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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research is to gain an understanding of the vitamin D - androgen interaction in prostate cancer. Calcitriol, the active hormonal form of vitamin D, induces androgen receptors (AR) in prostate cells. Major findings thus far involve studies in two areas. First, the ability of vitamin D and retinoids to regulate AR levels in LNCaP human prostate cancer cells. These studies have led to a publication which is included in the Appendix. The major conclusion is that the growth inhibitory activity of vitamin D and retinoid hormones is androgen-dependent and the activity can be blocked by the AR antagonist, Casodex. Second, is a study of prostate cancer cells derived from a bone metastasis in a patient whose prostate cancer had progressed to become androgen-independent. As detailed in a paper that is in press, the cells have a double mutation in the AR. Further study of these cells is in progress in an attempt to determine the nature of the changes that led to androgen-independence.

The significance of this work is that it will increase our understanding of factors that stimulate prostate cancer growth and will attempt to develop mechanisms to inhibit the growth and progression of prostate cancer.

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David Feldman 3/28/01

PI - Signature Date

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Introduction

1α, 25-dihydroxyvitamin D_3 (calcitriol or $1,25(OH)_2D_3$), the active hormonal form of Vitamin D, acts as a transcriptional regulator of target genes by interacting with a nuclear receptor known as the vitamin D receptor (VDR) [1]. The presence of VDR has been demonstrated in prostate cells, both primary cultures of normal prostate as well as in prostate cancer cell lines [2-5]. Calcitriol, acting via the VDR, inhibits prostate cancer cell growth as well as induces a number of target genes [6-10]. Of interest to this proposal, we have shown that calcitriol induces the androgen receptor (AR) in prostate cancer cells [11]. The basis of this grant is to investigate the role of vitamin D in prostate cancer with a focus on its role to increase AR abundance. We are pursuing several questions regarding the vitamin D - androgen interaction as they might impact on a number of aspects of prostate cancer biology: 1) androgen responsiveness, 2) effect of vitamin D on androgen ablation therapy, 3) the development of androgen-independence, and 4) the vitamin D axis as a determinant of prostate cancer risk.

Body

Original Statement of Work and Summary of Results

Task I. To investigate AR up-regulation by calcitriol in various prostate cancer cells (months 1-12).
1. AR-positive cells, LnCaP and ARCaP cells.
2. AR-negative cells, PC-3 and DU-145 cells.
3. Primary cultures of prostate cancer cells, both epithelial and stromal cells.

AR could not be regulated in any cells but LNCaP. The details of LNCaP regulation are discussed below in Section 2 and 4.

Task II. To examine less calcemic vitamin D analogs for potency in regulating AR expression compared to calcitriol to determine the optimal drug for regulating AR expression (months 6-12).
1. Test the effects of a series of less calcemic analogs on the regulation of AR and determine potency relative to calcitriol.

This work is discussed in section 2 and 4.

Task III. To investigate AR up-regulation in non-prostate cells (months 13-18).
1. MCF-7 and other breast cancer cells
2. Other androgen targets known to have VDR.

AR in non-prostate target glands were not regulated by vitamin D.

Task IV. To elucidate the mechanism of AR up-regulation (months 13-30).
1. Evaluate calcitriol for direct effects on the AR.
2. Study the VDR interaction with the AR promoter.

3. Identify and isolate the VDRE in the AR promoter.
4. Study post-transcriptional events in LNCaP cells if AR expression is not transcriptionally regulated by calcitriol.
5. Examine the regulation of transfected AR in PC-3 and DU-145 cells to elucidate calcitriol mediated post-transcriptional events in other prostate cancer cell lines.

AR up-regulation was evaluated and found to be indirect so that there was no evidence for a VDRE in the AR promoter. Transfected AR in PC-3 cells was studied but no target genes of interest were discovered. Transfection of AR into DU145 cells was not successful. This work is described in Sections 2 and 4.

Task V. To study the consequences of AR up-regulation on prostate cells (months 19-30).
1. Determine the effect of increased AR on androgen action to inhibit prostate growth and stimulate PSA expression in LNCaP cells.
2. Ascertain whether AR-negative cells can be converted to AR-positive cells and become androgen responsive after calcitriol treatment. Does calcitriol restore AR expression to AR-negative cells.
3. Evaluate androgen responses in PC-3 and DU-145 (AR-negative) that are transfected with AR to determine whether PSA expression is restored and whether androgens inhibit prostate cell growth.
4. Determine whether calcitriol augments androgen responses in PC-3 and DU-145 cells transfected with AR.
5. Determine whether androgen-independent cells can be converted to androgen dependence.

This subject is extensively discussed below in Sections 1, 2, 4 and 6.

New Projects Initiated

Several new areas were added to the research plan as the investigation moved forward. These were developed as new opportunities became apparent during the research. This is particularly true of the work on AR mutations and a novel mechanism for androgen-independence described below in Sections 3 and 5. Also, we made progress in the pathways by which vitamin D inhibits prostate cancer growth which is described in Section 6.

Research Progress

1. Liarozole acts synergistically with 1α, 25-dihydroxyvitamin D_3 to inhibit growth of DU-145 human prostate cancer cells by blocking 24-hydroxylase. by Lan H. Ly, Xiao-Yan Zhao, Leah Holloway, and David Feldman [12].

1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] inhibits the proliferation of many cancer cells in culture but not the aggressive human prostate cancer cell line DU145. We postulated that the $1,25(OH)_2D_3$-resistant phenotype in DU145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with $1,25(OH)_2D_3$. Since this P450 enzyme initiates $1,25(OH)_2D_3$ catabolism, we presumed a high level of enzyme induction could limit the effectiveness of the $1,25(OH)_2D_3$ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. Since imidizole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing $1,25(OH)_2D_3$ half-life, thereby enhancing $1,25(OH)_2D_3$ antiproliferative effects on DU145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. Neither $1,25(OH)_2D_3$ (1-10 nM) nor liarozole (1-10 μM) inhibited DU145 cell growth when given alone. However, together at 4 days, $1,25(OH)_2D_3$ (10 nM) and liarozole (1 μM) inhibited growth 65%. We used a thin layer chromatography method to assess 24-hydroxylase activity and demonstrated that liarozole (1-100 μM) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in $1,25-(OH)_2D_3$ half-life from 11 to 31 h. In addition, $1,25(OH)_2D_3$ can cause homologous up-regulation of the VDR and in the presence of liarozole, this effect was amplified thus enhancing $1,25(OH)_2D_3$ activity. Western blot analyses demonstrated that DU145 cells treated with $1,25(OH)_2D_3$/liarozole showed greater VDR up-regulation than with either drug alone. In summary, our data demonstrate that liarozole augments the ability of $1,25(OH)_2D_3$ to inhibit DU145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity leading to increased $1,25(OH)_2D_3$ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using $1,25(OH)_2D_3$ and liarozole or other inhibitors of 24-hydroxylase, both in non-toxic doses, might serve as an effective treatment for prostate cancer.

2. Induction of Androgen Receptor by 1α, 25-Dihydroxyvitamin D_3 and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells, by Xiao-Yan Zhao, Lan H. Ly, Donna M. Peehl, And David Feldman [11].

The study is based on our recent findings that $1,25(OH)_2D_3$ inhibits proliferation of LNCaP cells, an androgen-responsive human prostate cancer cell line. Also, $1,25(OH)_2D_3$ increases androgen receptor (AR) abundance and enhances cellular responses to androgen in these cells. In the current study, we have investigated the mechanism by which $1,25(OH)_2D_3$ regulates AR gene expression and the involvement of AR in the $1,25(OH)_2D_3$ and 9-cis retinoic acid (RA)-mediated growth inhibition of LNCaP. Northern blot analyses demonstrated that the steady-state

mRNA level of AR was significantly increased by $1,25(OH)_2D_3$ in a dose-dependent manner. Time course experiments revealed that the increase of AR mRNA by $1,25\text{-}(OH)_2D_3$ exhibited delayed kinetics. In response to $1,25(OH)_2D_3$, AR mRNA levels were first detected to rise at 8 h and reached a maximal induction of 10-fold over the untreated control at 48 h; the effect was sustained at 72 h. Furthermore, the induction of AR mRNA by $1,25(OH)_2D_3$ was completely abolished by incubation of cells with cycloheximide, a protein synthesis inhibitor. $1,25(OH)_2D_3$ was unable to induce expression of an AR promoter-luciferase reporter. These findings indicate that the stimulatory effect of $1,25(OH)_2D_3$ on AR gene expression was indirect. Western blot analyses showed an increase of AR protein in $1,25\text{-}(OH)_2D_3$-treated cells. This increased expression of AR was followed by an inhibition of growth in LNCaP cells by $1,25(OH)_2D_3$. Similar to $1,25(OH)_2D_3$, 9-cis RA also induced AR mRNA expression and the effect of both hormones was additive. Moreover, $1,25(OH)_2D_3$ and 9-cis RA acted synergistically to inhibit LNCaP cell growth. These anti-proliferative effects of $1,25(OH)_2D_3$ and 9-cis RA alone or in combination were blocked by the pure AR antagonist, Casodex. In conclusion, our results demonstrate that growth inhibition of LNCaP by $1,25(OH)_2D_3$ and 9-cis RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. This finding that differentiating agents such as vitamin D and vitamin A derivatives are potent inducers of AR and may have clinical implications for the treatment of prostate cancer.

3. Two Mutations Identified in the Androgen Receptor of a New Human Prostate Cancer Cell Line MDA PCa 2a. by Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone, Donna M. Peehl, and David Feldman [13].

In this study, we have characterized the AR in a new human prostate cancer cell line, MDA PCa 2a, that has recently been established from a bone metastasis of a patient whose cancer was exhibiting androgen-independent growth. These cells express abundant AR (Nmax=685±149 fmol/mg protein), as determined by equilibrium binding assays with [^{3}H]dihydrotestosterone (DHT). However, Scatchard analyses show the AR binding affinity for DHT in these cells to be only 25 nM, a 50-fold lower affinity than those of the mutated AR in LNCaP cells (Kd=0.5 nM) or the wildtype AR in MCF-7 cells (Kd=0.4 nM). DNA sequence analyses of the AR gene in MDA PCa 2a cells revealed two mutations in the ligand-binding domain, L701H and T877A, the latter being reported previously in LNCaP cells. Compared to LNCaP, the new cell line is 10 to 1000-fold less responsive to androgens in cell growth assays as well as in stimulation of PSA secretion. Interestingly, in the absence of added androgens, the new cell line expresses 15-fold higher baseline levels of PSA than LNCaP, suggesting constitutive expression of its PSA gene. In summary, we have identified two mutations in the AR gene of the MDA PCa 2a cell line that are likely responsible for decreased binding affinity for DHT and partial androgen insensitivity observed in these cells. Both androgen insensitivity and elevated baseline PSA levels exemplify the androgen-independent phenotype. Thus, this new cell line can serve as a functionally relevant model system of advanced prostate cancer and can be used to study important events related to androgen-independent growth.

4. 1α, 25-Dihydroxyvitamin D_3 Inhibits Prostate Cancer Cell Growth by Androgen-Dependent and Androgen-Independent Mechanisms, by Xiao-Yan Zhao, Donna M. Peehl, Nora M. Navone and David Feldman [14].

In this study, we examined the actions and interactions of $1,25(OH)_2D_3$ and the androgen DHT on two new human prostate cancer cell lines (MDA), MDA PCa 2a and MDA PCa 2b. Scatchard analysis revealed that these cell lines express high affinity vitamin D receptors (VDRs) with a binding affinity (K_d) for $[^3H]1,25(OH)_2D_3$ of 0.1 nM. However, the MDA cell lines contain low affinity ARs for $[^3H]$DHT binding, with a K_d of 25 nM. This is 50-fold lower than the AR in LNCaP cells (K_d = 0.5 nM). The MDA cell lines minimally respond to DHT. $1,25(OH)_2D_3$ causes a significant growth inhibition in both MDA cell lines, similar or greater than in the LNCaP cell line. Moreover, $1,25(OH)_2D_3$ significantly up-regulates AR mRNA in all three cell lines, as shown by Northern blot analysis. The growth inhibitory effect of $1,25(OH)_2D_3$ on LNCaP cells is blocked by the pure anti-androgen, Casodex, which we previously reported. But Casodex did not block the anti-proliferative activity of $1,25(OH)_2D_3$ in MDA PCa 2b cells and only partially blunted the effect in MDA PCa 2a cells. In conclusion, the growth inhibitory action of $1,25(OH)_2D_3$ in the MDA cell lines appears to be mostly androgen-independent, whereas $1,25(OH)_2D_3$ actions in LNCaP cells are androgen-dependent. Most importantly, the MDA cell lines, derived from a bone metastasis of human prostate carcinoma, remain sensitive to $1,25(OH)_2D_3$, a finding relevant to the therapeutic application of vitamin D and its low calcemic analogs in the treatment of advanced prostate cancer.

5. Glucocorticoids Can Promote Androgen-Independent Growth Of Prostate Cancer Cells Via a Mutated Androgen Receptor, by Xiao-Yan Zhao, Peter J. Malloy, Aruna V. Krishnan, Srilatha Swami, Nora M. Navone, Donna M. Peehl and David Feldman [15].

The AR has been implicated in the development, growth and progression of prostate cancer (CaP). CaP often progresses from an androgen-dependent to an androgen-independent tumor, making androgen ablation therapy ineffective. However, the mechanisms for the development of androgen-independent CaP are unclear. Over 80% of clinically androgen-independent prostate tumors show high levels of AR expression. In some CaPs, AR levels are elevated because of gene amplification and/or overexpression, while in others, the AR is mutated. Nevertheless, the role of the AR in CaP transition to androgen-independent growth and the subsequent failure of endocrine therapy are not fully understood. Here we show that, in CaP cells from a patient who failed androgen ablation therapy, a doubly mutated AR (L701H&T877A) functions as a high-affinity cortisol/cortisone receptor (AR^{CCR}). Cortisol, the major circulating glucocorticoid, and its metabolite, cortisone, both equally stimulate the growth of these CaP cells and increase prostate-specific antigen (PSA) secretion in the absence of androgens. Significantly, physiological concentrations of free cortisol (15-45 nM) and total cortisone (39-63 nM) in men greatly exceed the AR^{CCR} binding affinity (Kd = 4.8 nM) and would activate the receptor, promoting CaP cell proliferation. Our data demonstrate a novel mechanism for the androgen-independent growth of advanced CaP. Understanding this mechanism and recognizing the presence of glucocorticoid-responsive AR mutants are important for the development of new forms of therapy for the treatment of this subset of CaP.

6. Insulin-Like Growth Factor Binding Protein 3 (IGFBP-3) Mediates 1,25-Dihydroxyvitamin D_3 Growth Inhibition In The LNCaP Prostate Cancer Cell Line Through P21/WAF1, by Bryan J Boyle, Xiao-Yan Zhao, Pinchas Cohen, and David Feldman [16].

We have determined that IGFBP-3 induction by $1,25(OH)_2D_3$ is a necessary component of $1,25(OH)_2D_3$-mediated growth inhibition of the LNCaP human prostate cancer cell line. In addition, p21/WAF/CIP1 induction by $1,25(OH)_2D_3$ is mediated also by the presence of active IGFBP-3. Induction of IGFBP-3 by $1,25(OH)_2D_3$ was determined by ELISA assay (for IGFBP-3 protein), and Northern analysis (for IGFBP-3 mRNA). Growth assays for LNCaP cells were determined by measurement of DNA content. The contribution which IGFBP-3 makes towards $1,25(OH)_2D_3$-mediated growth inhibition was determined by both addition of either antisense oligonucleotides or immunoneutralization experiments to growth assays. Regulation of p21/WAF/CIP1 was determined by Western analysis. Addition of $1,25(OH)_2D_3$ to LNCaP prostate cancer cells demonstrated that $1,25(OH)_2D_3$ significantly up-regulates IGFBP-3 at both the mRNA and protein levels in these cells by approximately 3-fold over control levels. We have also shown that addition of IGFBP-3 protein to LNCaP cell growth medium itself has the capacity to inhibit LNCaP cell growth. Interestingly, addition of 10 μg/mL of IGFBP-3 antisense oligonucleotides or 10 ng/mL of antibodies directed towards IGFBP-3 reveals that the growth inhibitory actions of $1,25(OH)_2D_3$ are IGFBP-3-dependent. Finally, in order to connect the mechanisms of IGFBP-3 and $1,25(OH)_2D_3$-mediated growth inhibition, we have demonstrated that IGFBP-3 can up-regulate the expression of p21/WAF1 protein expression by approximately 2-fold over control levels. Addition of an IGFBP-3 immunoneutralizing antibody completely prevents the $1,25\text{-}(OH)_2D_3$-induced up-regulation of p21/WAF1. In conclusion, $1,25(OH)_2D_3$ up-regulates IGFBP-3 in the LNCaP cell line at both the mRNA and protein levels. The growth-inhibitory action of $1,25(OH)_2D_3$ on LNCaP cells is dependent upon active IGFBP-3, as evidenced by the loss of growth inhibition induced by IGFBP-3 antisense oligonucleotide and immunoneutralization experiments. A possible connection between IGFBP-3 and $1,25(OH)_2D_3$ lies in the cyclin-dependent kinase inhibitory protein, p21/WAF1, as both IGFBP-3 and $1,25\text{-}(OH)_2D_3$ up-regulate this protein, and both inhibit LNCaP cell growth. We hypothesize that the mechanism of action by which both IGFBP-3 and $1,25(OH)_2D_3$-induce growth inhibition is therefore by induction of p21/WAF1, as IGFPB-3 immunoneutralizing antibodies completely abrogates $1,25(OH)_2D_3$-mediated up-regulation of p21/WAF1.

Key Research Accomplishments

1. AR expression is increased in prostate cancer cells by vitamin D and retinoids.
2. The ability of vitamin D and retinoids to inhibit LNCaP cell growth is AR-dependent.
3. The MDA PCa 2A cell line has two mutations in the ligand binding domain of the AR which reduce its affinity for androgens.
4. The MDA cell lines are androgen-independent because the mutations alter the ligand binding pocket of the AR so that corticosteroids can act as "pseudo-androgens" and stimulate the growth of the cancer cells in an androgen-independent way.
5. The major action of $1,25(OH)_2D_3$ to inhibit prostate cancer cells is by cell cycle arrest. This action is mediated by stimulation of IGFBP-3 which in turn stimulates p21 that causes antiproliferative activity by inhibition of cells traversing the cell cycle.

