure 2 1,25-VD treatment of LNCaP and CWR22R cells results in accumulation in the G1 phase of the cell cycle. Cells were plated in 100 mm dishes and treated with either ethanol vehicle or 100 nM 1,25-VD for 1–6 days. Cells were fixed in 70% ethanol and stained with propidium iodide. Cell cycle profiles and distributions were then determined by flow cytometric analysis of 10000 cells. *Significant ($P < 0.05$) differences between control and 1,25-VD-treated groups

Figure 6B, the AR protein level was reduced significantly in AR RNAi-transfected cells, but not in vector-transfected cells. In contrast, there are no effects on VDR protein expression in either AR RNAi- or vector-transfected cells. Therefore, the AR RNAi can inhibit AR expression but not VDR.

Next, we cotransfected BS/U6 or AR RNAi together with GFP plasmids and sorted the cells into transfected (GFP-positive) and untransfected (GFP-negative) groups, and then analysed the cell cycle profiles by flow cytometry. As shown in Figure 6C, 1,25-VD fails to

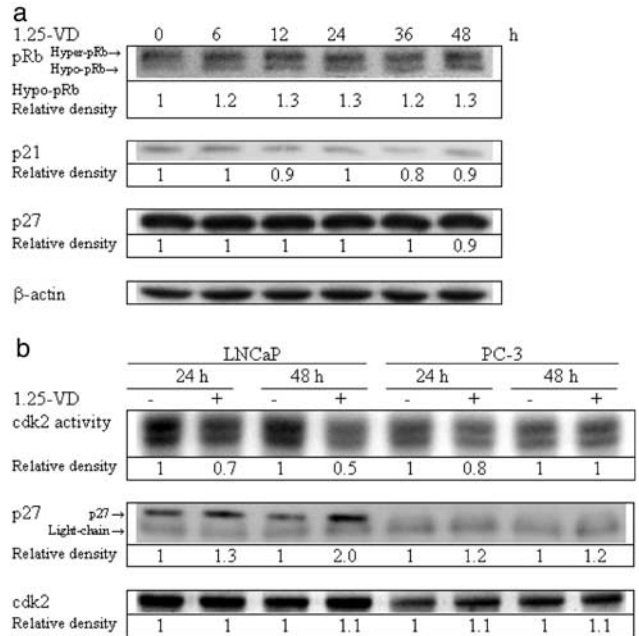


Figure 3 Effect of 1,25-VD on cell cycle regulatory molecules. (a) Western blot analysis of pRb, p21, and p27 in total cell lysates from mock or 1,25-VD-treated LNCaP cells. 1,25-VD (100 nM) was added to LNCaP cells, and the cells were incubated for 0, 6, 12, 24, 36 and 48 h. The total cellular proteins were isolated and subjected to Western blot analysis using 80 μ g protein per sample. (b) 1,25-VD increases the association of p27 with cdk2 and decreases the cdk2 activity in LNCaP cells. The cells were incubated with 100 nM 1,25-VD until the indicated time points and total cell lysates were immunoprecipitated with cdk2 antibody. Samples were immunoblotted with cdk2 and p27 antibodies. At the same time, *in vitro* kinase assays were carried out. The level of expression or activity was extrapolated by densitometric analysis after internal control correction

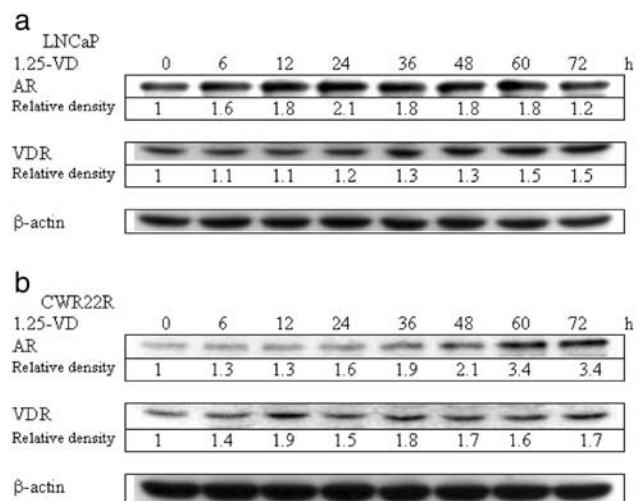
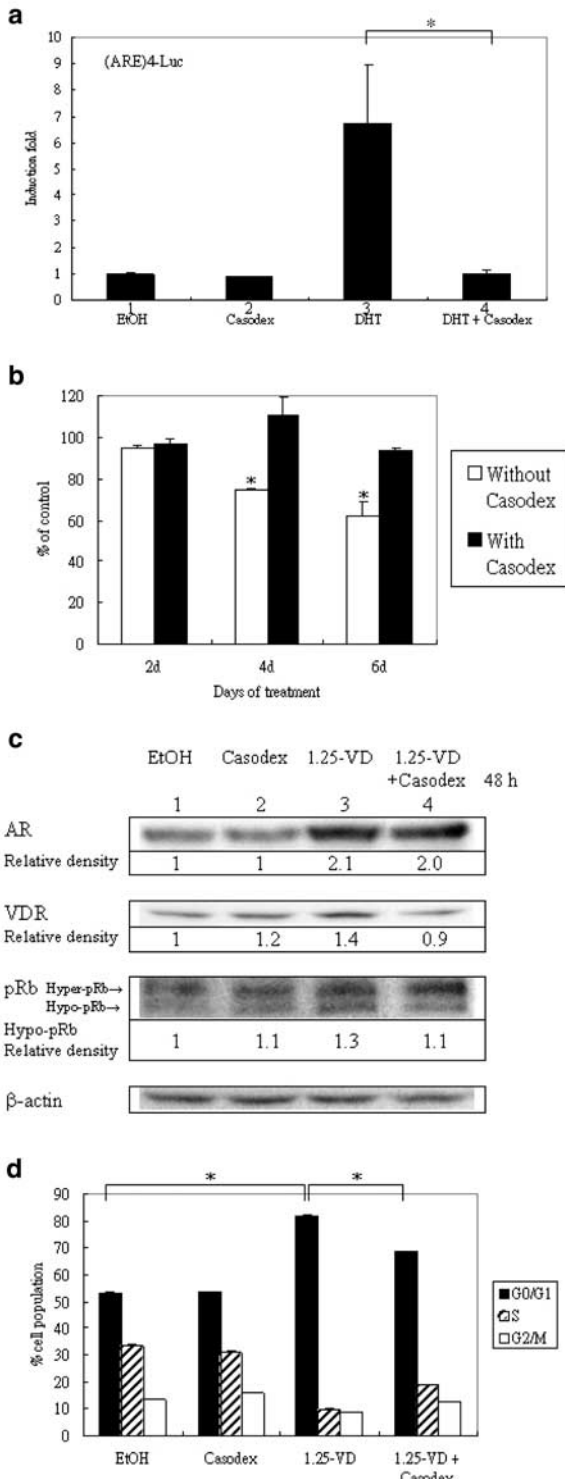