Reportable Outcomes
Manuscripts

1. L.H.. Ly, X.-Y. Zhao, L. Holloway, and D. Feldman, Liarozole acts synergistically with 1α, 25-dihydroxyvitamin D_3 to inhibit growth of DU145 human prostate cancer cells by blocking 24-hydroxylase [12].
2. X.-Y. Zhao, L. H. Ly, D. M. Peehl, and D. Feldman, Induction of Androgen Receptor by 1α, 25-Dihydroxyvitamin D_3 and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells. [11].
3. X.-Y. Zhao, B. Boyle, A. V. Krishnan, N. M. Navone, D. M. Peehl, and D. Feldman, Two Mutations Identified in the Androgen Receptor of a New Human Prostate Cancer Cell Line MDA PCa 2a [13].
4. X.-Y. Zhao, B. Boyle, A. Krishnan, N. Navone, D. M. Peehl and D. Feldman, Two mutations Identified in the Androgen Receptor of the New Human Prostate Cancer Cell Line MDA PCa 2a. [13].
5. X.-Y. Zhao, P.J. Malloy, A.V. Krishnan, S.R. Swami, N.M. Navone, D. M. Peehl and D.Feldman, Glucocorticoids Promote Androgen-Independent Prostate Cancer Cell Growth Via a Mutated Androgen Receptor [15].
6. X.-Y. Zhao, D.M. Peehl, N.M. Navone and D. Feldman, 1,25-Dihydroxyvitamin D_3 Inhibits Prostate Cancer Cell Growth by Androgen-Dependent and Androgen Independent Mechanisms. [14].
7. B. J. Boyle, X.-Y. Zhao, P. Cohen, and D. Feldman. Insulin-like growth factor binding protein 3 (IGFBP-3) mediates 1, 25 dihydroxyvitamin D_3 growth inhibition in the LNCaP prostate cancer cell line. [16].

Abstracts

1. X.-Y. Zhao, L. H. Ly, L. Holloway, and D. Feldman, Liarozole Acts Synergistically With 1α,25-Dihydroxyvitamin D_3 To Inhibit Growth of DU145 Human Prostate Cancer Cells by

Blocking 24-Hydroxylase. American Society of Bone & Mineral Research, Dec 1-6, 1998 San Francisco, Pg. S265, abstract T267.
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Conclusions/ "So What"

Prostate cancer (PCa) cells harbor receptors for vitamin D (VDR) as well as androgens (AR). The hormonally active form of vitamin D, $1,25(OH)_2D_3$, induces increased AR expression and enhances androgen actions [11], thus the two hormonal systems are linked. $1,25(OH)_2D_3$ mediates antiproliferative activity in both AR-positive and AR-negative PCa cells [14]. Less calcemic analogs of $1,25(OH)_2D_3$ have been designed that are more potent as antiproliferative agents while causing reduced calcemic side-effects [4]. Analog enhanced activity is related to improved RXR dimerization [17] and stabilization of the VDR in an active conformation [18]. The increased efficacy with decreased side-effects makes the analogs more likely then $1,25(OH)_2D_3$ to be clinically useful [19]. The pathways for growth inhibitory activity are diverse [9]. $1,25(OH)_2D_3$ and its analogs appear to predominantly exert antiproliferative activity by causing cell cycle arrest although some PCa cells exhibit apoptosis [6-8,10]. In our studies of the LNCaP cell, a major calcitriol pathway for growth arrest was via induction of IGFBP-3 expression [16]. In an IGF-independent action, IGFBP-3 induced synthesis of p21 which results in antiproliferative activity. Antisense and immunoneutralization experiments demonstrate that induction of p21 is essential for $1,25(OH)_2D_3$'s antiproliferative activity [16]. cDNA microarray analysis indicates that $1,25(OH)_2D_3$ induces many target genes in PCa cells. Some genes may inhibit PCa progression by inhibiting angiogenesis or metastases [20]. $1,25(OH)_2D_3$ also induces the enzyme 24-hydroxylase which metabolizes the active hormone into inactive products [2,5] so that inhibitors of 24-hydroxylase enhance $1,25(OH)_2D_3$'s antiproliferative activity [12]. Combination therapy with retinoids and other anti-cancer agents enhances PCa inhibitory activity and provides yet another potential therapeutic modality for $1,25(OH)_2D_3$ [21]. Small clinical trials have shown that $1,25(OH)_2D_3$ can slow the rate of PSA rise demonstrating proof of concept that $1,25(OH)_2D_3$ or its analogs will be clinically effective in PCa therapy [19]. Whether for therapy or chemoprevention, further investigation of the optimal role of calcitriol and its analogs for the treatment of PCa is currently being defined. However, our data suggest that vitamin D and its analogs will be effective additions to the treatment armamentarium as we move forward in developing new treatment stategies for prostate cancer.

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Induction of Androgen Receptor by 1α,25-Dihydroxyvitamin D_3 and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells*

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ABSTRACT

We have recently shown that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits proliferation of LNCaP cells, an androgen-responsive human prostate cancer cell line. Also, 1,25-$(OH)_2D_3$ increases androgen receptor (AR) abundance and enhances cellular responses to androgen in these cells. In the current study, we have investigated the mechanism by which 1,25-$(OH)_2D_3$ regulates AR gene expression and the involvement of AR in the 1,25-$(OH)_2D_3$- and 9-cis retinoic acid (RA)-mediated growth inhibition of LNCaP cells. Northern blot analyses demonstrated that the steady-state messenger RNA (mRNA) level of AR was significantly increased by 1,25-$(OH)_2D_3$ in a dose-dependent manner. Time-course experiments revealed that the increase of AR mRNA by 1,25-$(OH)_2D_3$ exhibited delayed kinetics. In response to 1,25-$(OH)_2D_3$, AR mRNA levels were first detected to rise at 8 h and reached a maximal induction of 10-fold over the untreated control at 48 h; the effect was sustained at 72 h. Furthermore, the induction of AR mRNA by 1,25-$(OH)_2D_3$ was completely abolished by incubation of cells with cycloheximide, a protein synthesis inhibitor. 1,25-$(OH)_2D_3$ was unable to induce expression of an AR promoter-luciferase reporter. Together, these findings indicate that the stimulatory effect of 1,25-$(OH)_2D_3$ on AR gene expression is indirect. Western blot analyses showed an increase of AR protein in 1,25-$(OH)_2D_3$-treated cells. This increased expression of AR was followed by 1,25-$(OH)_2D_3$-induced inhibition of growth in LNCaP cells. Similar to 1,25-$(OH)_2D_3$, 9-cis RA also induced AR mRNA expression, and the effect of both hormones was additive. Moreover, 1,25-$(OH)_2D_3$ and 9-cis RA acted synergistically to inhibit LNCaP cell growth. These antiproliferative effects of 1,25-$(OH)_2D_3$ and 9-cis RA, alone or in combination, were blocked by the pure AR antagonist, Casodex. In conclusion, our results demonstrate that growth inhibition of LNCaP cells by 1,25-$(OH)_2D_3$ and 9-cis RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. This finding, that differentiating agents such as vitamin D and A derivatives are potent inducers of AR, may have clinical implications in the treatment of prostate cancer. (*Endocrinology* 140: 1205–1212, 1999)

1α,25-DIHYDROXYVITAMIN D_3 [1,25-$(OH)_2D_3$], the active metabolite of vitamin D, regulates calcium homeostasis in the body by actions in the intestine, bone, kidney, and parathyroid glands (1, 2). Recently, 1,25-$(OH)_2D_3$ has also been shown to have nonclassical actions. For example, the hormone exerts antiproliferative and prodifferentiating effects on many cell types, including cells derived from myeloid, breast, colon, and prostate tissues (3–6). Biologic responses of target cells to 1,25-$(OH)_2D_3$ are mediated by its nuclear receptor, the vitamin D receptor (VDR) (7). The VDR belongs to the steroid/thyroid/retinoid receptor superfamily (1, 2). Numerous studies indicate that VDR controls target gene transcription by forming a heterodimeric complex with the retinoid X receptor (RXR), the receptor for 9-cis retinoic acid (RA), and binding to the vitamin D response element (VDRE) present in the promoter region of target genes.

Our group (8, 9), as well as others (10), have shown that VDRs are present in established human prostate cancer cell lines, as well as primary cultures of normal prostate and cancer cells (11). Moreover, 1,25-$(OH)_2D_3$ and its analogs significantly inhibit cellular proliferation of prostate cancer cells, including LNCaP (8, 9, 12–20). LNCaP cells express both the VDR and the androgen receptor (AR). Our recent studies (21) and those of others (15, 22) have demonstrated that cross-talk between 1,25-$(OH)_2D_3$ and androgens exists and that the antiproliferative actions of 1,25-$(OH)_2D_3$ in LNCaP cells are androgen-dependent. Blutt *et al.* (17) have shown that 9-cis RA acts synergistically with 1,25-$(OH)_2D_3$ to inhibit LNCaP cell growth.

Because cellular responsiveness to androgen depends on AR abundance, in the present study, we have analyzed the ability of 1,25-$(OH)_2D_3$ and 9-cis RA to regulate the level of AR gene expression in these cells. We found that 1,25-$(OH)_2D_3$ increased the levels of AR messenger RNA (mRNA) and AR protein in a concentration- and time-dependent manner. Such regulatory effects of 1,25-$(OH)_2D_3$ on AR gene expression required *de novo* protein synthesis. Furthermore, the stimulatory effect of 1,25-$(OH)_2D_3$ on AR mRNA was also enhanced by 9-cis RA. Because it has been reported that the antiproliferative effects of 1,25-$(OH)_2D_3$ on LNCaP cells can be synergistically enhanced by the addition of 9-cis RA (17), we examined the involvement of AR in the antiproliferative action of 9-cis RA, as well as 1,25-$(OH)_2D_3$. Using the pure AR antagonist, Casodex, we demonstrated that AR blockade prevented the growth inhibitory activity of both 1,25-$(OH)_2D_3$ and 9-cis RA. In contrast, Casodex did not affect the antiproliferative activity of dibutyrl cAMP, a well-known

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up-regulator of AR in LNCaP cells (23). Our studies demonstrate that both 1,25-$(OH)_2D_3$ and 9-cis RA up-regulate AR mRNA levels in LNCaP cells and that growth inhibition mediated by 1,25-$(OH)_2D_3$ and 9-cis RA requires the action of AR.

Materials and Methods

Materials

1,25-$(OH)_2D_3$ was the generous gift of Dr. M. Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Bicalutamide (Casodex or ICI 17,334) was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tissue culture media were purchased from Mediatech (Herndon, VA). All other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-AR monoclonal antibody F39.4 and the human AR complementary DNA (cDNA) were generous gifts from Dr. TH Van der Kwast (Erasmus University, Rotterdam, Netherlands) and Dr. M. McPhaul (University of Texas Southwestern Medical Center, Dallas, TX), respectively. FBS was obtained from Gibco BRL (Gaithersburg, MD). Charcoal-stripped FBS (CSS) was purchased from Sigma Chemical Co.

Cell culture and hormone treatment

The LNCaP human prostate carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI-1640 medium supplemented with 5% FBS and antibiotics (FBS medium), at 37 C in a humidified atmosphere of 5% CO_2. For experiments, LNCaP cells were trypsinized and seeded at an appropriate density, and hormonal treatments were initiated, the next day, in FBS medium or in RPMI-1640 medium supplemented with 5% CSS and antibiotics (CSS medium).

Hormone stocks [1,25-$(OH)_2D_3$, 9-cis RA, and Casodex] were prepared in 100% ethanol, at a concentration 1000-fold higher than the working concentrations. Fresh culture media were premixed with hormone stock and then added to triplicate wells. Media and hormone were replenished every 2 days. Controls received ethanol vehicle at a concentration equal to that in hormone-treated cells.

Assay of cell proliferation

Cell proliferation was assessed by measurement of attained cell mass using an assay of DNA content. As previously described (21), LNCaP cells were seeded in six-well tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ), at a density of 50,000 cells per well, in 3 ml RPMI-1640 containing 5% FBS. After incubation for 24 h, the medium was replaced with fresh medium containing 5% FBS (FBS medium). Cells were treated with vehicle (ethanol, final concentration 0.1%), 1,25-$(OH)_2D_3$, 9-cis RA, dibutyrl cAMP, or Casodex. On the sixth day, cell monolayers were processed for DNA assay using the method of Burton (24). DNA content of each treatment was derived from the mean value of triplicate wells in an experiment. Each experiment was repeated three times.

Western blot analysis

Cells were treated with ethanol or 1,25-$(OH)_2D_3$ (10 nM) in RPMI-1640 medium containing 5% CSS (CSS medium) for 2 days. They were harvested, and sonicated extracts were prepared as described. Aliquots of 100 μg protein were heated in SDS sample buffer at 95 C, for 5 min, before electrophoresis in an 8% SDS-polyacrylamide gel. After electrophoresis, the gels were transferred and processed as previously described (25). After transfer, the blots were incubated with anti-AR monoclonal antibody F39.4 (1:100 dilution) for 1 h, at room temperature, with gentle shaking. The blots were washed and then incubated with a horseradish peroxidase-conjugated rabbit antimouse IgG (1:1000 dilution) for 1 h at room temperature. Blots were rewashed and developed with the Enhanced Chemiluminesence (ECL) System system, according to the manufacturer's instructions (Amersham Chemical Co.).

Steroid receptor ligand-binding assay

LNCaP cells were seeded at a density of 150,000 cells per 100-mm dish in 10-ml medium containing 5% FBS or 5% CSS. At the end of the 6-day incubation with hormone (at concentrations of 0, 1, and 10 nM), cell monolayers were harvested, and high-salt nuclear extracts were made as previously described (21). Protein concentration of the extract was determined (26). In a typical binding experiment, 200 μl soluble extract (1–2 mg protein/ml) were incubated with 10 nM concentration of [^{3}H]-5α-dihydrotestosterone (DHT) for 16–20 h at 4 C. Bound and free hormone were separated by hydroxylapatite (21). Specific binding was calculated by subtracting nonspecific binding (obtained in the presence of a 250-fold excess of radioinert DHT) from the total binding (measured in the absence of radioinert steroid). Data were expressed as femtomoles [^{3}H]-DHT bound per milligram protein.

Northern blot analysis

Northern blot analysis was performed as previously described (8, 11). Briefly, semiconfluent LNCaP cells were treated with graded concentrations of 1,25-$(OH)_2D_3$, or 5 mM dibutyrl cAMP, or 9-cis RA in FBS medium and in CSS medium for 24 h before isolation of total RNA. Ten micrograms of total RNA were denatured, fractionated by electrophoresis, and transferred to Hybond-N nylon membrane (Amersham), as previously described (8, 11). The bound RNA was immobilized by UV cross-linking and then hybridized with a random primed [^{32}P]-labeled 0.8-kb *Hin*dIII-*Bam*HI fragment of the human AR cDNA at 60 C. To control for RNA sample loading and transfer, Northern blots were also hybridized with a [^{32}P]-labeled 0.9-kb *Eco*RI fragment of the human cDNA for the ribosomal protein gene L7 (8, 11). The silver grain pixel intensity of each AR and L7 band was scanned by a densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

AR promoter-luciferase reporter gene assay

LNCaP cells were seeded at 3×10^6 cells/dish in 60-mm tissue culture dishes (Corning, Inc., Corning, NY) in RPMI-1640 medium containing 5% FCS and antibiotics. A 6-kb promoter-luciferase reporter was transfected using a calcium-phosphate method (23). Each transfection contained 1 μg pAR-LUC DNA (Drs. G. Mora and D. Tindall, personal communication) and 0.1 μg pSV-Renilla DNA. The control plasmid pSV-Renilla was used to monitor transfection efficiency. Cells were harvested after 32 h of incubation with tested compounds at 37 C. Luciferase activity was employed to measure induction using Promega Corp. (Madison, WI) dual luciferase assay system on luminometer TD-20 (Turner Design, Sunnyvale, CA). The results were expressed as the ratio of luciferase activity to Renilla activity.

Statistical analysis

ANOVA was used to assess the statistical significance of the difference. $P < 0.05$ was considered significant.

Results

We have recently demonstrated that the antiproliferative action of 1,25-$(OH)_2D_3$ in LNCaP cells is androgen-dependent (21). Here, we investigate further the interaction between 1,25-$(OH)_2D_3$ and androgen signaling pathways by exploring the mechanism of 1,25-$(OH)_2D_3$ regulation of AR gene expression in these cells. We also examine the possible involvement of AR in the synergistic antiproliferative actions of 1,25-$(OH)_2D_3$ and 9-cis RA on LNCaP cells.

Dose response effect of 1,25-$(OH)_2D_3$ on AR mRNA

The effect of 1,25-$(OH)_2D_3$ on steady-state AR mRNA levels was assessed by Northern blot analysis. We have used two culture conditions (FBS medium and CSS me-

dium) in this set of experiments and have observed similar results. As shown in Fig. 1, LNCaP cells express a major transcript of AR at 11 kb. In Fig. 1A, the cells were treated in CSS medium for 24 h with increasing concentrations of 1,25-$(OH)_2D_3$ (0–100 nM), and AR mRNA transcripts increased in a dose-dependent manner. The increased AR mRNA levels became evident with a concentration of 1,25-$(OH)_2D_3$ at 1 nM (lane 3). Increasing the 1,25-$(OH)_2D_3$ concentration caused further induction of AR mRNA (lanes 4–5). The levels of AR mRNA were quantitatively determined by densitometric scanning of the autoradiographs, with correction for the L7 mRNA signal (Fig. 1B). At 100 nM of 1,25-$(OH)_2D_3$, more than 5-fold up-regulation of AR mRNA was detected (lane 5). When we carried out the experiment using FBS medium (Figs. 1, C and D), we also detected a significant up-regulation of AR mRNA in LNCaP cells in response to 1,25-$(OH)_2D_3$ treatment for 24 h. Hence, 1,25-$(OH)_2D_3$ increased AR mRNA expression in LNCaP cells in a dose-dependent manner in either CSS medium or FBS medium.

Time-course of AR mRNA expression in response to 1,25-$(OH)_2D_3$

In Fig. 2, time-course experiments using CSS medium revealed that addition of 10 nM 1,25-$(OH)_2D_3$ to LNCaP cells increased AR mRNA levels by 8 h. The AR mRNA levels peaked at 48 h in the treated cells, with a 10-fold higher level, compared with the untreated cells at the concurrent time point, and this inductive effect of 1,25-$(OH)_2D_3$ was sustained at 72 h. No change in AR mRNA could be detected at 4 h, suggesting a delayed primary response of AR gene expression to 1,25-$(OH)_2D_3$.

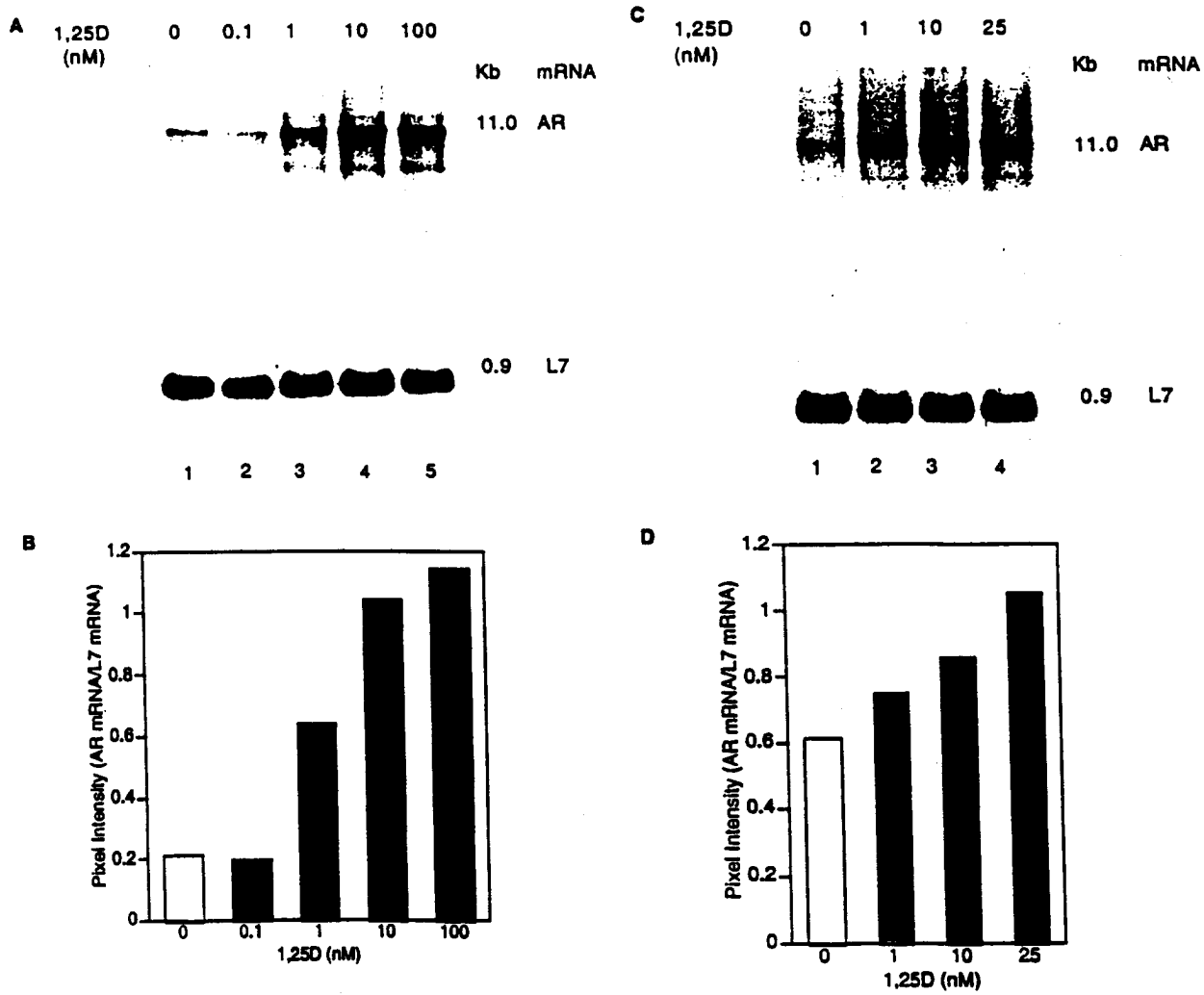

FIG. 1. Dose-dependent effect of 1,25-$(OH)_2D_3$ on AR mRNA levels in LNCaP cells. A, Northern blot analysis in CSS medium. LNCaP cells were treated with 1,25-$(OH)_2D_3$, at the indicated concentrations, for 24 h in RPMI medium containing 5% charcoal stripped serum. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P-labeled 712-bp *HindIII-EcoRI* fragment of the human AR cDNA at 60 C. The blot was simultaneously probed for expression of the L7 ribosomal protein gene as a control for sample loading and transfer. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA. C, Northern blot analysis in FBS medium. LNCaP cells were treated with 1,25-$(OH)_2D_3$, at the indicated concentrations, for 24 h in RPMI medium containing 5% FBS. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P-labeled human AR cDNA and the L7 gene. D, The pixel intensity of each AR band indexed to L7 in panel C.

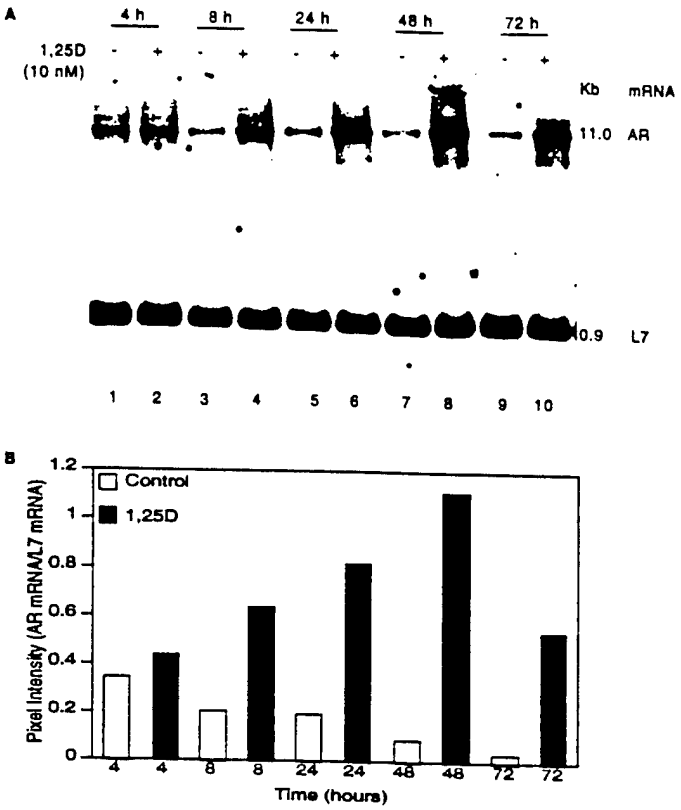

FIG. 2. Time-course of AR mRNA expression in LNCaP cells, in response to 1,25-$(OH)_2D_3$. A, Northern blot analysis. LNCaP cells were treated with 1,25-$(OH)_2D_3$, at 10 nM, for the indicated time period. Total RNA was isolated and analyzed by Northern blot using the human AR cDNA and L7 cDNA as probes. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

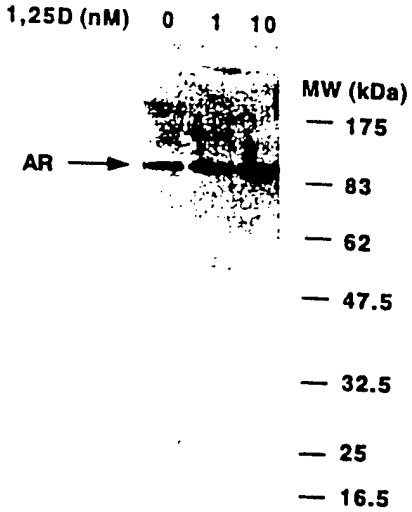

FIG. 3. Western blot analysis of AR protein in LNCaP cells. LNCaP cells were incubated, in RPMI medium containing 5% charcoal stripped serum, with the indicated dose of 1,25-$(OH)_2D_3$ for 2 days. High-salt protein extracts were electrophoresed in an 8% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with anti-AR monoclonal antibody F39.4. Immunoreactive bands were detected by incubation of blots with a secondary antibody (rabbit antimouse IgG), followed by ECL. Molecular weight standards are indicated. hAR is indicated by an *arrow*. The experiment was repeated twice, with similar results.

Up-regulation of the AR protein by 1,25-$(OH)_2D_3$

1,25-$(OH)_2D_3$ also caused a concentration-dependent stimulation of AR protein expression in LNCaP cells cultured in CSS medium, as measured by Western blot analysis (Fig. 3). The major species of AR in LNCaP cells was detected as a single band, at 108 kDa, by monoclonal antibody F39.4. There was no detectable increase in AR protein levels after 24 h treatment (data not shown), but levels rose approximately 4-fold in cells treated with 10 nM 1,25-$(OH)_2D_3$ for 48 h. As seen for AR mRNA levels (Fig. 1), a detectable increase in AR protein was evident with 1 nM 1,25-$(OH)_2D_3$.

[^{3}H]DHT-binding analyses demonstrated that 1,25-$(OH)_2D_3$ increased the AR content in LNCaP cells when they were cultured in either CSS medium or FBS medium. As we have reported (21), cells treated with 1 nM 1,25-$(OH)_2D_3$ in CSS medium showed a more than 2-fold increase in DHT-binding (from 197 ± 17.4 to 430 ± 9.6 fmol/mg protein, n = 3). Addition of 10 nM of 1,25-$(OH)_2D_3$ further up-regulated the AR content (from 197 ± 17.4 to 532 ± 60 fmol/mg, n = 3). Meanwhile, cells cultured in FBS medium exhibited a higher baseline DHT-binding than in CSS medium (378 ± 33.9 fmol/mg *vs.* 197 ± 17.4 fmol/mg, n = 3). 1,25-$(OH)_2D_3$ at 1 nM in FBS medium also increased the AR content (from 378 ± 33.9 to 451 ± 54 fmol/mg protein, n = 3). Therefore, 1,25-$(OH)_2D_3$ up-regulates the AR content in LNCaP cells in either growth condition. Interestingly, CSS medium allowed us to detect a clear up-regulation of AR because charcoal treatment removes endogenous steroids in serum that may interfere with [^{3}H]DHT-binding.

Requirement of new protein synthesis for 1,25-$(OH)_2D_3$ regulation of AR

To determine whether 1,25-$(OH)_2D_3$ affected AR mRNA levels via a direct mechanism, LNCaP cells in CSS medium were treated for 24 h with 1,25-$(OH)_2D_3$ in the presence of the protein synthesis inhibitor cycloheximide (CHX) at various doses (0, 2, 5, and 10 μg/ml). As shown in Fig. 4, CHX blocked the 1,25-$(OH)_2D_3$-induced increase in AR mRNA levels, such that in the presence of CHX and 1,25-$(OH)_2D_3$ (lane 3), AR mRNA levels were no higher than those in untreated cells (lane 1). The extent of blockade depended upon the concentration of CHX included in the media (lanes 3–5). Moreover, the effect of CHX could be detected at either 16 h (lane 6) or 24 h (lane 4).

In other studies, using a 6-kb AR promoter-luciferase reporter transfected into LNCaP cells, we attempted to directly induce expression of AR with 1,25-$(OH)_2D_3$. No increase in luciferase could be detected with 1,25-$(OH)_2D_3$, whereas dibutyrl cAMP induced a 5-fold rise in luciferase (data not shown). Taken together, these findings indicate that 1,25-$(OH)_2D_3$ regulates AR mRNA expression via an indirect mechanism requiring new protein synthesis.

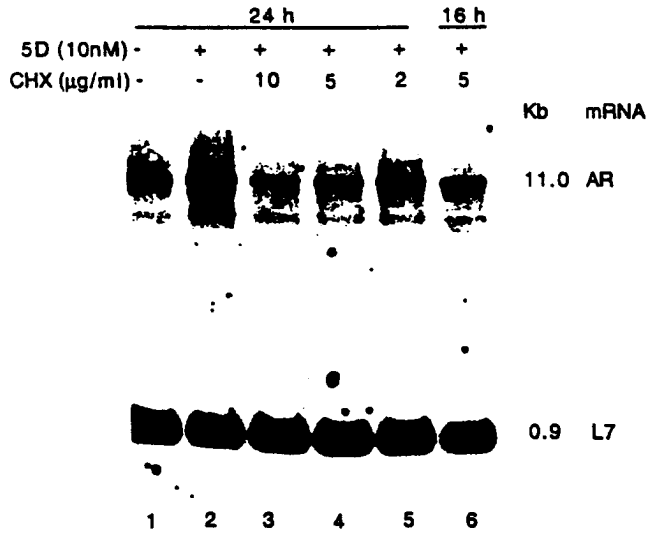

FIG. 4. Effect of CHX on the 1,25-$(OH)_2D_3$ induction of AR mRNA in LNCaP cells. Cells were treated with ethanol (lane 1) or 10 nM 1,25-$(OH)_2D_3$ (lanes 2–6), in the presence of CHX, at the indicated concentrations, for 24 h (lanes 1–5) or 16 h (lane 6). Total RNA was isolated and analyzed by Northern blot using the human AR cDNA and L7 cDNA as probes.

Enhancement of 1,25-$(OH)_2D_3$-mediated up-regulation of AR by 9-cis RA

It recently has been reported that 1,25-$(OH)_2D_3$ acts synergistically with 9-cis RA to inhibit LNCaP cell proliferation [7]. We therefore investigated the effect of 9-cis RA on 1,25-$(OH)_2D_3$ regulation of AR mRNA. Both culture conditions (FBS medium and CSS medium) gave similar results. As shown in Fig. 5, 1,25-$(OH)_2D_3$, at a dose of 10 nM, induced a 5-fold increase in AR mRNA levels (lane 1) over the control at 24 h (lane 2). LNCaP cells, treated with 100 nM 9-cis RA for 24 h, expressed 3-fold more AR mRNA (lane 3) than the untreated cells (lane 2). Combination treatment of 1,25-$(OH)_2D_3$ and 9-cis RA gave a more than 8-fold induction of AR mRNA (lane 4). Thus, although 1,25-$(OH)_2D_3$ was more effective than 9-cis RA in up-regulating AR mRNA, both hormones acted additively to increase AR gene expression in LNCaP cells.

Involvement of AR action in the antiproliferative response of 1,25-$(OH)_2D_3$ and 9-cis RA

We further tested the possible involvement of AR action in the antiproliferative effect of 1,25-$(OH)_2D_3$ and its synergism with 9-cis RA. As shown in Fig. 6A, 10 nM 1,25-$(OH)_2D_3$ inhibited LNCaP cell growth 50%, whereas 100 nM 9-cis RA only reduced proliferation by 10%. However, combination treatment with 1,25-$(OH)_2D_3$ and 9-cis RA caused 80% growth inhibition. Casodex was used to determine whether the 9-cis RA action was also AR-dependent. In the presence of Casodex, neither 1,25-$(OH)_2D_3$ nor 9-cis RA inhibited cell growth individually or in combination (Fig. 6A). On the other hand, dibutyrl cAMP, a known up-regulator of AR in LNCaP cells, inhibited cell proliferation in a dose-dependent manner (Fig. 6B). With maximal growth inhibition of 90% occurring at a dose of 5 mM dibutyrl cAMP, addition of Casodex, however, did not block this effect (Fig. 6B). These data imply

FIG. 5. Enhancement of 1,25-$(OH)_2D_3$ action by 9-cis RA in the induction of AR mRNA in LNCaP cells. A, Northern blot analysis. LNCaP cells were treated with 1,25-$(OH)_2D_3$ at 10 nM, or 9-cis RA at 100 nM, individually or in combination for 24 h. Total RNA was extracted and analyzed by Northern blot. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

that the actions of 1,25-$(OH)_2D_3$ and 9-cis RA on LNCaP cell growth are both AR-dependent. In contrast, cAMP, although an inducer of AR (23), inhibits LNCaP cell proliferation by an AR-independent mechanism.

Model of 1,25-$(OH)_2D_3$ and 9-cis RA action in LNCaP

The hormonal action of 1,25-$(OH)_2D_3$ is mediated by the VDR present in LNCaP cells. Figure 7 depicts the possible events in the 1,25-$(OH)_2D_3$ signaling pathway in these cells. 1,25-$(OH)_2D_3$ binds to the VDR and activates the receptor. The activated VDR controls target gene transcription by forming a heterodimer with the partner RXR and binding to the VDRE in the promoter region of a target gene. A 1,25-$(OH)_2D_3$ target gene (or genes) encodes protein(s) X, mediators of the up-regulation of AR mRNA in response to 1,25-$(OH)_2D_3$. The production of protein(s) X is CHX-sensitive. Both 1,25-$(OH)_2D_3$ and 9-cis RA, individually or in combi-

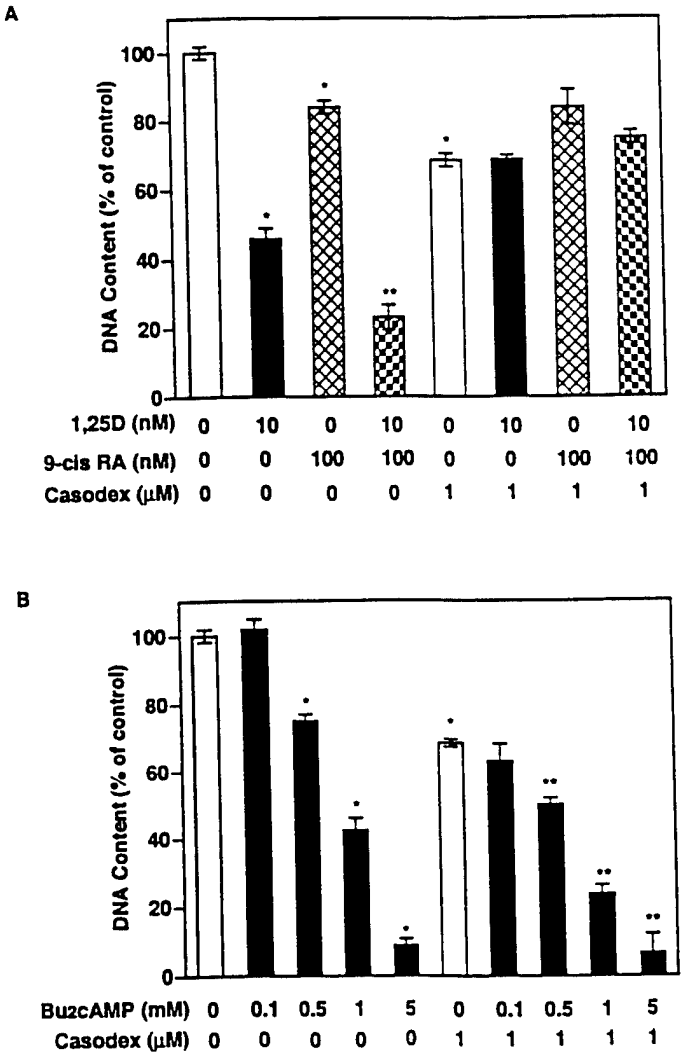

FIG. 6. Effect of Casodex on 1,25-$(OH)_2D_3$ and 9-cis RA-induced growth inhibition on LNCaP cells. A, LNCaP cells were treated with ethanol, 1,25-$(OH)_2D_3$ at 10 nM, or 9-cis RA at 100 nM, individually or both in the presence or absence of Casodex at 1 μM, for 6 days. Cellular DNA contents were determined by Burton's method. The data are expressed as percent of control, a mean of three triplicate samples ± SEM. *, $P < 0.01$, compared with the untreated control group; **, $P < 0.05$, compared with the single-treatment group. B, LNCaP cells were treated with dibutyrl cAMP [Bu2cAMP], from 0–5 mM, in the presence or absence of Casodex at 1 μM, for 6 days.

nation, induce AR mRNA expression. The increased AR mRNA causes an increase in AR protein levels, which mediates the action of androgens on LNCaP cell growth. As we reported previously (23), either 1,25-$(OH)_2D_3$ or DHT was growth inhibitory on LNCaP when cells were cultured in FBS-containing medium. The androgen action is blocked by the AR antagonist, Casodex. Although both the AR mRNA and the AR protein are also induced in CSS medium, in the absence of androgens, 1,25-$(OH)_2D_3$ does not exhibit an antiproliferative effect on LNCaP cells in this culture system (21).