Figure 4 1,25-VD upregulates the expression of AR and VDR protein in LNCaP and CWR22R cells. 1,25-VD (100 nM) was added to LNCaP (a) and CWR22R (b) cell cultures, and the cells were incubated for 0, 6, 12, 24, 36, 48, 60, and 72 h. The total cellular proteins were isolated and subjected to Western blot analysis using 80 μ g protein per sample. The level of expression was extrapolated by densitometric analysis after internal control correction

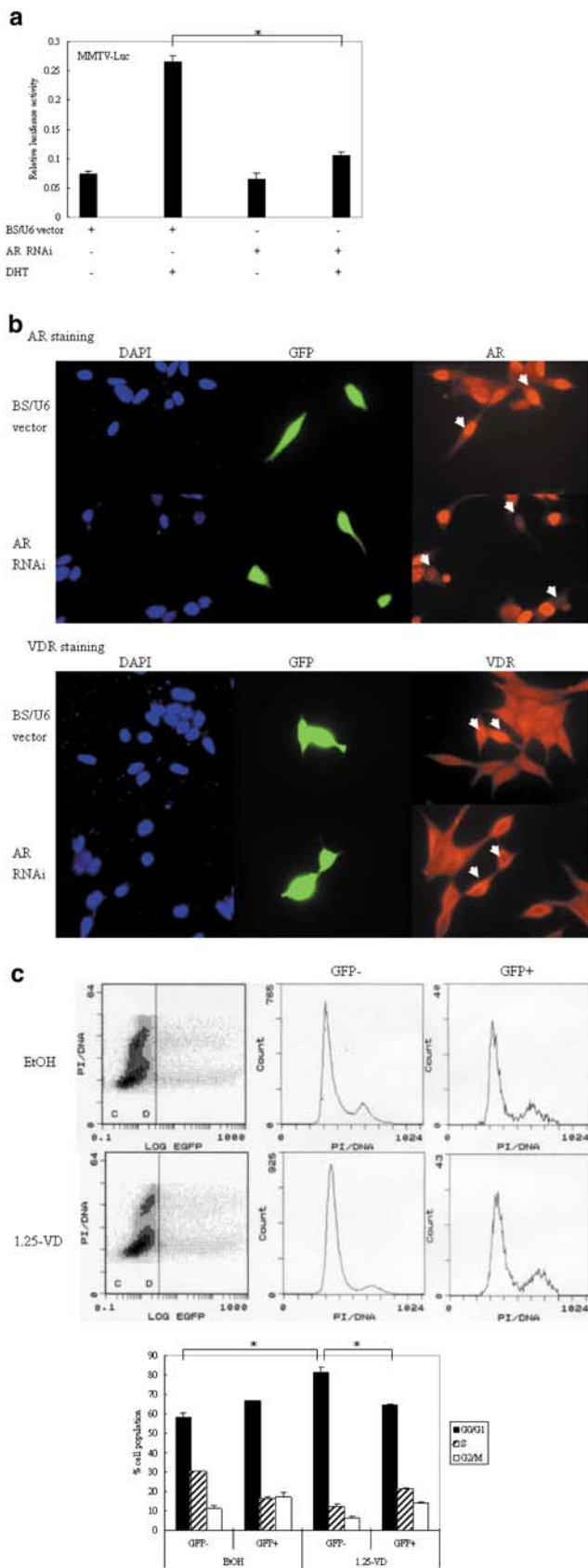
induce the G0/G1 phase arrest in AR RNAi-transfected LNCaP cells (GFP positive), whereas 1,25-VD mediates the G0/G1 phase accumulation in untransfected cells (GFP negative). Our control experiment demonstrated no change of 1,25-VD-induced G0/G1 phase arrest in either BS/U6 vector-transfected cells (GFP positive) or untransfected cells (GFP negative) (data not shown).