Discussion

The AR is the key element in the androgen signal transduction cascade, and it plays a critical role in the regulation of growth and differentiation of the prostate. The data presented here demonstrate that 1,25-$(OH)_2D_3$ up-regulates AR gene expression at both mRNA and protein levels in LNCaP cells, an androgen-responsive human prostate cancer cell line. This inductive action of 1,25-$(OH)_2D_3$ was enhanced by 9-cis RA, which by itself also up-regulates AR expression in LNCaP cells. Our data show that growth inhibition induced by 1,25-$(OH)_2D_3$ alone or in combination with 9-cis RA was accompanied by increased AR expression. Moreover, the antiproliferative actions of 1,25-$(OH)_2D_3$ and 9-cis RA were AR-dependent and could be blocked by the AR antagonist, Casodex. Although androgens are not added in these experiments, androgens are present in the serum supplement to the culture medium (FBS medium), and we hypothesize that the amplitude of the androgen response is augmented by the increased levels of AR expressed in these cells after treatment with 1,25-$(OH)_2D_3$ and/or 9-cis RA. Support for the effect of androgens, in FBS medium, on cell growth was presented in our earlier studies (21). In the absence of androgens (for example, in CSS medium), 1,25-$(OH)_2D_3$ does not exhibit an antiproliferative action on LNCaP cells.

Nonetheless, our finding that androgen mediates the antiproliferative activity of 1,25-$(OH)_2D_3$ in LNCaP cells is not the situation in all prostate cancer cells. 1,25-$(OH)_2D_3$ inhibits the growth of AR-negative prostate cancer cell line PC-3, as well as primary cultures of human prostate cells. In contrast to LNCaP cells, mechanisms other than androgen signaling are responsible for the growth inhibitory effect of 1,25-$(OH)_2D_3$ on these cells.

It is of interest to consider whether increasing the abundance of a steroid receptor, such as the AR, will cause an increased amplitude of response, *i.e.* antiproliferation. Although it has been well demonstrated that the level of receptors in LNCaP does not necessarily predict the ligand potency of a hormonal response (8, 14, 19, 27), it is clear that the presence of a receptor is essential for a response (1, 2, 13, 14, 28). In a given cell, in the presence of a constant level of hormone, up-regulation of the receptor does cause an enhanced response, whereas down-regulation of the receptor diminishes the response (13, 14, 28–30). Therefore, we believe that up-regulation of AR, in these studies, is the mechanism of the enhanced antiproliferative effect.

The expression of the AR gene has been found to be induced by a number of agents in several systems, such as the rat ventral prostate (31), and in LNCaP human prostate cancer cells (32, 33). Growth factors such as FSH, EGF, and TGF-β regulate AR gene expression (34, 35). Activators of protein kinase A, such as forskolin and dibutyrl cAMP, are the known up-regulators of AR in LNCaP cells (23). The induction of AR by these reagents was not detected in the two other commonly studied prostate cancer cell lines, PC-3 and DU 145, which do not express basal levels of AR mRNA (36).

1,25-$(OH)_2D_3$ is the most potent inducer of AR in LNCaP cells, among the three agents that we tested. Consistent with the reported data (23), we found that treatment of LNCaP cells with dibutyrl cAMP for 24 h caused a 2-fold increase in AR mRNA levels (data not shown). In the same experiment, we observed an increase of 5-fold in AR mRNA, with 1,25-$(OH)_2D_3$ at 10 nM. 9-cis RA induced 3-fold induction of AR mRNA. It has been reported that dibutyrl cAMP increases

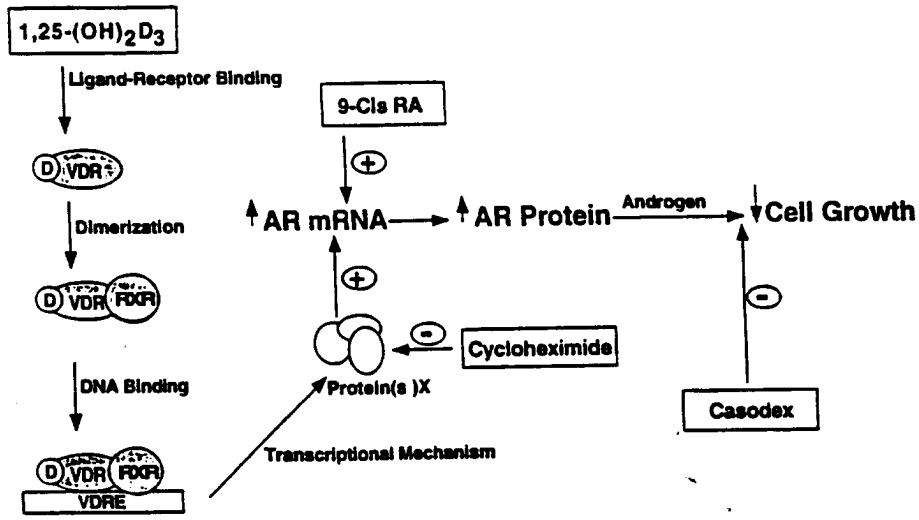

FIG. 7. A tentative model of 1,25-$(OH)_2D_3$ and 9-cis RA action on LNCaP cells. Both 1,25-$(OH)_2D_3$ and 9-cis RA induce AR mRNA expression. The increased AR mRNA leads to an increase in AR protein levels. AR protein mediates androgen action in cell proliferation. The pure AR antagonist, Casodex, blocks AR action; in turn, it blocks the growth-inhibitory action of 1,25-$(OH)_2D_3$ and 9-cis RA.

AR gene transcription via the cAMP-response elements present in the 2.3-kb promoter region of the human AR gene (23). In contrast, the same promoter region of the AR gene seems to lack a VDRE and an RXRE. Computer searching of the 2.3-kb promoter region failed to identify a consensus sequence for these regulatory elements. Moreover, the luciferase reporter construct, driven by a 6-kb promoter region of the human AR gene, did not respond to 1,25-$(OH)_2D_3$ or 9-cis RA but did respond to dibutyrl cAMP.

An indirect mechanism for 1,25-$(OH)_2D_3$ action to induce AR was supported by several findings taken together: the delayed time of AR mRNA rise in time-course experiments (Fig. 2), the CHX studies (Fig. 3), and the failure of the promoter to respond to 1,25-$(OH)_2D_3$ (data not shown). We refer to the indirect, CHX-inhibited mediator(s) of 1,25-$(OH)_2D_3$ action to up-regulate AR as protein(s) X. We surmise that, in the presence of CHX, 1,25-$(OH)_2D_3$ was unable to induce protein(s) X, and as a consequence, 1,25-$(OH)_2D_3$ failed to up-regulate AR mRNA (Fig. 4). It is interesting to speculate on the nature of protein(s) X. Protein(s) X may be related to the chaperon proteins, given the fact that several chaperons have been identified in the regulation of steroid receptors (37). Further studies are needed to elucidate this mechanism.

We did not detect AR up-regulation by 1,25-$(OH)_2D_3$ in two human breast cancer cells, either MCF-7 or T47D (unpublished data). Both MCF-7 and T47D cells express the VDR, as well as the AR. However, the levels of AR protein did not change in both cell lines when treated with 1,25-$(OH)_2D_3$. Therefore, induction of protein(s) X by 1,25-$(OH)_2D_3$ may be tissue-specific. At present, it is difficult to examine this point because of the limited number of human prostate cancer cells that exhibit the AR. We have, thus far, been unable to induce AR in cells that lack the AR, including primary cultures of prostate cancer cells and established cell lines PC-3 or DU 145. To determine whether AR induction by 1,25-$(OH)_2D_3$ is LNCaP cell-specific, we hope to study other AR-positive human prostate cancer cell lines as they become available.

The action of androgens to inhibit proliferation of cultured prostate cancer cells is an interesting finding. We and others (21, 38) showed that LNCaP cells exhibit a biphasic growth response to DHT in charcoal-stripped serum-containing medium, with a growth stimulatory effect at a low concentration (less than 1 nM) and an inhibitory effect at a high concentration (greater than 1 nM). The levels of AR protein in LNCaP cells determine the concentration of DHT at which the stimulatory effect crosses over to an inhibitory effect. In other words, the stimulatory effect is favored at low abundance of AR, and an inhibitory effect at high abundance of AR (21). Liao and co-workers (39–41) found that high-passage LNCaP cells in an androgen-depleted medium express 10- to 20-fold higher AR levels and are growth inhibited by androgens *in vitro* and in an *in vivo* mouse model. Moreover, they demonstrated that G1 arrest of the high AR-expressing cells by androgen is caused by the induction of $p27^{kip1}$, which in turn inhibits Cdk2, a factor critical for cell cycle progression and proliferation (41). There are two additional examples to document the role of AR in the inhibition of growth of prostate cancer cells. Yuan *et al.* (42) have reported that PC-3 cells, stably transfected with the human AR cDNA, were growth inhibited by androgen. Recently, Zhau *et al.* (43) have established an androgen-repressed human prostate cancer cell line (ARCaP) derived from the ascites fluid of a patient with advanced metastatic disease, which is growth inhibited by androgens. Cumulatively, these findings support the hypothesis that higher levels of AR in cultured prostate cancer cells cause increased sensitivity to growth inhibition.

In summary, we have shown that the hormonally active forms of vitamin D and vitamin A are potent inducers of AR in LNCaP cells. Both 1,25-$(OH)_2D_3$ and 9-cis RA act in synergy to inhibit cell proliferation; moreover, their antiproliferative actions can be blocked by the AR antagonist, Casodex. In conclusion, our study provides direct evidence for an important role of the AR in mediating the growth inhibitory actions of 1,25-$(OH)_2D_3$ and 9-cis RA in LNCaP cells. More importantly, the newly discovered AR-inducing property of both vitamins A and D suggests a possible application of these potential chemo-preventive agents in increasing androgen sensitivity of prostate cancer cells. An understanding of the mechanisms of AR gene

regulation may be of great importance in efforts to restore androgen responsiveness to the patients with androgen-independent prostate cancer, because this type of cancer is commonly unresponsive to most conventional therapies.

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Liarozole Acts Synergistically with 1α,25-Dihydroxyvitamin D_3 to Inhibit Growth of DU 145 Human Prostate Cancer Cells by Blocking 24-Hydroxylase Activity*

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ABSTRACT

1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits the proliferation of many cancer cells in culture, but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-$(OH)_2D_3$-resistant phenotype in DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-$(OH)_2D_3$. As this P450 enzyme initiates 1,25-$(OH)_2D_3$ catabolism, we presumed that a high level of enzyme induction could limit the effectiveness of the 1,25-$(OH)_2D_3$ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. As imidizole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing the 1,25-$(OH)_2D_3$ half-life, thereby enhancing 1,25-$(OH)_2D_3$ antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. When used alone, neither 1,25-$(OH)_2D_3$ (1–10 nM) nor liarozole (1–10 μM) inhibited DU 145 cell growth. However, when added together, 1,25-$(OH)_2D_3$ (10 nM)/liarozole (1 μM) inhibited growth 65% after 4 days of culture. We used a TLC method to assess 24-hydroxylase activity and demonstrated that liarozole (1–100 μM) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-$(OH)_2D_3$ half-life from 11 to 31 h. In addition, 1,25-$(OH)_2D_3$ can cause homologous up-regulation of the vitamin D receptor (VDR), and in the presence of liarozole, this effect was amplified, thus enhancing 1,25-$(OH)_2D_3$ activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-$(OH)_2D_3$/liarozole showed greater VDR up-regulation than cells treated with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-$(OH)_2D_3$ to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity, leading to increased 1,25-$(OH)_2D_3$ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-$(OH)_2D_3$ and liarozole or other inhibitors of 24-hydroxylase, both in nontoxic doses, might serve as an effective treatment for prostate cancer. (*Endocrinology* **140:** 2071–2076, 1999)

THE MAJOR biological action of 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the active metabolite of vitamin D, is to maintain calcium homeostasis in the body (1). Recent findings indicate that 1,25-$(OH)_2D_3$ is also involved in regulating cellular proliferation and differentiation in various target tissues that possess vitamin D receptors (VDR) (1–4). 1,25-$(OH)_2D_3$ and less calcemic analogs have been shown to inhibit cell growth in both human prostate carcinoma cell lines (5–11) and primary cultures of normal and prostate cancers (12). However, 1,25-$(OH)_2D_3$ showed only minimal inhibition of cell proliferation of DU 145, a human prostate cancer cell line derived from a brain metastasis, despite the presence of substantial amounts of VDR in this cell type (5, 7). The mechanism for the relative unresponsiveness of DU 145 to the antiproliferative action of 1,25-$(OH)_2D_3$ is not known.

DU 145 cells have been shown to express high levels of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) after treatment with 1,25-$(OH)_2D_3$ (5, 7). LNCaP cells can be induced to express low levels of 24-hydroxylase activity [12.6 ± 3.1 × 10^{-9} μmol/2 × 10^6 cells·30 min of 24,25$(OH)_2D_3$ produced] and are substantially growth inhibited by 1,25-$(OH)_2D_3$, whereas DU 145 cells can be induced to express very high levels of 24-hydroxylase activity (96.7 ± 39.5 × 10^{-9} μmol/2 × 10^6 cells·30 min) and are minimally growth inhibited (5, 7). As this P450 enzyme initiates the 1,25-$(OH)_2D_3$ inactivation pathway (1), we (8) and others (7, 13) have considered the possibility that rapid breakdown of 1,25-$(OH)_2D_3$ by 24-hydroxylase might be the cause of the resistant phenotype in DU 145 cells. In this study, we examine the premise that combination treatment with 1,25-$(OH)_2D_3$ and an inhibitor of 24-hydroxylase might render DU 145 cells more sensitive to the antiproliferative action of 1,25-$(OH)_2D_3$.

Combination therapy is often used to enhance the anticancer activity of various agents. Ketoconazole, liarozole, and other inhibitors of P450 enzymes may exhibit anticancer properties via several pathways, including actions on critical enzyme pathways (13, 14). In this study, we examined the possibility that combination treatment with 1,25-$(OH)_2D_3$ and liarozole, an imidazole derivative with antiprostate cancer properties (15, 16), might result in enhanced growth inhibition of DU 145 cells. Liarozole is known to inhibit

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several cytochrome P-450 enzymes, including retinoic acid 4-hydroxylase and aromatase (15, 17, 18). It is suspected that the former activity prolongs the half-life of retinoic acid and thereby increases the antiproliferative activity of endogenous retinoic acid when liarozole is administered to patients with prostate cancer (15, 16, 18). Here we show that 1,25-$(OH)_2D_3$ and liarozole interact synergistically to inhibit DU 145 cell growth. Our data demonstrate, for the first time, the ability of liarozole to directly inhibit 24-hydroxylase activity. The mechanism of liarozole action on DU 145 cells appears to be via inhibition of 24-hydroxylase, which causes a dual effect to prolong 1,25-$(OH)_2D_3$ half-life and to enhance up-regulation of VDR levels. Additional mechanisms may also play a role.

Materials and Methods

Materials

25-Hydroxy-[^{3}H]vitamin D_3 (SA, 12.7 Ci/mmol) was obtained from Amersham Chemical Co. (Arlington Heights, IL). Liarozole fumarate (5-[(3-chlorophenyl)(1*H*-imidazol-1-yl)methyl]1*H*-benzimidazole fumarate) was a gift from Dr. C. Bowden (Janssen Research Foundation, Spring House, PA), and 1,25-$(OH)_2D_3$ was a gift from Dr. M. Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture media were purchased from Mediatech (Herndon, VA). FBS was obtained from Life Technologies (Gaithersburg, MD). CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS reagent) was purchased from Promega Corp. (Madison, WI). Silica gel TLC plates were purchased from E. M. Science (Darmstadt, Germany). All other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

The DU 145 human prostate carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely cultured in RPMI 1640 medium supplemented with 5% FBS and antibiotics at 37 C in a humidified atmosphere of 5% CO_2.

Assay of cell growth

Cell growth was assessed by measurement of viable cells using the MTS assay. DU 145 cells were trypsinized and seeded at a density of approximately 2,000 cells/well in 96-well tissue culture plates (Falcon, Lincoln Park, NJ) in 200 μl culture medium. The cells were allowed to attach for 24 h, and the medium was replaced with fresh medium containing 5% FBS. Cells were then treated with vehicle (ethanol), 1,25-$(OH)_2D_3$, and/or liarozole. Triplicate wells were used for each experimental condition. The medium containing vehicle or test compounds was renewed every 2 days during the course of the experiment. After the appropriate incubation period, the cells were processed by replacing them with fresh RPMI 1640 medium containing MTS reagent (100 μl medium plus 20 μl MTS reagent/well). The plates were incubated at 37 C in a humidified atmosphere of 5% CO_2 for approximately 3–4 h. The absorbance at 490 nm was read using an automatic plate reader (Emax Precision Microplate Reader, Molecular Devices, Menlo Park, CA) and was linear up to the highest cell concentration tested (40,000 cells/well).

Induction of 24-hydroxylase activity

24-Hydroxylase enzyme activity was assayed in a cell suspension system slightly modified from the method previously described (19). A 100-mm^2 confluent DU 145 culture, growing under standard conditions, was treated for various times (0.5, 3, 6, 16, and 20 h) with either vehicle (ethanol) or 10 nM 1,25-$(OH)_2D_3$. Cells were then rinsed with 10 ml PBS and incubated with 10 ml culture medium at 37 C in a humidified atmosphere of 5% CO_2 for approximately 30 min to remove 1,25-$(OH)_2D_3$. Cells were then trypsinized and resuspended at 10^6 cells/200 μl RPMI 1640 containing 10 mM HEPES with 1% FBS. The cells were incubated for 30 min at 37 C with 1.0 nM [^{3}H]25-OHD_3 and 1.0 μM 25-$(OH)D_3$. The reaction was terminated by the addition of 750 μl methanol-chloroform (2:1) and 20 μl 24,25-$(OH)_2D_3$. The metabolites were extracted three times with 200 μl chloroform. The organic extracts were combined, dried with a Speed-Vac (Savant Instruments, Farmingdale, NY) and dissolved in a 90:10 mixture of hexane-isopropanol. The production of [^{3}H]24,25-$(OH)_2D_3$ was quantitated by TLC on silica gel/aluminum foil plates developed in methylene chloride-ethyl acetate (1:1) run with authentic standards. The TLC strips were cut into 14 fractions and placed individually in minicounting vials. This TLC system produced good separation of [^{3}H]25-$(OH)_2D_3$ from [^{3}H]24,25-$(OH)_2D_3$.

Inhibition of 24-hydroxylase activity

Time-course studies indicate that induction of 24-hydroxylase activity could be detected at 3 h by 10 nM 1,25-$(OH)_2D_3$ treatment with a plateau at approximately 20 h. Therefore, the conditions selected for studying the inhibition of 24-hydroxylase activity by liarozole were 20-h induction, 10^6 cells, 1 nM [^{3}H]25-$(OH)D_3$, and 30-min incubation with various concentrations of liarozole (1, 10, 50, and 100 μM).

Determination of 1,25-$(OH)_2D_3$ half-life

The half-life of 1,25-$(OH)_2D_3$ in DU 145 cells was determined by measuring the residual unmetabolized [^{3}H]1,25-$(OH)_2D_3$ in the conditioned medium at various time points after addition. Confluent DU 145 cells were treated for various times with [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of liarozole. Two hundred microliters of conditioned medium were mixed with 750 μl methanol-chloroform (2:1), and the metabolites were extracted with 200 μl chloroform. Chloroform extraction was repeated three times. The organic extracts were dried with a Speed-Vac and dissolved in a 90:10 mixture of hexane-isopropanol. The disappearance of [^{3}H]1,25-$(OH)_2D_3$ and the production of [^{3}H]1,24,25-$(OH)_3D_3$ were quantitated by a TLC system using methylene chloride-ethyl acetate (1:3). After 145 min of development, the TLC strips were dried and fractionated by cutting regions identified as 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ by comigration of authentic standards. This TLC system gave good separation between [^{3}H]1,25-$(OH)_2D_3$ and [^{3}H]1,24,25-$(OH)_3D_3$. The Rf value for 1,25-$(OH)_2D_3$ was 0.667, and that for 1,24,25-$(OH)_3D_3$ was 0.333.

Western blot analysis of vitamin D receptor (VDR)

Cell monolayers grown in RPMI 1640 supplemented with 5% charcoal-stripped serum in 100-mm dishes were incubated with ethanol vehicle, 1,25-$(OH)_2D_3$ (0.1, 1, and 10 nM), and/or liarozole (10 μM) for 4 days. After 4 days of incubation, cells were harvested, and Western blot analysis was performed as described previously using anti-VDR monoclonal antibody (9A7) (20). The experiment was repeated twice with similar results.

Results

Combination effect of 1,25-$(OH)_2D_3$ and liarozole on DU 145 cell growth

DU 145 cells are only minimally responsive to the antiproliferative effect of 1,25-$(OH)_2D_3$ (5, 7, 8). In our current studies, DU 145 cells were treated with increasing concentrations of 1,25-$(OH)_2D_3$ (1, 10, and 100 nM) over a time course of 2, 4, and 6 days (Fig. 1, A and B). The growth of DU 145 cells was not significantly inhibited by the lower concentrations of 1 and 10 nM; however, at the highest concentration (100 nM), there was a slight growth inhibition of approximately 20% on day 6. Similarly, as shown in Fig. 1, C and D, liarozole failed to inhibit the proliferation of DU 145 cells at 1 and 10 μM, but 100 μM resulted in 50% growth inhibition at 4 days and 60% growth inhibition at 6 days. However, 100 μM liarozole is a toxic dose and when administered to patients at these concentrations it causes hypervi-

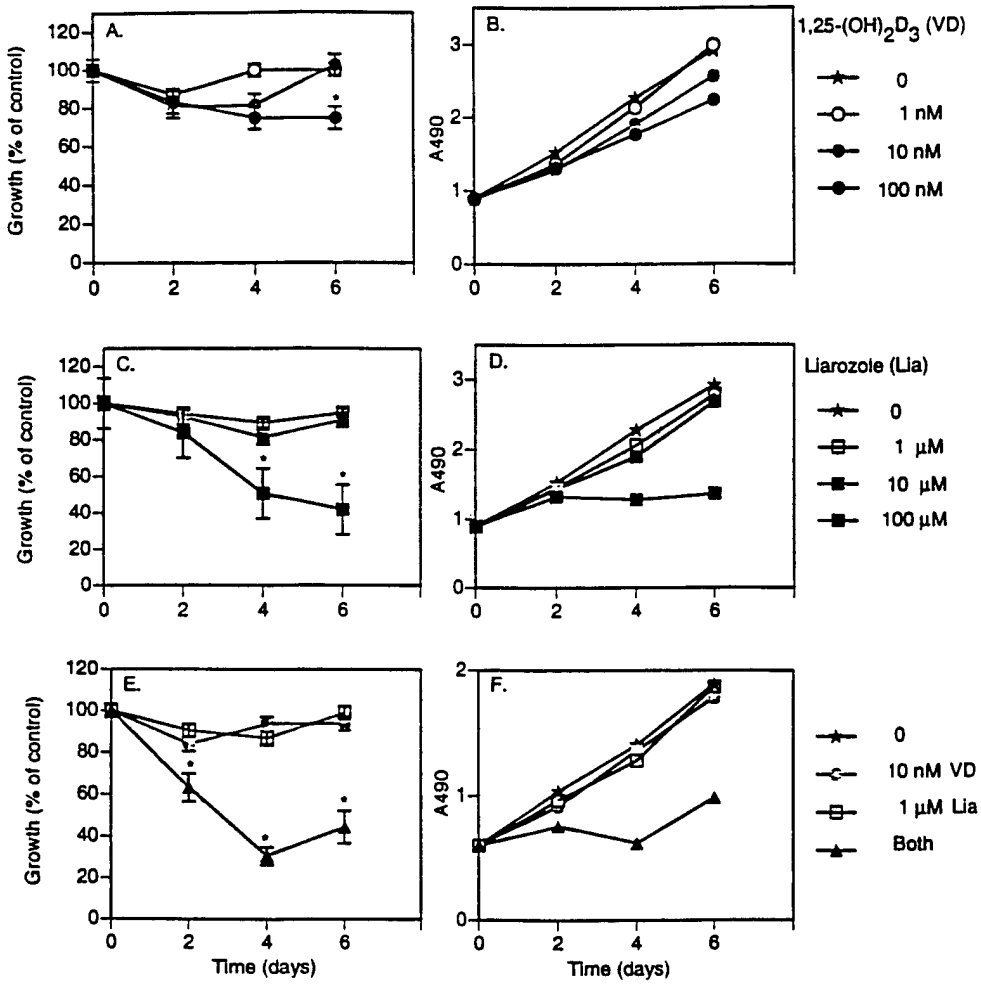

FIG. 1. Dose-response effect of 1,25-$(OH)_2D_3$, liarozole, and the combination on DU 145 cell growth over a time course of 6 days. Cells were plated at approximately 2000 cells/well in 96-well tissue culture plates in 200 μl medium with the indicated concentrations of hormone. Media were changed every 2 days. Cell proliferation was estimated using the MTS assay. Data are expressed as the mean ± SD (n = 3) in the *left panels*. The *right panels* show a single representative experiment comparing treatment to vehicle and expressed in absorbance units. *, Significant changes ($P < 0.05$) compared with the ethanol control. A and B, Treatment with 1,25-$(OH)_2D_3$. C and D, Treatment with liarozole. E and F, Treatment with a combination of 1,25-$(OH)_2D_3$ (10 nM) and liarozole (1 μM).

taminosis A. Neither 10 nM 1,25-$(OH)_2D_3$ nor 1 μM liarozole had any antiproliferative effect when used alone. However as shown in Fig. 1, E and F, the combination treatment caused 60% growth inhibition. These data indicate that 1,25-$(OH)_2D_3$ and liarozole interact synergistically to inhibit DU 145 cell growth.

Inhibition of 24-hydroxylase activity by liarozole

We next investigated the possible mechanisms by which liarozole enhanced the ability of 1,25-$(OH)_2D_3$ to inhibit DU 145 cell proliferation. As shown in many other cell culture systems, we found here that 1,25-$(OH)_2D_3$ induced 24-hydroxylase activity in DU 145 cells in a time-dependent manner (Fig. 2). The level of 24-hydroxylase activity in DU 145 cells is much higher than that in other cell types, particularly compared with LNCaP cells, which are substantially inhibited by 1,25-$(OH)_2D_3$ alone (5, 7). Liarozole had no intrinsic ability to induce 24-hydroxylase activity. However, liarozole can inhibit 24-hydroxylase activity. After treating cells with 10 nM 1,25-$(OH)_2D_3$ for 20 h to induce 24-hydroxylase activity, we examined the abilities of various concentrations of liarozole to inhibit enzyme activity by blocking the conversion of [^{3}H]25-OHD_3 to 24,25-$(OH)_2D_3$. As shown in Fig. 3, liarozole (1, 10, 50, and 100 μM) was able to directly inhibit 24-hydroxylase activity in a dose-dependent manner, such that 10 μM liarozole resulted in approximately 80% inhibition of enzyme activity compared with the activity of the induced cells in the absence of liarozole.

Effect of liarozole on 1,25-$(OH)_2D_3$ half-life

As our data indicated that liarozole was capable of directly inhibiting 24-hydroxylase activity, the enzyme involved in the first step of 1,25-$(OH)_2D_3$ inactivation, we next investigated the effect of liarozole on the 1,25-$(OH)_2D_3$ half-life. We treated two group of cells at various times, one with the single addition of 10 nM 1,25-$(OH)_2D_3$ and the other with a combination of 10 nM 1,25-$(OH)_2D_3$ and 1 μM liarozole. As anticipated, in cultures treated with 1,25-$(OH)_2D_3$ alone, the half-life of 1,25-$(OH)_2D_3$ was shorter (~10 h) compared with that of cells treated with the combination (~30 h; Fig. 4). Therefore, these data are consistent with the observation that liarozole directly inhibits 24-hydroxylase activity, thereby prolonging 1,25-$(OH)_2D_3$ half-life. This finding provides one possible mechanism for the synergistic growth inhibitory effect of combination therapy with 1,25-$(OH)_2D_3$ and liarozole.

Effect of 1,25-$(OH)_2D_3$ and liarozole on VDR protein level

As previously shown in other cell culture systems, 1,25-$(OH)_2D_3$ and other vitamin D analogs induce homologous

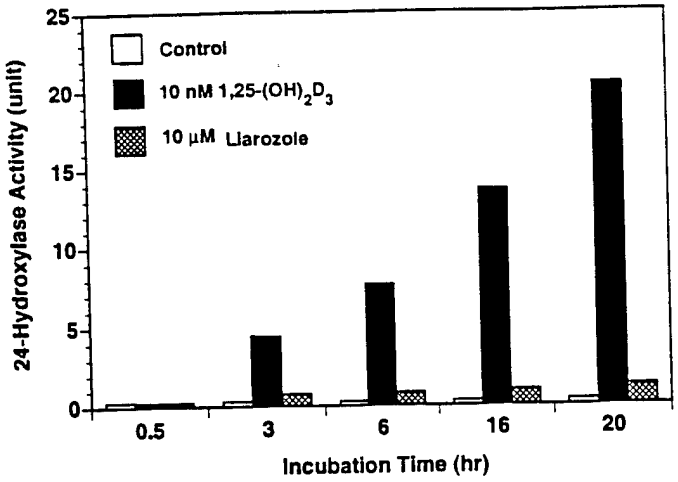

FIG. 2. Time course of the effect of 1,25-$(OH)_2D_3$ or liarozole on 24-hydroxylase activity in DU 145 cells. Cells were treated with 10 nM 1,25-$(OH)_2D_3$, 10 μM liarozole, or ethanol vehicle, and enzyme activity was measured at 0.5, 3, 6, 16, and 20 h. At 20 h, 10 nM 1,25-$(OH)_2D_3$ induced a 27-fold rise in 24-hydroxylase activity compared with the effect of vehicle. This is a representative experiment that was performed twice with similar results.

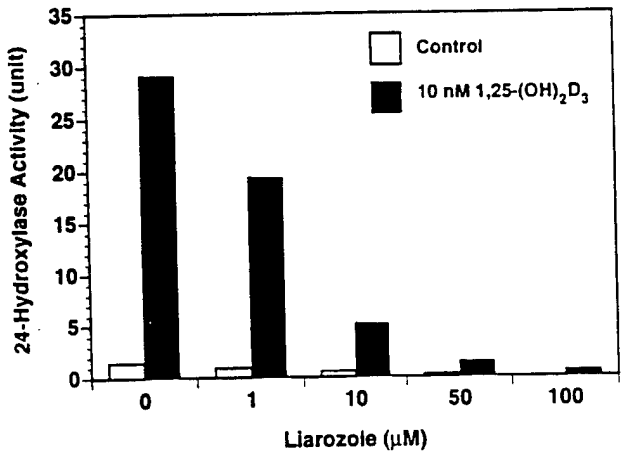

FIG. 3. Dose-dependent effect of liarozole on 24-hydroxylase activity in DU 145 cells. Cells were treated with 10 nM 1,25-$(OH)_2D_3$ for 20 h. Treated cells were subsequently incubated with liarozole at various concentrations (0, 1, 10, 50, and 100 μM) for 30 min before enzyme activity was measured. This is a representative experiment that was performed three times with similar results.

up-regulation of the VDR (21, 22). We next investigated whether liarozole, by inhibiting 24-hydroxylase activity and prolonging 1,25-$(OH)_2D_3$ half-life, is also capable of augmenting VDR up-regulation. Cells were treated with various concentrations of 1,25-$(OH)_2D_3$ (0.1, 1, and 10 nM) with and without the addition of 10 μM liarozole over a time course of 4 days. Protein extracts were made from these treated cells and were subjected to Western blot analysis to evaluate VDR content. Using the monoclonal antibody 9A7, the 50-kDa VDR protein was visualized. As shown in Fig. 5A, 1,25-$(OH)_2D_3$ alone resulted in a slight up-regulation of the VDR protein level (2-fold). Liarozole alone had no significant effect on VDR abundance. However, the combination of 1,25-$(OH)_2D_3$ and liarozole resulted in a 1,25-$(OH)_2D_3$ dose-dependent increase in the VDR protein level. This effect was

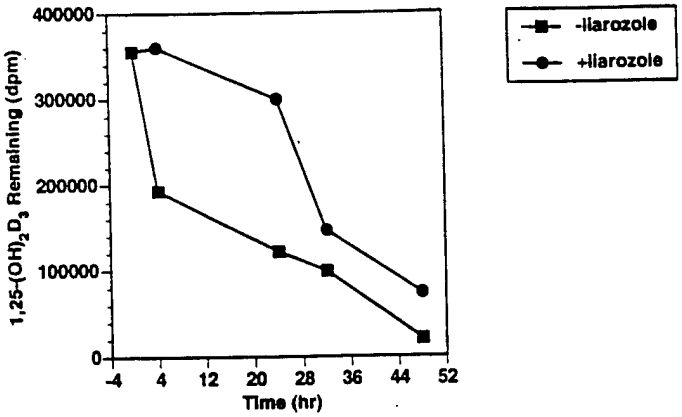

FIG. 4. Effect of liarozole on 1,25-$(OH)_2D_3$ half-life. DU 145 cells were incubated with [^{3}H]1,25-$(OH)_2D_3$ (0.5 nM) plus unlabeled 1,25-$(OH)_2D_3$ (10 nM) in the absence or presence of 10 μM liarozole. Conditioned media were collected at various time points (0, 4, 24, 32, and 48 h), and the residual amount of unmetabolized [^{3}H]1,25-$(OH)_2D_3$ was determined by TLC. This is a representative experiment performed twice with similar results.

observed most profoundly after a combination treatment with 10 nM 1,25-$(OH)_2D_3$ and 10 μM liarozole. This combination, as shown in Fig. 5B, resulted in a 5-fold increase in VDR abundance. Our data suggest that an increase in the VDR protein level may serve as a second and related mechanism, added to the prolonged 1,25-$(OH)_2D_3$ half-life, that contributes to the synergistic effect of 1,25-$(OH)_2D_3$ and liarozole inhibition of DU 145 cell growth.

Discussion

Our study was designed to investigate the combination of 1,25-$(OH)_2D_3$ and liarozole as a possible treatment for prostate cancer. We carried out our studies using an aggressive human prostate cancer cell line, DU 145, because of its resistance to the growth inhibitory effects of 1,25-$(OH)_2D_3$. We successfully inhibited DU 145 cell growth by 60% using the combination treatment of 1,25-$(OH)_2D_3$ and liarozole. In addition, we gained insight into the mechanism of the DU 145 cell unresponsiveness and the possibility of reversing the resistance with combination therapy.

As discussed earlier, at concentrations that are nontoxic, neither liarozole nor 1,25-$(OH)_2D_3$ alone substantially inhibited cell growth. Yet the combination of 1 μM liarozole and 10 nM 1,25-$(OH)_2D_3$ resulted in significant synergistic antiproliferative effects. Furthermore, this synergy is observed at a pharmacologically relevant concentration for both compounds. Based on this observation, we explored the possible mechanisms behind this synergy. We discovered that liarozole directly inhibited 24-hydroxylase activity in addition to inhibiting the already known P-450 enzymes, such as 4-hydroxylase and aromatase (15, 17, 18). As 24-hydroxylase is the initial enzyme for inactivating 1,25-$(OH)_2D_3$, we measured 1,25-$(OH)_2D_3$ half-life, and indeed, it was prolonged from 11 to 31 h. Therefore, by preventing rapid inactivation of 1,25-$(OH)_2D_3$ and prolonging the exposure time of cells to active hormone, DU 145 cells were able to respond to its antiproliferative effect. This provided the first possible mechanism for the synergistic activity of the 1,25-$(OH)_2D_3$/liarozole combination.

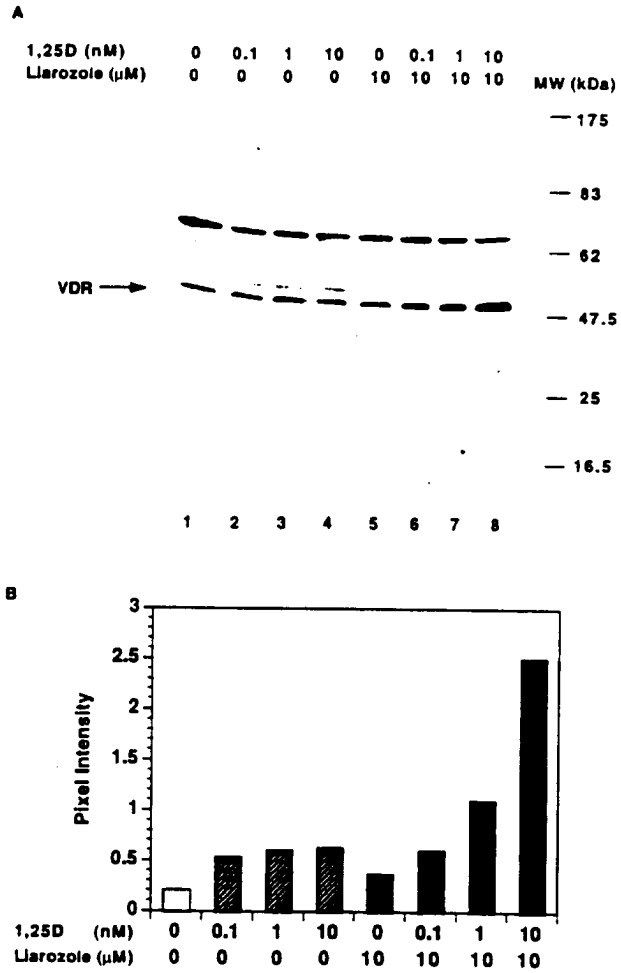

FIG. 5. A, Western blot analysis of VDR levels in DU 145 cells in response to various treatments. Cells were treated with ethanol or increasing concentrations of 1,25-$(OH)_2D_3$, liarozole, or 1,25-$(OH)_2D_3$ and liarozole for 4 days. High salt extracts were prepared, and 100 μg protein were loaded onto an 8% SDS-PAGE. After gel transfer, the blot was probed with anti-VDR monoclonal antibody 9A7, and the signal was detected using the enhanced chemiluminescence method. B, Densitometric analysis of Western blot. The pixel intensities of VDR bands were quantitated using a laser densitometer.