CWR22R cells with reduced AR content show less sensitivity to 1,25-VD-induced growth inhibition

By targeted disruption of the AR gene exon 1, we generated several CWR22R sublines in which AR expression was knocked down. Details of the AR targeting vector construction were described previously (Yeh *et al.*, 2003). Briefly, a promoterless neomycin cassette was inserted in frame with the first ATG of AR. Upon homologous recombination, transcription from the AR promoter results in the expression of the neomycin cassette and termination of transcription within exon 1, preventing transcription of the remainder of the AR gene. The AR targeting vector was transfected into CWR22R cells and cell clones were selected with G418 (400 $\mu\text{g}/\text{ml}$). The genotypes of surviving clones were determined by Southern blotting analysis, which showed that selected clones were all heterozygotes. The untargeted locus found at 9.0 kb represents the wild-type AR, as found in parental CWR22R cells (Figure 7A, lane 1), and the locus band at 3.5 kb represents targeted recombination of the other AR locus (Figure 7A, lanes 2–5). To further confirm that the selected CWR22R clones were AR heterozygotes, the levels of AR and VDR protein expression were measured. As shown in Figure 7B, AR protein expression was reduced significantly (from 60 to 90%), while VDR expression was intact in the AR-reduced CWR22R sublines (CWR22R AR \downarrow -3 and CWR22R AR \downarrow -5). We then tested the antiproliferative effect of 1,25-VD on parental CWR22R cells and CWR22R

Figure 5 Effect of Casodex on 1,25-VD-induced inhibition of LNCaP cell growth. **(a)** The magnitude of the antagonistic effect of Casodex on LNCaP cells. Cells were transiently transfected with 1.5 $\mu\text{g}/\text{well}$ of ARE reporter construct and treated (10⁻⁸ M DHT, 10⁻⁶ M Casodex), as indicated, for 24 h. Reporter gene expression was measured via the luciferase assay. The fold induction of luciferase activity is presented relative to the transactivation observed upon ethanol treatment. *Significant ($P < 0.05$) differences between DHT-treated and DHT + Casodex groups. The effect of Casodex on 1,25-VD-induced growth inhibition **(b)**, protein expression of AR, VDR, and pRb **(c)**, and on cell cycle distribution **(d)** in LNCaP cells. In the cell growth assay **(b)**, cells were plated at a density of 5600 cells/cm² and treated 24 h later with 100 nM 1,25-VD or vehicle (ethanol, 0.1%), in the presence or absence of 10⁻⁶ M Casodex. At the indicated time points, the medium was changed and the level of cell proliferation was determined by MTT assay. Data are expressed as the mean \pm s.d. of triplicate samples. *Significant ($P < 0.05$) differences between control and 1,25-VD-treated groups. In the protein expression assay **(c)**, 100 nM 1,25-VD or vehicle (ethanol, 0.1%), in the presence or absence of 10⁻⁶ M Casodex, was added to LNCaP cells. The cells were incubated for 48 h, and the total cellular proteins were isolated and subjected to Western blot analysis using 80 μg protein per sample. The level of expression was extrapolated by densitometric analysis after internal control correction. In the cell cycle analysis **(d)**, cells were plated in 100 mm dishes and treated with either ethanol vehicle or 100 nM 1,25-VD, with or without 10⁻⁶ M Casodex, for 48 h. Cells were then fixed in 70% ethanol and stained with propidium iodide. Cell cycle profiles and distributions were determined by flow cytometric analysis of 10 000 cells. *Significant ($P < 0.05$) differences between control and 1,25-VD-treated groups, as well as between 1,25-VD-treated and 1,25-VD + Casodex groups



AR \downarrow sublines. Cells were cultured in 10% FBS medium, treated with 10 nM 1,25-VD, and then harvested for the cell proliferation assay at the times indicated. As we expected, 1,25-VD inhibited CWR22R cell growth up to 40%, but cell growth was only inhibited by 25–30% in CWR22R AR \downarrow sublines (Figure 7C). Consistent with our findings, decreased AR content in the cells correlates with decreased 1,25-VD-mediated growth inhibitory effects. The doubling times of CWR22R and CWR22R AR \downarrow sublines are similar, at 1.3 and 1.6 days, respectively. The difference in the antiproliferative effects of 1,25-VD is not due to differences in growth rates among these cell lines. Therefore, our results strongly support the idea that intact androgen/AR signaling is required to attain the antiproliferative effects of 1,25-VD.