It is known that receptor regulation is an important mechanism for modulating target cell responsiveness to hormone (20, 23, 24). We explored the possibility that homologous up-regulation of the VDR would be enhanced in the presence of liarozole. Western blot analysis demonstrated a 5-fold increase in VDR protein level when cells were treated with 10 nM 1,25-$(OH)_2D_3$ and 10 μM liarozole compared with that after treatment with 1,25-$(OH)_2D_3$ alone. DU 145 cells treated with 1,25-$(OH)_2D_3$ alone only demonstrated a slight up-regulation of the VDR protein level. The possible explanation for this observation is that 1,25-$(OH)_2D_3$ is a potent stimulus of 24-hydroxylase activity; therefore, this would induce rapid degradation of 1,25-$(OH)_2D_3$, causing only a transient homologous up-regulation of VDR in DU 145 cells. Augmentation of VDR up-regulation has previously been reported using ketoconazole to inhibit 24-hydroxylase in a similar manner (25, 26). Enhanced VDR up-regulation is the second contributing mechanism explaining the liarozole synergistic interaction with 1,25-$(OH)_2D_3$. The increase in both ligand and receptor is a plausible mechanism for the enhanced antiproliferative activity of the 1,25-$(OH)_2D_3$/liarozole combination therapy in DU 145 cells. It is of interest that analogs of 1,25-$(OH)_2D_3$ designed to prevent 24-hydroxylation, such as 19-nor-25,26-hexafluoro-1,25-$(OH)_2D_3$, inhibit DU145 cell proliferation (9).

Our data suggest that DU 145 cells are more responsive to the antiproliferative effect of 1,25-$(OH)_2D_3$ when both its hormone and receptor are increased. Although the presence of VDR is essential for 1,25-$(OH)_2D_3$ activity (27), the level of VDR abundance in different prostate cancer cell lines by itself is not necessarily predictive of the amplitude of hormonal response (5, 28). However, in a given cell, increased abundance of receptor does appear to predict the extent of hormonal responsiveness, and increased or decreased receptor levels are usually correlated with increased and decreased responsiveness, respectively (20, 23, 24).

It should be noted that Zhao *et al.* have shown that combination therapy with either ketoconazole or liarozole and 1,25-$(OH)_2D_3$ or its analogs is cell type specific (13). That finding supports the concept that differences in cellular metabolism can at least partially explain the different potencies of various vitamin D analogs and differences in antiproliferative activity between different cancer cells. The fact that some cells are substantially growth inhibited by 1,25-$(OH)_2D_3$ alone (LNCaP and primary cultures) and other cells are not (DU 145) depends on a combination of factors, including, but not limited to, VDR abundance and inducible 24-hydroxylase activity (5, 7, 28). Liarozole in combination with 1,25-$(OH)_2D_3$ improves both parameters; by increasing VDR abundance and inhibiting 24-hydroxylase activity, it allows the otherwise resistant DU 145 cell to be growth arrested by 1,25-$(OH)_2D_3$. In preliminary experiments, liarozole also augmented the growth inhibitory activity of 1,25-$(OH)_2D_3$ in PC-3 and LNCaP cells, but to a much lesser extent (data not shown) than shown here for DU145 cells. The smaller augmentation was probably due to the greater antiproliferative activity of 1,25-$(OH)_2D_3$ alone in these cells (5) as well as the lesser induction of 24-hydroxylase in these cell lines (7), making the liarozole action to inhibit 24-hydroxylase less essential for 1,25-$(OH)_2D_3$-mediated growth inhibition.

Another possible mechanism for the enhanced antiproliferative effect in the presence of liarozole is its ability to inhibit retinoid metabolism, leading to increased retinoid levels (15, 18, 29). Retinoids have been shown to inhibit various cancer cell lines, including prostate cancer (30), and to be synergistic with 1,25-$(OH)_2D_3$ in inhibiting prostate cancer cell growth (10, 31). In fact, the beneficial effect of liarozole in patients with prostate cancer is attributed to this activity (15, 18, 29). We investigated the possibility that this activity might be contributing to the growth inhibition in our experiments. We treated DU 145 cells with a combination of 1,25-$(OH)_2D_3$ and increasing concentrations of retinoic acid to mimic the liarozole effect. We observed only a slight enhancement of growth inhibition (data not shown). As liarozole alone had no antiproliferative activity, and retinoids were not added in our standard combination experiments, we believe that the inhibition of retinoid metabolism does not substantially contribute to the effects that we have seen in cultured cells.

However, in patients, the ability of liarozole to inhibit retinoid metabolism would be expected to further enhance the synergistic activity that we have demonstrated.

The mechanism(s) by which 1,25-$(OH)_2D_3$ inhibits the growth of prostate cancer cells is complex, multifactorial, and different in different cell lines. Several investigators have reported that treatment with 1,25-$(OH)_2D_3$ causes LNCaP cells to accumulate in the G_1 phase of the cell cycle (10, 11). 1,25-$(OH)_2D_3$ also elicits a reduction of cyclin-dependent kinase 2 activity and an increase in the level of hypophosphorylated retinoblastoma (Rb) protein, which is a critical regulator of the G_1/S checkpoint (11). Interestingly, DU 145 cells lack functional Rb protein. However, ectopic expression of functional Rb in DU 145 cells was not sufficient to restore the growth response to 1,25-$(OH)_2D_3$ (32). Others have found that growth inhibition of prostate cancer cells by a potent vitamin D analog involves the induction of $p21^{waf1}$, $p27^{kip1}$, and E-cadherin (9). In addition, we have demonstrated that 1,25-$(OH)_2D_3$ significantly regulates androgen receptor gene expression, which contributes to the regulation of LNCaP cell growth (33). Therefore, the mechanism by which 1,25-$(OH)_2D_3$ inhibits cell proliferation involves multiple signaling pathways and differs in various prostate cancer cell lines.

In summary, our data suggest that liarozole directly inhibits 24-hydroxylase activity, thereby effectively prolonging the 1,25-$(OH)_2D_3$ half-life. The increase in the 1,25-$(OH)_2D_3$ half-life resulted in enhanced up-regulation of VDR protein levels. We believe that this combination of increased 1,25-$(OH)_2D_3$ hormone levels as well as augmented VDR abundance represents the principal mechanism for the synergistic effect of 1,25-$(OH)_2D_3$ and liarozole in our experiments. However, additional mechanisms may play a role in the synergistic effect of this combination in DU 145 cells. In conclusion, the novel combination of liarozole and 1,25-$(OH)_2D_3$ therapy may serve as an effective treatment regimen for prostate cancer patients.

Acknowledgments

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mechanism of action of AA, the effect of AA on PKC activity was examined by adding inhibitors of PKA (H8 and H89), lipoxygenase (NDGA and esculetin), or cyclooxygenase (indomethacin) to the cultures in the presence or absence of 24,25. The identification of which PKC isoform is activated by AA was determined by PKC isoform-specific antibodies. AA stimulated [^{3}H]-thymidine incorporation and inhibited the activity of alkaline phosphatase and PKC, but had no effect on matrix production. In contrast, 24,25 inhibited [^{3}H]-thymidine incorporation and stimulated alkaline phosphatase and PKC as well as matrix production. In cultures treated with both agents, the effects of 24,25 were abrogated by AA. Studies using cyclooxygenase and lipoxygenase inhibitors indicated that the effects of AA were due in part to prostaglandins but not leukotrienes. AA did not alter the translocation of PKC from the cytosol to the membrane. Direct addition of AA to isolated matrix vesicles activated PKC, while addition to plasma membranes inhibited PKC. This was opposite to the effect seen with 24,25; in addition, when added to the membranes at the same time, AA abrogated the effect of 24,25. Studies with PKC isoform-specific antibodies indicated that the predominant PKC isoform affected by AA treatment was PKCα. This study shows that AA regulates RC proliferation, differentiation, and matrix production. The effect of AA on the cultures was the opposite of that observed with 24,25. We conclude that 24,25 mediates its effects on RC cells by inhibiting AA production. AA also had a direct effect on membranes isolated from the cultures, suggesting that there may be a unique membrane receptor for AA.

T267

Liarozole Acts Synergistically With 1,25-Dihydroxyvitamin D3 to Inhibit DU 145 Human Prostate Cancer Cell Growth by Blocking 24-Hydroxylase Activity. X. Y. Zhao, Lan H. Ly, Leah Holloway, David Feldman. Department of Medicine, Stanford University, Stanford, CA.

1α,25-Dihydroxyvitamin D3 [1,25-(OH)2D3] inhibits the proliferation of many cancer cells in culture but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-(OH)2D3-resistant phenotype of DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-(OH)2D3. Since this P450 enzyme initiates 1,25-(OH)2D3 catabolism, we presumed a high level of enzyme induction could limit the effectiveness of the 1,25-(OH)2D3 antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. Since imidizole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing 1,25-(OH)2D3 half-life, thereby enhancing 1,25-(OH)2D3 antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. Neither 1,25-(OH)2D3 (1-10 nM) nor liarozole (1-10 μM) inhibited DU 145 cell growth when given alone. However, together at 4 days, 1,25-(OH)2D3 (10 nM)/liarozole (1 μM) inhibited growth 65%. We used a thin layer chromatography method to assess 24-hydroxylase activity and demonstrated that liarozole (1-100 μM) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-(OH)2D3 half-life from 11 to 31 h. In addition, 1,25-(OH)2D3 can cause homologous up-regulation of the VDR and in the presence of liarozole, this effect was augmented thus enhancing 1,25-(OH)2D3 activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-(OH)2D3/liarozole showed greater VDR up-regulation than with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-(OH)2D3 to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity leading to increased 1,25-(OH)2D3 half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-(OH)2D3 and liarozole or other inhibitors of 24-hydroxylase, both in non-toxic doses, might serve as an effective treatment for prostate cancer.

T268

Expression of 11β-Hydroxysteroid Dehydrogenase Type 2 in Rat Osteosarcoma Cells: Autocrine Regulation of Glucocorticoid Responses in Bone. M. Hewison, L. J. Eyre,* R. Bland,* M. C. Sheppard,* P. M. Stewart.* Department of Medicine, The University of Birmingham, Birmingham, UK.

The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) acts as a prereceptor signaling mechanism for corticosteroids, by regulating the access of active glucocorticoids to both glucocorticoid (GR) and mineralocorticoid receptors (MR). To examine a possible relationship between endogenous glucocorticoid metabolism and osteoblast activity, we have characterized the expression of 11β-HSD isozymes in rat osteosarcoma cells. Analysis of mRNA from ROS 25/1, UMR106 and ROS 17/2.8 cells revealed transcripts for both 11β-HSD type 1 (11β-HSD1) and type 2 (11β-HSD2) in all three cell lines. However, enzyme activity studies showed only high affinity (Km = 45 nM) dehydrogenase activity (inactivation of corticosterone to 11-dehydrocorticosterone), characteristic of 11β-HSD2. Conversion of corticosterone to 11-dehydrocorticosterone in ROS 25/1 > UMR106 > ROS 17/2.8 cells. Activity in all three cell lines was up-regulated in an autocrine fashion following treatment with natural glucocorticoids, but not dexamethasone (dex). Induction of 11β-HSD2 was unaffected by co-treatment with the GR antagonist RU486 or the MR antagonist RU752, but was completely inhibited by treatment with 11β-HSD inhibitor glycyrrhetinic acid. Analysis of ^{3}H-dex binding revealed approximately 60,000 GR/cell in all three cell lines, with much lower levels of MR detected using ^{3}H-aldosterone (aldo). Parallel studies of alkaline phosphatase activity indicated that dex and corticosterone stimulated activity in ROS 17/2.8 cells but had no effect on ROS 25/1 cells or UMR106; the classical MR ligand, aldo, was without effect in all three cell lines. Up-regulation of alkaline phosphatase activity in ROS 17/2.8 by dex was completely inhibited by RU486 whereas RU752 had no effect. In contrast, induction of alkaline phosphatase activity by corticosterone was inhibited by both RU486 and RU752, suggesting different receptor mechanisms for osteoblast responses to synthetic versus naturally occurring glucocorticoids. These studies demonstrate capacity for local inactivation of glucocorticoids in osteoblasts. The presence of GR and lack of functional response to aldo suggest that 11β-HSD2 in rat osteoblasts acts in a protective fashion by regulating GR occupancy; cells with relatively high levels of 11β-HSD2 activity (ROS 25/1 and UMR106) were insensitive to glucocorticoids, whilst cells with low levels demonstrated functional responses to both dex and corticosterone. We therefore postulate that pre-receptor regulation of ligand availability is a major determinant of glucocorticoid effects on bone.

T269

Glucocorticoids Inhibit the Expression of Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in Osteoblasts. Frederic Blanquaert, Ernesto Canalis. Saint Francis Hospital and Medical Center, Hartford, CT.

Glucocorticoids have profound effects on skeletal metabolism, and delay wound and possibly fracture healing. However, the mechanisms involved are not known. Hepatocyte growth factor/scatter factor (HGF/SF) plays a central role in tissue regeneration and healing and enhances the replication of cells of the osteoblastic lineage. Expression of HGF/SF in osteoblasts is limited, but it is enhanced by factors that accelerate fracture repair, confirming the role of HGF/SF in healing. We examined the effects of cortisol on HGF/SF expression in osteoblast enriched cells from 22 day fetal rat calvariae (Ob cells). Cortisol at 1 uM for 24 to 48 h decreased the basal expression of HGF/SF transcripts in Ob cells by approximately 50%. In contrast, fibroblast growth factor (FGF) -2 at 2 nM and platelet derived growth factor (PDGF) BB at 3 nM for 24 h significantly increased HGF/SF transcripts by 2 to 8 fold in osteoblast cultures. Cortisol at 1 uM significantly decreased the stimulatory effect of FGF-2 and PDGF BB on HGF/SF expression in Ob cells. An analogous effect was observed in the MC3T3 osteoblastic cell line. Cortisol also caused a time and dose dependent increase in the level of expression of c-met, a protooncogene encoding for the HGF/SF receptor. This effect was observed at cortisol concentrations of 10 to 1,000 nM and after 6 to 24 h cortisol increased c-met transcripts by 3 fold. In conclusion, FGF-2 and PDGF BB increase HGF/SF mRNA expression, and cortisol decreases basal and growth factor-induced HGF/SF transcripts. This effect may be critical to the inhibitory actions of glucocorticoids on fracture healing. Cortisol induction of c-met mRNA levels may be a compensatory mechanism to maintain HGF/SF function in osteoblasts in conditions of glucocorticoid excess.

T270

Calreticulin Regulates Integrin $\alpha_v\beta_3$ Response To 1α,25(OH)$_2$D$_3$. Zhijie Chang,[1] K. A. Hruska,[2] S. Dedhar, S. L. Teitelbaum,[2] F. P. Ross,[2] Xu Cao.[1] [1]Department of Pathology, University of Alabama, Birmingham, AL. [2]Washington University, St. Louis, MO.

1α,25(OH)$_2$D$_3$ plays an important role in osteoclast differentiation. Calreticulin, a calcium-binding protein, can activate gene transcription such as $\alpha_v\beta_3$. 1 α,25(OH)$_2$D$_3$ transactivition activity is modulated by calreticulin, which binds to the DNA binding domain of 1 α,25(OH)$_2$D$_3$ receptor.

In this study we demonstrated that calreticulin dose-dependently inhibited both VDR binding and its transactivation by transfection of integrin β_3 and VDRE and by gel-shift. Further more, we showed that over-expression of calreticulin blocks 1 α,25(OH)$_2$D$_3$-induced integrin β_3 mRNA level by Northern blot analysis. Whereas, the over-expression of anti-sense calreticulin cDNA enhanced β_3 mRNA levels in HD11 cells treated with 1 α,25(OH)$_2$D$_3$. To further examine the function of calreticulin in osteoclast differentiation, we constructed calreticulin-adenovirus expression vector which can express calreticulin at very high level in transfected osteoclast like cells. The bone resorption and cell attachment was inhibited in the osteoclast precursors infected with adenovirus bearing calreticulin, compared with cells infected with adenovirus alone. Finally, we use immunostaining assay to demonstrate that calreticulin was involved in VDR translocation into nucleus. In summary, we conclude that calreticulin inhibits integrin β_3 expression by direct interacting with VDR, and affects attachment and bone resorption mediated by osteoclasts.

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TWO MUTATIONS IDENTIFIED IN THE ANDROGEN RECEPTOR OF THE NEW HUMAN PROSTATE CANCER CELL LINE MDA PCA 2A

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ABSTRACT

Purpose: We have characterized the androgen receptor (AR) in a new human prostate cancer cell line, MDA PCa 2a, that has recently been established from a bone metastasis of a patient whose cancer exhibited androgen-independent growth.

Materials and Methods: Androgen responsiveness of these cells was assessed by measuring the effect of DHT and R1881 on cell growth and PSA secretion. Scatchard analysis was used to characterize the affinity and abundance of AR protein. Using a PCR based strategy, genomic DNA of the entire coding region of AR gene was sequenced to identify possible mutations.

Results: These cells express abundant AR (Nmax = 685 ± 149 fmol./mg. protein), but the AR binding affinity (K_d) for DHT is only 25 nM, ~50-fold lower affinity than the mutated AR in LNCaP prostate cancer cells (K_d = 0.5 nM) or the wildtype AR in MCF-7 breast cancer cells (K_d = 0.4 nM). Two mutations, L701H and T877A, were identified in the ligand binding domain of the AR gene. Compared with LNCaP cells, the new cell line is significantly less responsive to DHT and R1881 as well as to other androgens such as testosterone, androstenedione, and DHEA. Similar to LNCaP cells, the ligand specificity of the AR in MDA PCa 2a cells appears to be relaxed and non-androgens such as progesterone and estradiol act as agonists although with less potency than in LNCaP cells. Interestingly, in the absence of androgens, the new cell line expresses 15-fold higher baseline levels of PSA than LNCaP.

Conclusions: Two mutations were identified in the AR gene of the MDA PCa 2a cell line that are likely responsible for the decreased androgen sensitivity and altered ligand specificity observed in these cells. Thus, this new cell line with partial androgen responsiveness and PSA expression can serve as a functionally relevant model system of bone metastatic prostate cancer, and can be used to investigate the role of AR mutations in prostate cancer and its progression to androgen independence.

KEY WORDS: androgen receptor mutations, PSA, MDA PCa 2a, LNCaP, prostate

In recent years, prostate cancer has become the most common cancer and the second leading cause of cancer death in men in the United States. Since the growth of prostate cancer cells is initially androgen-dependent, androgen ablation therapy has been the most effective treatment for patients with metastatic prostate cancer. However, androgen ablation appears to be effective for only a limited duration of time due to the progression of prostate cancer from an androgen-dependent to an androgen-independent state.[1] The mechanism(s) underlying the transition to androgen-independence are not well-understood.

Androgen actions are mediated by the androgen receptor (AR), a member of the steroid-thyroid-retinoid receptor superfamily of ligand-dependent transcription factors. The AR has been implicated in the development and growth of prostate cancer as well as in its progression to an androgen-independent phenotype.[2] In vivo, androgens stimulate prostate cell growth and androgen withdrawal leads to tumor regression and apoptosis.[3] Recent findings indicate that the AR gene in some prostate cancers has altered expression or function due to amplification, overexpression, or mutations.[2] Mutations in the AR gene have so far been reported in 27 prostate cancer cases and most of them were found in metastases at the later stages of the disease.[4] Unfortunately, the biological properties of the majority of the mutant ARs discovered in metastatic prostate cancer specimens have not been characterized due to a limited supply of tissue. Therefore, AR-positive human prostate cancer cell lines derived from metastases can be a useful tool for such studies. For example, the LNCaP cell line has been an excellent model for prostate cancer, and the T877A AR mutation in these cells has been well characterized.[5] This mutation, which has been found in a proportion of different metastatic prostate cancer specimens,[6] confers upon the AR a broadening of ligand specificity and allows non-androgens and commonly used anti-androgens to act as AR agonists.[5]

Navone et al have established a new AR-positive cell line designated MDA PCa 2a from a bone metastasis of prostate carcinoma in a 63-year-old black male who died of complications from his cancer in 1995 following orchiectomy in 1993.[7] Although these cells express abundant AR protein of normal size,[7] our findings described here show that MDA PCa 2a cells are less responsive to androgens than LNCaP cells and that the AR exhibits a very low binding affinity for DHT. Therefore, we suspected that mutations in the ligand binding domain (LBD) of the AR gene would explain these findings. We used PCR to amplify and sequence the entire coding region of the AR gene and identified two mutations (L701H and T877A). Although double mutations in the AR have been reported six times,[4] to our knowledge this is the only report

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of a double mutation in the AR LBD of a human prostate cancer cell line derived from a bone metastasis and studied in culture. Steroid specificity of the doubly mutated AR is reduced and like LNCaP cells, MDA PCa 2a cells exhibit an agonist response to progesterone and estradiol. However, the double mutant AR is less responsive to androgens, antiandrogens, progesterone and estradiol than the singly mutated AR in LNCaP cells. Interestingly, the MDA PCa 2a cell line secrets 15-fold higher baseline levels of PSA than the LNCaP cell line.

MATERIALS AND METHODS

Materials. 5α-dihydro-[1α, 2α-^{3}H]-testosterone (DHT) (specific activity, 40 to 70 Ci/mmol.) was obtained from Amersham Chemical Co. (Arlington Heights, IL). Radioinert DHT, dehydroepiandrosterone (DHEA), progesterone, 17-β-estradiol, androstenedione (ASD), and testosterone (T) were ordered from Steraloids, Inc. (Wilton, NH). The synthetic androgen, methyltrienolone (R1881), was purchased from Dupont NEN Life Science Products (Boston, MA). Hydroxyflutamide and bicalutamide (Casodex or ICI17,334) were provided by Schering-Plough Corporation (Bloomfield, NJ) and Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK), respectively. Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tissue culture media RPMI-1640 and BRFF-HPC-1 were obtained from Mediatech (Herndon, VA) and Biological Research Faculty Facility, Inc. (Ijamsville, MD) respectively. Fetal bovine serum (FBS) and antibiotics were obtained from GIBCO/BRL (Gaithersburg, MD). Charcoal stripped FBS and other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. The human prostate carcinoma cell line LNCaP and human breast cancer cell line MCF-7 were obtained from the American Type Culture Collection (Rockville, MD). LNCaP and MCF-7 cells were routinely cultured in RPMI-1640 medium, supplemented with 5% FBS and antibiotics, at 37C in a humidified atmosphere of 5% CO_2. The human prostate cancer cell line MDA PCa 2a was maintained in BRFF-HPC-1 medium, supplemented with 20% FBS and gentamicin.

For growth experiments, cells were trypsinized and plated at an appropriate density in RPMI-1640 medium containing 5% charcoal-stripped FBS. Hormonal treatments were initiated one day after plating. Hormone stocks were prepared in 100% ethanol at a concentration 1000-fold higher than working concentrations. Fresh culture medium was premixed with hormone stock and then added to triplicate wells. Media and hormone were replenished every three days. Controls received ethanol vehicle at a volume equal to that in hormone-treated cells.

Assay of cell growth. Cell growth was assessed by measurement of attained cell mass using an assay of DNA content.[8] Cells were seeded in six-well tissue culture plates (Becton Dickinson & Co. Lincoln Park, NJ) at a density of 50,000 cells per well in 3 ml. of RPMI-1640 containing 5% FBS. After incubation for 24 hours, the medium was replaced with RPMI-1640 medium containing 5% charcoal-stripped FBS. Cells were treated with vehicle (ethanol, final concentration 0.1%), androgens or other ligands at indicated concentrations. Triplicate wells were used for each treatment. The medium containing vehicle or test compounds was subsequently changed every three days during the course of the experiment. On the 6th day, conditioned medium was collected for PSA analysis and cell monolayers processed for DNA assay. Cells were precipitated with ice-cold 5% trichloroacetic acid. After a wash with 100% ice-cold ethanol, the cell pellet was solubilized with 0.2 N NaOH and the DNA content determined using the diphenylamine assay of Burton.[9] DNA content of each treatment was derived from the mean value of the triplicate wells in one experiment. Each experiment was repeated two or more times.

Assay of PSA secretion. Both the LNCaP and MDA PCa 2a cell lines express PSA.[8] The conditioned media collected in cell growth assays were subjected to a low-speed centrifugation to remove cell debris. PSA levels in the supernatant were determined by the TOSOH assay, an automated immunoenzymometric assay system (TOSOH Medics Inc., Foster City, CA) as previously described.[8]

[^{3}H]DHT binding assay. Cells were cultured in medium containing FBS. Cell monolayers were harvested and high salt nuclear extracts were made as previously described.[8] Protein concentration of the extract was determined by the method of Bradford.[10] In a typical binding assay, 200 μl. of soluble extract (0.5 to 1 mg. protein/ml.) were incubated with 0 to 100 nM concentration of [^{3}H]DHT for 16 to 20 hours at 0C. Bound and free hormones were separated by hydroxylapatite.[8] Specific binding was calculated by subtracting nonspecific binding obtained in the presence of a 250-fold excess of radioinert DHT from the total binding measured in the absence of radioinert steroid. Data were expressed as fmoles of [^{3}H]DHT bound per mg. protein.

DNA sequence analysis. Genomic DNA was isolated from LNCaP cells or MDA PCa 2a cells using the QIAamp Blood Kit (QIAGEN Inc., Valencia, CA). One microgram of DNA was used for each PCR reaction. PCR was performed with AR intronic primers and run for 30 cycles according to the following conditions: 94C for 30 seconds, 60C for 1 minute, and 72C for 1 minute (5 minutes for the last extension). The AR intronic primers for each exon were designed as previously described[11] and synthesized by Operon Inc. (Foster City, CA). PCR products were subcloned into the pCR™ vector (Invitrogen Corp., San Diego, CA) and plasmids containing insert DNAs were sequenced on an ABI automatic sequencer by the protein and nucleic acid core facility (Stanford University School of Medicine) using either M13 reverse or forward primers. Sequencing data were analyzed using the Genetics Computer Group sequence analysis software package (Madison, WI). At least 3 independent clones and two different samples of DNA were tested. The same mutations were found each time.

Statistical analysis. ANOVA was used to assess statistical significance of difference. $p < 0.05$ was considered significant.

RESULTS

Effect of androgens on MDA PCa 2a and LNCaP cell growth. To test whether MDA PCa 2a cells are responsive to androgens, cell growth assays were performed. We used both the major prostatic androgen DHT and the synthetic androgen R1881. Cells were treated with increasing doses (0 to 10,000 nM) of DHT or R1881 for 6 days in RPMI-1640 medium containing 5% charcoal-stripped FBS. DNA content was determined from obtained cell mass. As shown in fig. 1, under the same experimental conditions, both MDA PCa 2a and LNCaP cells exhibited a biphasic growth response to DHT or R1881, with a growth-stimulatory effect at low concentrations and a reversal to a growth-inhibitory effect at higher concentrations. However, MDA PCa 2a cells were less responsive than LNCaP cells. For example, the maximal stimulation of LNCaP growth was seen at 0.1 nM for both DHT and R1881. In contrast, 0.1 nM DHT had no effect on the growth of MDA PCa 2a cells, with stimulation of growth evident only at 1000 nM DHT or 1 nM R1881. We observed a greater difference of growth response between LNCaP and MDA PCa 2a with DHT (1000-fold) than R1881 (10-fold). Some of this difference might result from DHT having a shorter half-life than R1881 in the culture medium. Decreased androgen sensitivity of MDA PCa 2a cells was also observed when cells were cultured in FBS-containing me-

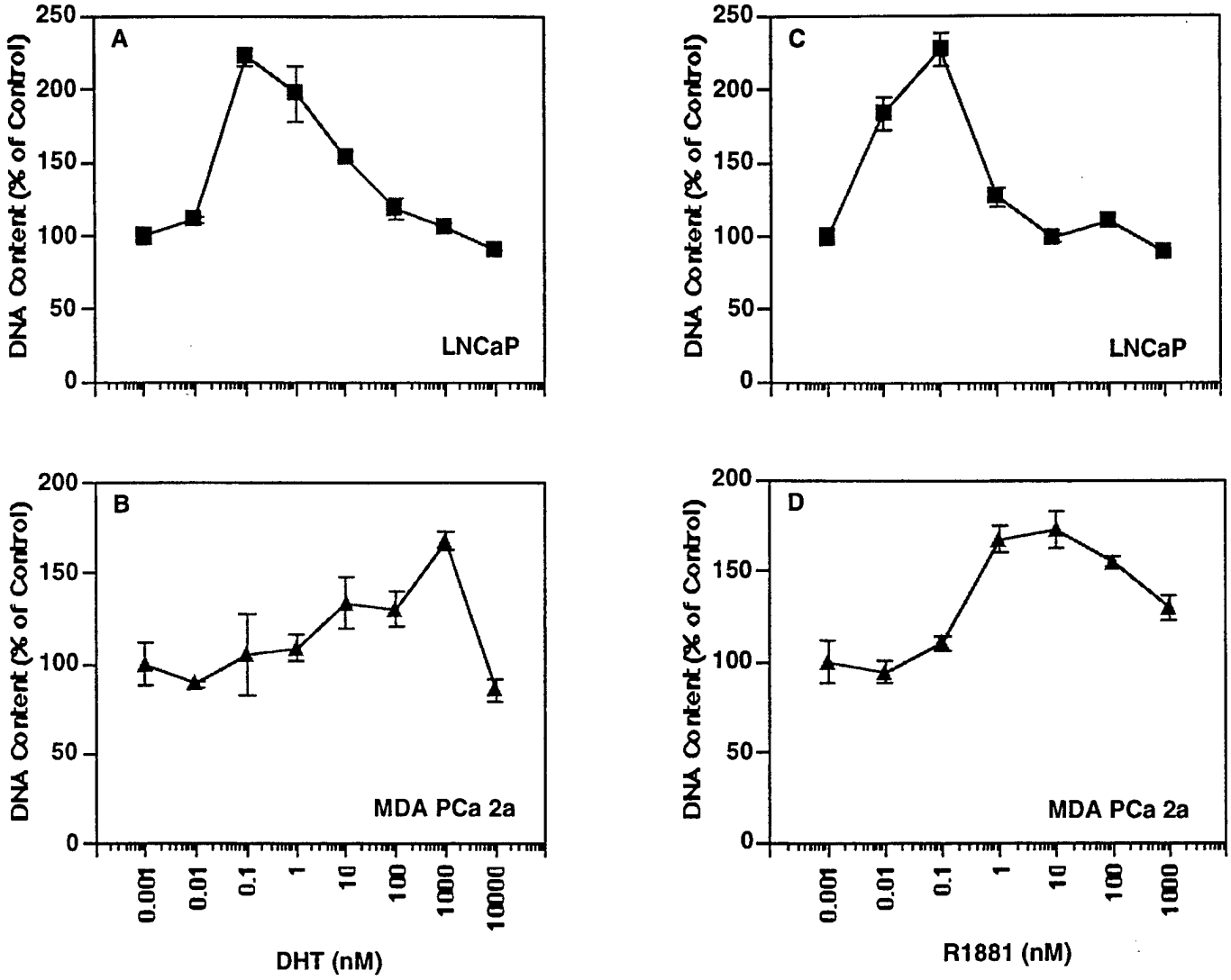

FIG. 1. Dose response effect of DHT or R1881 on growth of MDA PCa 2a and LNCaP cells in 6 day assay. Data are expressed as percent of control value. Control (100%) levels corresponded to 8.25 ± 0.47 μg. DNA per well for LNCaP cells and 2.496 ± 0.29 μg. DNA per well for MDA PCa 2a cells. *A*, growth response of LNCaP to DHT. *B*, growth response of MDA PCa 2a to DHT. *C*, growth response of LNCaP to R1881. *D*, growth response of MDA PCa 2a to R1881.

dium (data not shown). In summary, MDA PCa 2a cells were substantially (10 to 1,000-fold) less sensitive to androgens than LNCaP cells in growth assays.

[^{3}H]DHT-binding in MDA PCa 2a and LNCaP cells. Equilibrium ligand binding analyses were carried out on extracts of the MDA PCa 2a cell line in an effort to evaluate both the abundance and the affinity of the AR for [^{3}H]DHT. Figure 2 shows representative binding data and Scatchard analysis. Three independent experiments indicated that MDA PCa 2a cells expressed a higher abundance of AR (685 ± 149 fmol./mg. protein, n = 3) than LNCaP cells (457 fmol./mg. protein) or MCF-7 cells (30 fmol./mg. protein). However, ~50-fold decrease in the affinity of the receptor for DHT was found in MDA PCa 2a cells (K_d=25 ± 4.1 nM), when compared with LNCaP (K_d =0.5 nM) or the wildtype AR in MCF-7 human breast cancer cells (K_d =0.4 nM). The K_d values for LNCaP and MCF-7 obtained in our study are consistent with values reported previously.[12,13] Attempts to analyze the ligand binding specificity of the AR by competition binding assay were unsuccessful due to the low affinity for [^{3}H]DHT.

Sequence analysis of the AR gene in MDA PCa 2a and LNCaP cells. Because of reduced binding affinity for DHT and decreased responses to DHT in cell growth experiments, we suspected a mutation in the ligand binding domain of the AR gene. We therefore embarked on an analysis of the DNA sequence of the entire coding region of the AR gene. We amplified every exon (A-H) of the AR by PCR using genomic DNA extracted from both MDA PCa 2a and LNCaP cells. A comparison of the regions where the nucleotide sequences differed is shown in fig. 3. MDA PCa 2a cells exhibited a T to A missense mutation at position 2258 in exon D, which replaces a hydrophobic leucine residue with a hydrophilic histidine residue at amino acid 701 (L701H). This mutation was found only in MDA PCa 2a cells and not in LNCaP cells. In addition, a second mutation was identified in exon H in which an A to G mutation occurred at nucleotide position 2785 in DNA from both MDA PCa 2a and LNCaP cells. This alteration replaces a threonine with alanine at position 877 (T877A). This mutation previously has been described in the AR gene of LNCaP cells.[5] Sequencing of the opposite DNA strand confirmed these results for both mutations. All other nucleotides sequenced (exons A-H) were identical to wild type, indicating that the mutant AR in MDA PCa 2a cells differed from the normal AR only by these two separate base substitutions.

PSA stimulation in response to androgens in MDA PCa 2a and LNCaP cells. We next examined the androgen regulation of PSA secretion as another marker of androgen responsiveness. The level of PSA secretion by MDA PCa 2a and LNCaP cells was measured in the conditioned media collected from

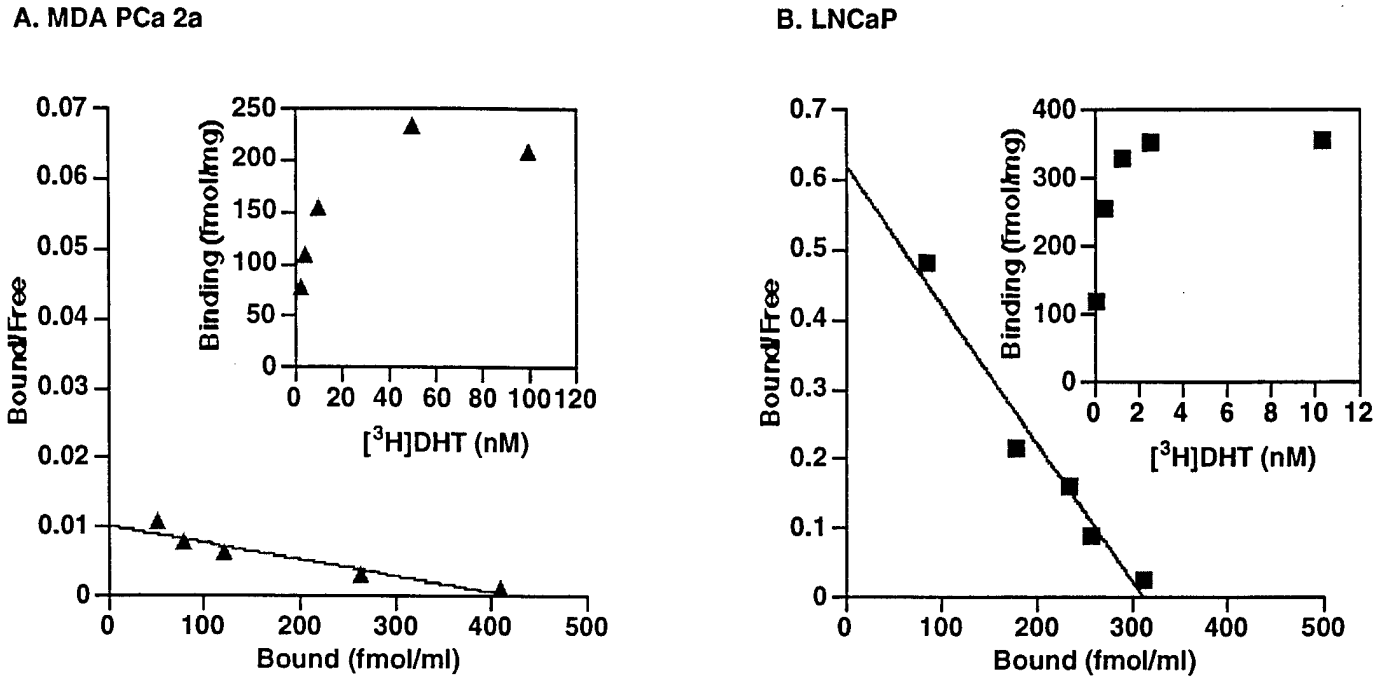

FIG. 2. Saturation analysis and Scatchard plot of specific [^{3}H]DHT binding in MDA PCa 2a and LNCaP cells. High salt protein extracts from MDA PCa 2a and LNCaP cells were incubated with various doses of [^{3}H]DHT at 0C in equilibrium binding assays as described in Materials and Methods. *A*, saturation plot of specific [^{3}H]DHT binding in MDA PCa 2a and Scatchard plot of the binding data. The protein concentration was 0.575 mg./ml. *B*, saturation plot of specific [^{3}H]DHT binding in LNCaP and Scatchard plot of the binding data. The protein concentration was 0.670 mg./ml.

the growth assays performed in fig. 1 and the values are expressed as ng. PSA/ml. of medium and shown in fig. 4. DHT stimulated PSA secretion by LNCaP cells in a dose-dependent manner, with a clear induction of PSA beginning at 0.1 nM DHT. In contrast, MDA PCa 2a cells were responsive to DHT only at concentrations of 100 nM or higher. A similar profile was observed when R1881 was used to treat the cells. The effective dose for the half maximal induction of PSA in LNCaP (EC_{50}) was 0.05 nM for DHT and 0.05 nM for R1881. The EC_{50} values in MDA PCa 2a cells were 40 nM for DHT and 0.2 nM for R1881. Thus, the androgen sensitivity between these two cell lines differs significantly. Remarkably, the baseline PSA level in MDA PCa 2a cells was 15-fold higher than in LNCaP (8.8 ng./ml. versus 0.6 ng./ml.) in androgen-depleted medium with charcoal-stripped FBS. Maximal levels of PSA stimulation were similar in both cell lines (40 to 60 ng./ml.). Considering that the total number of cells at the end of the experiment was lower with MDA PCa 2a than LNCaP (based on the DNA content) because of slower growth of MDA PCa 2a cells, the baseline PSA levels in MDA PCa 2a were actually 48-fold higher than in LNCaP when expressed on the basis of DNA (10.6 ng. PSA/μg. DNA versus 0.22 ng. PSA/μg. DNA). These data indicate that PSA can be stimulated by androgens in MDA PCa 2a cells. However, MDA PCa 2a cells were less responsive to androgens than LNCaP cells.