Discussion

Studying the effects of 1,25-VD on human prostate cancer cell lines shows that various cell lines respond differently to the antiproliferative effect of 1,25-VD. In this study, we investigated VDR mRNA expression and its transcriptional activity among the most commonly used human prostate cancer cell lines (LNCaP, CWR22R, PC-3, and DU 145). Surprisingly, PC-3 cells have high 1,25-VD sensitivity but low cell growth inhibitory response. These data suggest that the existence of VDR is necessary, yet not sufficient for growth inhibition by 1,25-VD, which is consistent with previous studies (Skowronski *et al.*, 1993; Zhuang *et al.*, 1997; Zhuang and Burnstein, 1998). Since both AR-positive cell lines are more sensitive to 1,25-VD growth inhibition, androgen/AR signaling might be important for 1,25-VD antiproliferative actions.

Figure 6 Effect of AR RNAi on 1,25-VD-induced inhibition of LNCaP cell growth. **(a)** The effect of AR RNAi on AR transcriptional activity in LNCaP cells. Cells were transiently transfected with 0.2 μ g/well of MMTV reporter construct and 0.8 μ g/well of AR RNAi or empty vector, and were then treated (10⁻⁸ DHT), as indicated, for 24 h. Reporter gene expression levels were measured by luciferase assay. *Significant ($P < 0.05$) differences between BS/U6 vector- and AR RNAi-transfected groups after DHT treatment. **(b)** Immunofluorescence analysis of AR and VDR expression in AR RNAi-transfected cells. LNCaP cells were cotransfected with 0.3 μ g GFP expression plasmid and 1.2 μ g AR RNAi or empty vector. DAPI (nuclear staining), GFP fluorescence, and AR or VDR expression in cells was visualized by fluorescent microscopy 48 h after transfection. The arrowheads indicate GFP-positive (transfected) cells. **(c)** LNCaP cells were cotransfected with a GFP expression plasmid (2 μ g) and 8 μ g AR RNAi or empty vector. 1,25-VD (100 nM) or vehicle (ethanol, 0.1%) was added to LNCaP cells 24 h after transfection, and the cells were incubated for another 48 h. Cells were fixed and stained with propidium iodide. Cell cycle profiles and distributions were then determined by flow cytometric analysis with at least 4000 GFP-positive cells. *Significant ($P < 0.05$) differences between control and 1,25-VD-treated groups in GFP-negative (untransfected) cells, also between GFP-negative (untransfected) and GFP-positive (transfected) cells after 1,25-VD treatment

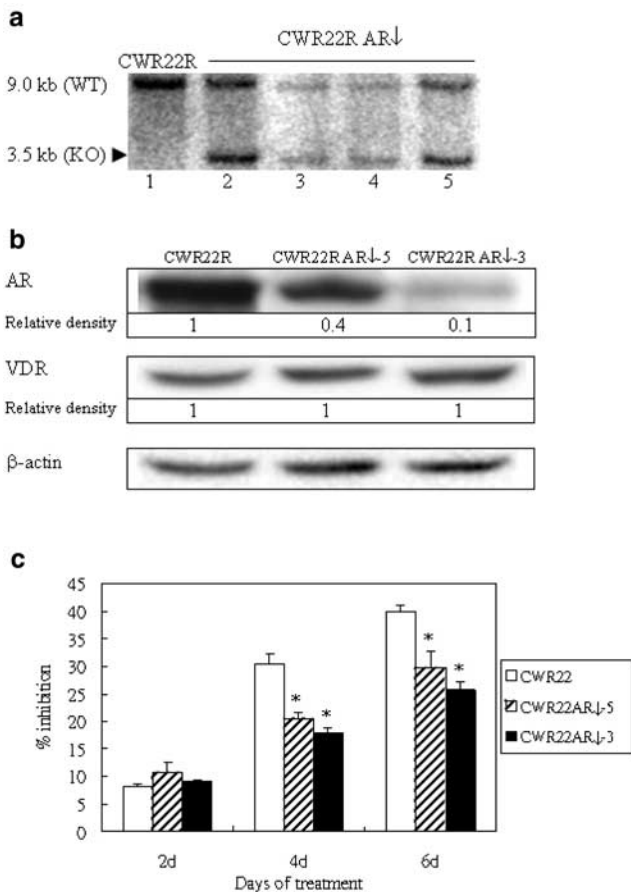


Figure 7 The antiproliferative effects of 1,25-VD on CWR22R and CWR22R AR↓ cells. (a) Analysis of recombination events by Southern blot. Lane 1, CWR22R (parental cell line); lanes 2–5, four independent cell lines where AR gene is targeted with a neomycin cassette. The position of the targeted band is indicated by the arrowhead on the left. (b) Expression of AR and VDR protein in CWR22R and CWR22R AR↓ cells. Total cellular protein was isolated and subjected to Western blot analysis using 80 μg protein per sample. The level of expression was extrapolated by densitometric analysis after internal control correction. (c) Assay of CWR22R and CWR22R AR↓ cell proliferation. Cells were plated at a density of 5600 cells/cm² and, after 24 h, treated with 10 nM 1,25-VD or vehicle (ethanol, 0.1%). At the indicated time points, the media were changed and cell proliferation was determined by MTT assay. The percent growth inhibition observed is reported. Data are expressed as the mean ± s.d. of triplicate samples. *Significant ($P < 0.05$) differences between CWR22R (parental cell line) and CWR22R AR↓ cells after 1,25-VD treatment

Several lines of evidences from our studies indicate the involvement of androgen action in the antiproliferative effects of 1,25-VD on prostate cancer cells. First, we found that 1,25-VD inhibited both AR-positive LNCaP and CWR22R cell lines to a similar degree; however, 1,25-VD had much less effect on AR-negative PC-3 and DU 145 cells, which is consistent with the G₀/G₁ cell cycle arrest. In addition, the expression of AR induced by 1,25-VD was concurrent with the accumulation of cells in the G₀/G₁ phase of cell cycle. Second, we applied a pure antiandrogen, Casodex, to block AR function in LNCaP cells and

found that in the presence of Casodex, the addition of 1,25-VD has no further inhibitory effect on LNCaP cells, which is consistent with the previous reports (Zhao *et al.*, 1997). Third, we applied the RNAi technique to knock down the expression of AR in LNCaP cells transiently, which allowed us to elucidate the androgen/AR function in 1,25-VD-mediated antiproliferative effects. As we show in our results (Figure 6C), the blockage of AR expression in LNCaP cells results in no accumulation of cells in the G₀/G₁ phase of cell cycle. Finally, we applied the homologous recombination technique to knock down AR expression and evaluated the role of AR in 1,25-VD-mediated antiproliferative effects. As expected, 1,25-VD can inhibit parental cell growth up to 40%, but only inhibits growth by 25–30% in the CWR22R AR↓ sublines, further supporting the role of AR in 1,25-VD-mediated antiproliferative pathways.

However, it has recently been shown that the antiproliferative effects of 1,25-VD do not require androgen signaling (Zhao *et al.*, 2000b; Yang *et al.*, 2002). In their study, stable overexpression of AR in the AR-negative prostate cancer cell line ALVA31 does not increase the sensitivity to 1,25-VD growth inhibition. It is reasonable to argue that AR-negative prostate cancer cell lines have a relative deficiency of transcription factors and that there is downregulation by hypermethylation of AR and its target genes' promoters (Chlenski *et al.*, 2001). Therefore, restoring AR in AR-negative cells may not be sufficient to turn on its target genes. Meanwhile, it was also demonstrated that 1,25-VD can inhibit the growth of an androgen-independent prostate cancer derivative of LNCaP (LNCaP-104R1), even in the absence of androgen, and that the growth inhibition was not blocked by Casodex (Yang *et al.*, 2002). However, during the development of androgen independence in prostate cancer, some mitogenic pathways, such as epidermal growth factor (EGF) receptor, ErbB-2, mitogen-activated protein kinases, and transforming growth factor- α (TGF- α) signaling, are upregulated and crosstalk with androgen signaling in regulating androgen-independent prostate cancer growth (Myers *et al.*, 1999; Lee *et al.*, 2003). Therefore, Casodex might not be sufficient to block AR actions at this stage. In the second study, it was shown that the antiproliferative effects of 1,25-VD were not blocked by Casodex in MDA PCa 2a and 2b cells. However, there are two mutations in the ligand-binding domain (L701H and T877A) of AR that have been identified in these two cell lines (Zhao *et al.*, 2000a), which might alter ligand specificity. It has been reported that the doubly mutated AR in MDA PCa 2a and 2b cells can be activated by glucocorticoids and can stimulate androgen-independent growth. It is possible that this doubly mutated AR is no longer responsive to Casodex, or if there is a response to Casodex, it can still be activated by glucocorticoids in MDA PCa 2a and 2b cells. The antagonist activity of Casodex in these two prostate cancer cell lines needs to be examined further.

The sensitivity of androgen/AR signaling is the major factor controlling cell growth in prostate cancer cells. Several reports have already demonstrated that the human prostate cancer cell line LNCaP shows a biphasic response to androgen. Low concentrations (up to 0.1 nM) of androgen stimulate cell proliferation, whereas high concentrations (higher than 1 nM) of androgen result in strong inhibition of proliferation and increase the expression of PSA and other differentiation markers (Henttu *et al.*, 1992; Esquenet *et al.*, 1996; Knudsen *et al.*, 1998; Tsihlias *et al.*, 2000). Studies have shown that the effect of androgen on LNCaP cell proliferation is closely reflected in the degree of Rb phosphorylation and in the level of E2F activity (Hofman *et al.*, 2001). At androgen concentrations up to 0.1 nM, E2F activity increases in parallel with Rb phosphorylation, and a similar stimulation of E2F target genes, cyclin A and E2F-1, is observed. However, at higher concentrations of androgen, the remarkable dephosphorylation of Rb is accompanied by lower E2F activity and lower expression of E2F target genes. The mechanisms by which androgen signaling affects the phosphorylation status of Rb and cell proliferation are still poorly understood, but may be due to increased expression of some cdk inhibitors, such as p21 (Lu *et al.*, 1999; Lu *et al.*, 2000) and p27 (Knudsen *et al.*, 1998; Tsihlias *et al.*, 2000). While examining the changes in cell cycle regulators after 1,25-VD treatment, we found decreasing cdk2 activity and increasing hypophosphorylated Rb in LNCaP cells, yet no change in the expression of total p21 and p27 (Figure 3). p27 is present in both proliferating and arrested cells, but it only interacts with cyclin E-cdk2 complexes in arrested cells (Polyak *et al.*, 1994). Indeed, we found that the amount of cdk2-associated p27 increased after 1,25-VD treatment in LNCaP cells, but not in PC-3 cells. Thus, this increased association provides a basis of G0/G1 cell cycle arrest. Our unpublished data also demonstrated that 1,25-VD can significantly increase androgen-induced AR transcriptional activity. Therefore, higher levels of AR activity may contribute to the antiproliferative effects of 1,25-VD, which might explain why 1,25-VD modulates androgen/AR activity and then triggers cell arrest in AR-positive cells.

In this study, we have demonstrated four lines of evidence to support our hypothesis that the antiproliferative effects of 1,25-VD in human prostate cancer require androgen signaling. Our data demonstrate that 1,25-VD-mediated growth inhibition requires androgen signaling in AR-positive human prostate cancer cell lines (LNCaP and CWR22R). Further study of the relationship between androgen/AR and 1,25-VD signaling, as well as elucidation of other factors in the process of 1,25-VD-mediated growth inhibition will allow better evaluation of 1,25-VD as a treatment modality for prostate cancer. More importantly, increased androgen responsiveness in androgen-independent prostate cancer patients with 1,25-VD treatment might be useful as this type of cancer is relatively unresponsive to conventional therapies.

Materials and methods

Materials

1,25-VD was the generous gift of Dr Lise Binderup from Leo Pharmaceutical Products. 5 α -dihydrotestosterone (DHT) was obtained from Sigma. Antibodies to p21, VDR, and β -actin were purchased from Santa Cruz Biotechnology. The anti-AR polyclonal antibody, NH27, was produced as described (Yeh and Chang, 1996). Antibodies specific for p27 and phosphorylated Rb (14001A) were obtained from BD Transduction Laboratories and Pharmingen, respectively. The plasmid pEGFPC1, encoding GFP, was purchased from Clontech. The LNCaP cells were obtained from the American Type Culture Collection. The CWR22R cells were a generous gift of Dr Franky Chan from the University of Hong Kong. AR RNAi molecules were constructed into the BS/U6 vector as described previously (Yeh *et al.*, 2003). Cell culture media (RPMI-1640) was obtained from Gibco-BRL.

Cell culture, transfection, and luciferase assays

The LNCaP, CWR22R, PC-3, and AR-reduced CWR22R subline (CWR22R AR \downarrow) cells were maintained in RPMI-1640 containing penicillin (100 IU/ml), streptomycin (100 mg/ml), and 10% FBS in 5% CO $_2$ at 37°C. DU 145 cells were maintained in DMEM under the same conditions described above. Transfections were performed by using SuperFect according to the manufacturer's suggested procedures (Qiagen). After transfection, cells were treated with charcoal-stripped FBS medium containing either ethanol or ligands for 24 h. Cell lysates were prepared, and the luciferase activity was normalized for transfection efficiency using pRL-CMV as an internal control. Luciferase assays were performed using the dual-luciferase reporter system (Promega, Madison, WI, USA).

Cell proliferation assay

Cells were seeded in 24-well tissue culture plates at a density of 5600 cells/cm 2 in RPMI-1640 containing 10% FBS. After incubation for 24 h, the medium was replaced with fresh medium containing 10% FBS and treated with vehicle (ethanol, final concentration 0.1%), 1,25-VD, or DHT at indicated concentrations. At the indicated time points, the medium was replenished and cell proliferation was determined by MTT assay (Sigma). Serum-free medium containing MTT (0.5 mg/ml) was added into each well. After 4 h incubation at 37°C, the stop solution was added to solubilize the formazan product and the absorbance was recorded. Data are expressed as the mean \pm s.d. of triplicate samples.

Real-time PCR analysis

Total RNA was extracted from LNCaP, CWR22R, PC-3, and DU 145 cells using Trizol (Invitrogen). We carried out reverse transcription with the Super Script II kit (Invitrogen) and PCR amplifications with SYBR Green PCR Master Mix on an iCycler IQ multicolor real-time PCR detection system (Bio-Rad). The PCR was performed as follows: initial denaturation at 95°C for 10 min, and 45 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. Primer sequences were: VDR, sense 5'-CGCTCCAATGAGT CCTTACC-3' and antisense 5'-GCTTCATGCTGCACT CAGGC-3'; 24-hydroxylase, sense 5'-CAGCAGCATCT CACTACC-3' and antisense 5'-GACAGCCTCAGAGCA TTG-3'; β -actin, sense 5'-TGTGCCCATCTCAGAGGG TATGC-3' and antisense 5'-GGTACATGGTGGTGGCCG

CAGACA-3'. Δ CT values were calculated by subtracting the threshold (CT) value from the corresponding β -actin CT (internal control) value from each time point. Then $\Delta\Delta$ CT values were calculated by subtracting the Δ CT value of untreated controls from the Δ CT value of 1,25-VD-treated samples. The absence of nonspecific amplification products was confirmed by agarose-gel electrophoresis.

Flow cytometric analysis

After indicated treatments, cells were harvested and fixed in 70% ethanol overnight. Cells were then pelleted and incubated in PBS containing 0.05 mg/ml RNase A for 30 min at room temperature. After washing, the cells were stained with 10 μ g/ml propidium iodide. Cell cycle profiles were determined by using the FACSscan flow cytometer, and cell cycle analysis of DNA histograms was performed with ModFitLT software. For the analysis of GFP protein expression by flow cytometry, cells were fixed in 1% formaldehyde for 1 h at 4°C, before the 70% ethanol fixation, as described (Chu *et al.*, 1999).

Western blot analysis

Cells were lysed in ice-cold RIPA buffer (1 \times PBS, 1% Ipegal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS) containing proteinase inhibitor (Roche), and lysates were clarified by centrifugation. The protein concentration of the supernatant was evaluated with the Bio-Rad reagent kit. From each sample, 80 μ g protein was separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked in TBS (10 mM Tris-Cl/pH 7.4, 150 mM NaCl containing 4% nonfat dry milk) for 1 h at room temperature. Primary antibodies in TBS were added and incubated at 4°C overnight, and then the HRP-conjugated secondary antibodies (Santa Cruz) in TBS were added and incubated for 1 h at room temperature. The membranes were washed three times in TBS (5 min at room temperature), and immunoblots were detected by electrochemiluminescence, according to the manufacturer's instructions (Amersham).

Immunoprecipitation and in vitro kinase assay

Cells were lysed in ice-cold RIPA buffer containing proteinase inhibitor (Roche) and lysates were clarified by centrifugation. Total cell lysates were precleared by incubating with 35 μ l protein A/G plus agarose (Santa Cruz Biotechnology) for 1 h at 4°C. Protein (500 μ g) were incubated with 2 μ g cdk2 antibody overnight at 4°C with agitation. Protein A/G plus agarose (35 μ l) was then added to each sample and incubated for 1 h. After washing three times with RIPA buffer and once with kinase assay buffer (50 mM Tris-HCl/pH 7.4, 10 mM MgCl₂, and 1 mM DTT), 30 μ l kinase assay mix (0.25 μ l (2.5 μ g) of histone H1 (Roche), 0.5 μ l (5 mCi) of [γ -³²P]ATP, 0.5 μ l 0.1 mM ATP and 28.75 μ l kinase assay buffer) was added, and the complexes were incubated for 30 min at 37°C.

References

Bikle DD. (1992). *Endocr. Rev.*, **13**, 765–784.
Blutt SE, Allegretto EA, Pike JW and Weigel NL. (1997). *Endocrinology*, **138**, 1491–1497.
Chlenski A, Nakashiro K, Ketels KV, Korovaitseva GI and Oyasu R. (2001). *Prostate*, **47**, 66–75.
Chu YW, Wang R, Schmid I and Sakamoto KM. (1999). *Cytometry*, **36**, 333–339.
Darwish H and DeLuca HF. (1993). *Crit. Rev. Eukaryot. Gene Expr.*, **3**, 89–116.

The reactions were stopped by addition of 4 \times sample buffer. After boiling for 5 min, the reactions were subjected to standard SDS-PAGE. The phosphorylation signals were visualized by PhosphorImager.

Immunocytofluorescence

LNCaP cells were seeded on two-well Lab Tek Chamber slides (Nalge), in RPMI-1640 with 10% FBS, for 24 h before being transfected with 1.2 μ g/well. BS/U6 vector, or AR RNAi plasmids, together with 0.3 μ g/well of GFP expression vector pEGFPc1, using SuperFect. After 24 h, transfected cells were fixed in 3% formaldehyde and 10% sucrose in PBS for 15 min on ice, and then permeabilized with methanol. Immunostaining was performed by incubating slides with blocking solution (2% BSA in PBS) for 15 min at room temperature, followed by a 1:200 dilution of anti-AR polyclonal antibody (NH27) or anti-VDR polyclonal antibody (Santa Cruz Biotechnology) for 1 h (room temperature) or overnight (4°C). A Texas red-conjugated goat anti-rabbit secondary antibody (ICN) was then applied for 45 min at room temperature. Stained slides were washed and mounted (Vectashield; Vector Laboratories). The slides were photographed via fluorescent microscopy at \times 400 magnification.

Statistical and densitometric analysis

The results are the mean \pm s.d. of values obtained from two or three separate experiments. ANOVA was used to analyse cell growth curve data. After flow cytometric analysis and 1,25-VD-induced 24-hydroxylase mRNA experiments, the Student's *t*-test was used to assess the statistical significance of the difference between control and 1,25-VD-treated groups. A statistically significant difference was considered to be present at $P < 0.05$. Autoradiograms/bands were scanned and the mean density of each band was analysed by the Quantity one program (Bio-Rad). Densitometric data presented below bands are the fold changes compared with control sample band densities for each treatment time.

Abbreviations

1,25-VD, 1 α ,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; AR, androgen receptor; Rb, retinoblastoma; RNAi, RNA interference.

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Esquenet M, Swinnen JV, Heyns W and Verhoeven G. (1996). *Prostate*, **28**, 182–194.
Hanchette CL and Schwartz GG. (1992). *Cancer*, **70**, 2861–2869.
Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE and Jurutka PW. (1998). *J. Bone Miner. Res.*, **13**, 325–349.
Hedlund TE, Moffatt KA and Miller GJ. (1996). *J. Steroid Biochem. Mol. Biol.*, **58**, 277–288.

- Henttu P, Liao SS and Vihko P. (1992). *Endocrinology*, **130**, 766–772.
- Hofman K, Swinnen JV, Verhoeven G and Heyns W. (2001). *Biochem. Biophys. Res. Commun.*, **283**, 97–101.
- Inoue T, Kamiyama J and Sakai T. (1999). *J. Biol. Chem.*, **274**, 32309–32317.
- Jones G, Strugnell SA and DeLuca HF. (1998). *Physiol. Rev.*, **78**, 1193–1231.
- Kliwer SA, Umesono K, Noonan DJ, Heyman RA and Evans RM. (1992). *Nature*, **358**, 771–774.
- Knudsen KE, Arden KC and Cavenee WK. (1998). *J. Biol. Chem.*, **273**, 20213–20222.
- Lee MS, Igawa T, Yuan TC, Zhang XQ, Lin FF and Lin MF. (2003). *Oncogene*, **22**, 781–796.
- Liao J, Ozono K, Sone T, McDonnell DP and Pike JW. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 9751–9755.
- Liu M, Lee MH, Cohen M, Bommakanti M and Freedman LP. (1996). *Genes Dev.*, **10**, 142–153.
- Lu S, Jenster G and Epner DE. (2000). *Mol. Endocrinol.*, **14**, 753–760.
- Lu S, Liu M, Epner DE, Tsai SY and Tsai MJ. (1999). *Mol. Endocrinol.*, **13**, 376–384.
- MacDonald PN, Dowd DR and Haussler MR. (1994). *Semin. Nephrol.*, **14**, 101–118.
- MacDonald PN, Dowd DR, Nakajima S, Galligan MA, Reeder MC, Haussler CA, Ozato K and Haussler MR. (1993). *Mol. Cell. Biol.*, **13**, 5907–5917.
- Miller GJ, Stapleton GE, Ferrara JA, Lucia MS, Pfister S, Hedlund TE and Upadhyaya P. (1992). *Cancer Res.*, **52**, 515–520.
- Miller GJ, Stapleton GE, Hedlund TE and Moffat KA. (1995). *Clin. Cancer Res.*, **1**, 997–1003.
- Munker R, Kobayashi T, Elstner E, Norman AW, Uskokovic M, Zhang W, Andreeff M and Koeffler HP. (1996). *Blood*, **88**, 2201–2209.
- Muto A, Kizaki M, Yamato K, Kawai Y, Kamata-Matsushita M, Ueno H, Ohguchi M, Nishihara T, Koeffler HP and Ikeda Y. (1999). *Blood*, **93**, 2225–2233.
- Myers RB, Oelschlager D, Manne U, Coan PN, Weiss H and Grizzle WE. (1999). *Int. J. Cancer*, **82**, 424–429.
- Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA and Feldman D. (1994). *Cancer Res.*, **54**, 805–810.
- Pols HA, Birkenhager JC, Foekens JA and van Leeuwen JP. (1990). *J. Steroid Biochem. Mol. Biol.*, **37**, 873–876.
- Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM and Koff A. (1994). *Genes Dev.*, **8**, 9–22.
- Schwartz GG and Hulka BS. (1990). *Anticancer Res.*, **10**, 1307–1311.
- Schwartz GG, Oeler TA, Uskokovic MR and Bahnson RR. (1994). *Anticancer Res.*, **14**, 1077–1081.
- Skowronski RJ, Peehl DM and Feldman D. (1993). *Endocrinology*, **132**, 1952–1960.
- Sui G, Soohoo C, Affar el B, Gay F, Shi Y and Forrester WC. (2002). *Proc. Natl. Acad. Sci. USA*, **99**, 5515–5520.
- Tsihlias J, Zhang W, Bhattacharya N, Flanagan M, Klotz L and Slingerland J. (2000). *Oncogene*, **19**, 670–679.
- Yang ES, Maiorino CA, Roos BA, Knight SR and Burnstein KL. (2002). *Mol. Cell. Endocrinol.*, **186**, 69–79.
- Yeh S and Chang C. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 5517–5521.
- Yeh S, Hu YC, Wang PH, Xie C, Xu Q, Tsai MY, Dong Z, Wang RS, Lee TH and Chang C. (2003). *J. Exp. Med.*, **198**, 1899–1908.
- Zhao XY, Ly LH, Peehl DM and Feldman D. (1997). *Endocrinology*, **138**, 3290–3298.
- Zhao XY, Ly LH, Peehl DM and Feldman D. (1999). *Endocrinology*, **140**, 1205–1212.
- Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM and Feldman D. (2000a). *Nat. Med.*, **6**, 703–706.
- Zhao XY, Peehl DM, Navone NM and Feldman D. (2000b). *Endocrinology*, **141**, 2548–2556.
- Zhuang SH and Burnstein KL. (1998). *Endocrinology*, **139**, 1197–1207.
- Zhuang SH, Schwartz GG, Cameron D and Burnstein KL. (1997). *Mol. Cell. Endocrinol.*, **126**, 83–90.