Hormone response specificity of MDA PCa 2a and LNCaP cells. Since our attempts to examine ligand binding specificity of the mutated AR in MDA PCa 2a cells did not succeed due to the very low affinity for DHT, we investigated hormone response specificity of the mutant AR. In these experiments, we determined the effect of various hormones on cell growth and PSA secretion by MDA PCa 2a cells and compared the results to LNCaP cells. Since MDA PCa 2a cells were responsive to DHT only at concentrations of 100 nM or higher as shown in fig. 4, we treated cells with various ligands at 100 nM for 6 days in charcoal-stripped FBS-containing medium. At the conclusion of the experiment, measurements of DNA content and PSA were obtained. Overall, MDA PCa 2a cells were less responsive to tested ligands than LNCaP cells in both the growth and PSA assays (fig. 5). As shown in fig. 5, *A*, the growth of LNCaP cells was stimulated by testosterone (T), androstenedione (ASD), DHEA, progesterone, estradiol, and the anti-androgen hydroxyflutamide but not bicalutamide. Moreover, LNCaP cells increased PSA secretion more than 10-fold in response to T, ASD, DHEA, progesterone, estradiol, and the anti-androgen hydroxyflutamide (fig. 5, *C*). The anti-androgen bicalutamide decreased the baseline level of PSA secretion by LNCaP cells 3-fold (fig. 5, *C*). In contrast, the growth of MDA PCa 2a cells did not change significantly following treatment with the same set of ligands at 100 nM (fig. 5, *B*). MDA PCa 2a cells are stimulated to increase PSA in response to T, ASD, and progesterone (less than 4-fold) (fig. 5, *D*). Estradiol causes minimal stimulation. The anti-androgens hydroxyflutamide and bicalutamide have no agonist (fig. 5, *D*) or antagonist effect (data not shown) on MDA PCa 2a cells. Hence, the mutant AR of MDA PCa 2a cells is less responsive to androgens than the singly mutated AR in LNCaP cells. Moreover, it is also less promiscuous than that of LNCaP cells to the non-androgens we tested.

DISCUSSION

In this study, we first investigated the responses to androgens of a new human prostate cancer cell line, MDA PCa 2a, established from a bone metastasis of prostate carcinoma in a patient following failure of androgen ablation therapy.[7] Two parameters of cellular response to androgens, cell growth and PSA secretion, were examined. As compared with the well-established prostate cancer cell line LNCaP, MDA PCa 2a showed a decrease in androgen responsiveness. For example, although MDA PCa 2a cells expressed abundant AR, they required 10- to 1000- fold higher concentrations of DHT or R1881, respectively, than LNCaP cells to elicit functional responses. In fact, Scatchard analysis revealed that the AR in the MDA PCa 2a cell line had a 50-fold lower

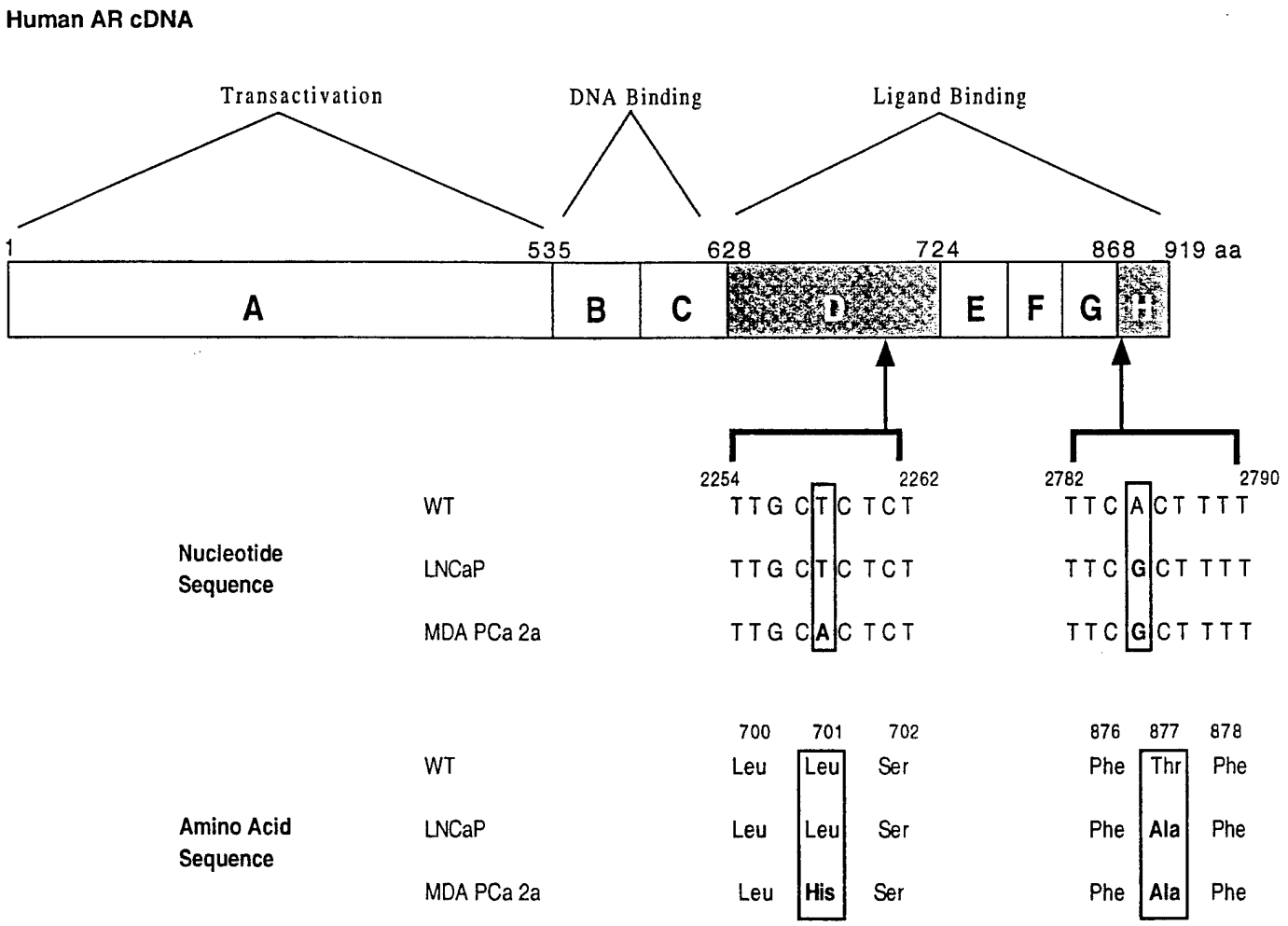

FIG. 3. DNA sequence analysis of AR gene. Exons A to H from MDA PCa 2a or LNCaP cells were amplified by PCR and sequenced. Both nucleotide sequence and amino acid sequence of wildtype (WT) AR gene from GenBank (accession number M21748) are shown in comparison with AR genes in LNCaP and MDA PCa 2a cells. Positions of mutations are indicated by bold type in boxes.

binding affinity for DHT than the mutated AR in LNCaP or the wildtype AR in the MCF-7 cell line.

Suspecting that a mutation in the AR gene was responsible for the decreased binding affinity for DHT in MDA PCa 2a cells, we then sequenced the entire coding region of the AR gene of MDA PCa 2a cells and compared it to the sequence in LNCaP and the wildtype AR. Analysis of the DNA sequences demonstrated the presence of double amino acid substitutions (L701H and T877A) in the AR LBD of MDA PCa 2a cells. Extrapolating from the crystal structure of the unliganded RXRα LBD,[14] both mutations appear to be located in the vicinity of ligand contact sites and situated in helix 3 and 11 of the AR, respectively.

The threonine residue at codon 877 is unique to the AR among steroid receptors including AR, PR, GR, MR, and ER.[15] The T877A mutation has been reported in LNCaP cells as well as in metastatic prostate cancer specimens,[6] and thus appears to be a mutational hot spot in the AR gene. The LNCaP mutation does not alter ligand binding affinity of androgens for the AR, but does result in a broadened ligand specificity. For example, T877A mutant ARs can be activated by estrogen, progesterone, and even the antiandrogen hydroxyflutamide.[5] We found that MDA PCa 2a cells containing the same mutation plus an additional second mutation in the AR gene also respond to progesterone (fig. 5) and minimally to estradiol. Hence this T877A mutation could provide the tumor cells with a growth advantage in an androgen-depleted environment.

On the other hand, the leucine at position 701 is a moderately conserved residue among steroid receptors and is also present in PR and MR.[15] The L701H mutation is not present in the AR gene of LNCaP cells. It should be noted that the L701H and T877A mutations have been found separately in different tumor foci of a cancer patient who died after androgen ablation failure.[16] However, the properties and biological characteristics of the AR harboring the L701H mutation were not presented. Interestingly, a mutation at position 702 of the AR identified in a patient with complete androgen insensitivity syndrome (AIS) resulted in loss of androgen binding.[17] Along these lines, we show that the mutated AR in MDA PCa 2a cells resulted in a 50-fold decrease in ligand binding affinity for DHT compared with the AR in LNCaP cells with a single T877A mutation. The L701H mutation would thus cause a decrease in the ability of MDA PCa 2a cells to respond to low concentrations of androgen. MDA PCa 2a cells harbor two mutations in the AR gene. The interaction between these two mutations seems to be additive so that these cells do respond to progesterone but they are much less responsive than LNCaP cells which contain only the T877A mutation. According to the AR mutation database,[4] there have been six double amino acid substitutions found in prostate tumor samples, but their corresponding phenotypes have not been examined.

Our comparative studies of the two AR-positive prostate cancer cell lines, LNCaP and MDA PCa 2a, lead to several interesting findings common to both. First, both cell lines respond to androgens and exhibit qualitatively similar biphasic growth responses, evidencing a growth stimulatory re-

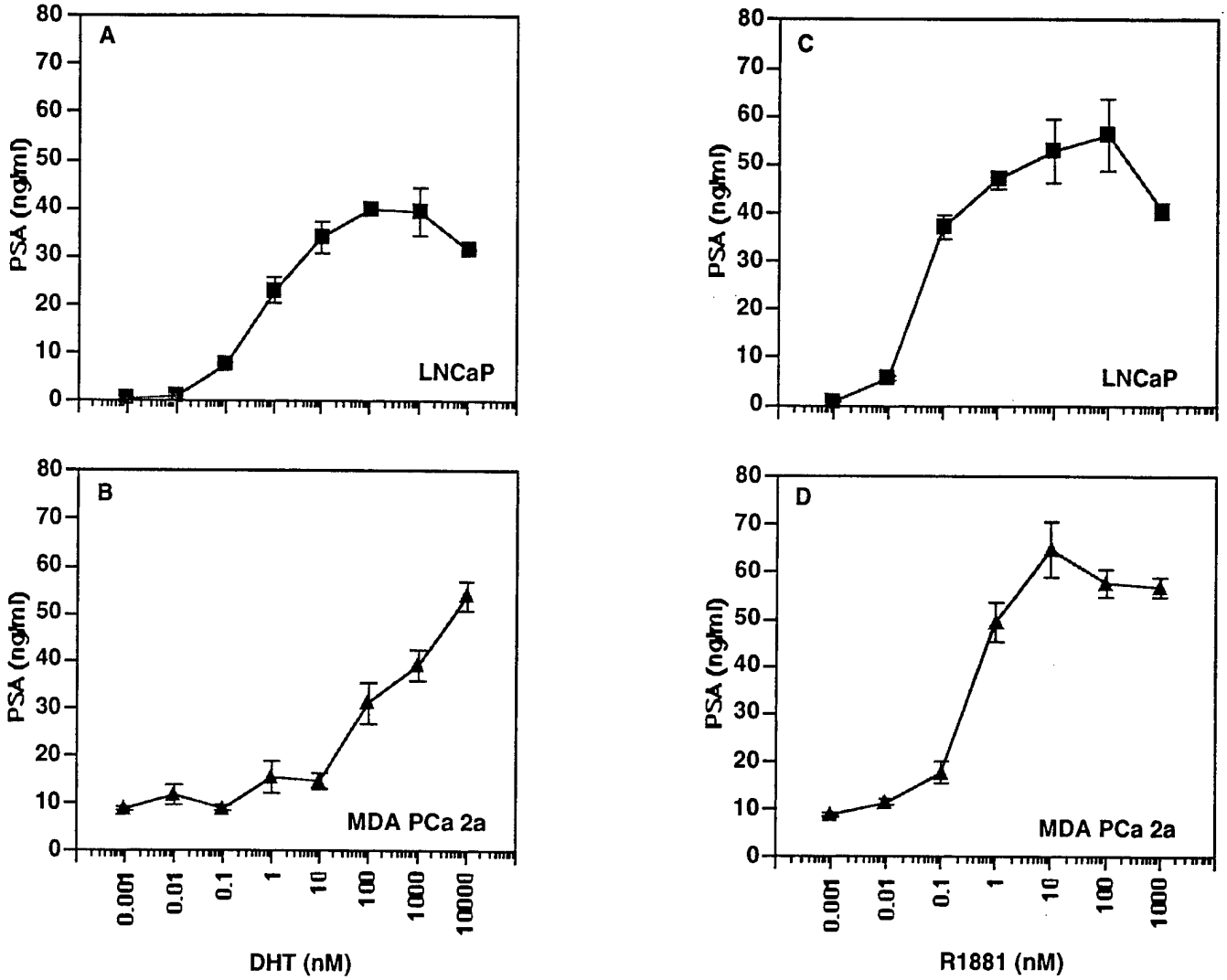

FIG. 4. Dose response effect of DHT and R1881 on secretion of PSA by MDA PCa 2a and LNCaP cells after 6 day treatment. PSA levels are expressed as ng. per ml. of conditioned medium. Values are given as mean ± SEM from triplicate assays (n = 3). *A*, PSA secretion by LNCaP in response to DHT. Baseline PSA level was 0.6 ng./ml. Maximal amount of PSA was 39.98 ng./ml. at 100 nM DHT. *B*, PSA secretion by MDA PCa 2a in response to DHT. The baseline PSA level was 8.812 ng./ml. The maximal amount of PSA was 53.86 ng./ml. at 10000 nM DHT. *C*, PSA secretion by LNCaP in response to R1881. The maximal level of PSA was 56.16 ng./ml. at 100 nM R1881. *D*, PSA secretion by MDA PCa 2a in response to R1881. Maximal level of PSA was 64.7 at 10 nM R1881.

sponse at low doses and a growth inhibitory response at high doses. Therefore, the biphasic growth pattern is not unique to LNCaP cells although the mechanism for such a pattern is not well understood. Secondly, the secretion of PSA by both cell lines is responsive to androgens in a dose-dependent manner. However, it does not follow a biphasic curve, demonstrating dissociation between the growth response to androgens and androgen-stimulated PSA. Both cell lines showed similar maximal amounts of PSA secretion at a range of 40 to 60 ng./ml. Thirdly, MDA PCa 2a and LNCaP cells express comparably high levels of the AR protein which may be a feature of metastatic prostate cancer since both lines were established from metastatic tumors. AR amplification occurs in 28 to 30% of prostate cancer patients that have been treated with androgen ablation.[18,19] The persistent expression of AR in androgen-independent prostate cancers raises the possibility of continued AR activity even in the absence of androgen. Finally, both cell lines respond to progesterone, and the broader ligand specificity may provide the tumor cells with a growth advantage in an androgen-depleted environment.

It is of interest to note that MDA PCa 2a cells express an elevated basal level of PSA even when cultured in charcoal-stripped FBS-containing medium that was depleted of androgens (fig. 4). This finding correlates with the clinical course of the patient who had rising PSA levels after orchiectomy. It is also consistent with the mice grafted with MDA PCa 2a cells. The mice showed a constant level of PSA per gram of tumor both before and after orchiectomy.[7] The mechanism for the high basal PSA levels in MDA PCa 2a cells is not clear. Further investigation is warranted to examine this interesting issue.

In summary, we have characterized the AR in a new human prostate cancer cell line MDA PCa 2a and compared it to the AR in the well-established LNCaP cell line. Although they are androgen responsive, MDA PCa 2a cells show a decreased affinity for DHT binding as well as a decreased androgen sensitivity. MDA PCa 2a cells also exhibit a widened ligand specificity with agonist responses to progesterone and also minimally to estradiol. Such a phenotype may be explained by the two mutations in the AR LBD in this cell line. One of the mutations (T877A) in this bone metastasis-derived cell line is identical to that in LNCaP, a cell line derived from a lymph node metastasis. It is likely that the MDA PCa 2a cell line with two mutations may represent a later stage of prostate cancer. We presume that the second

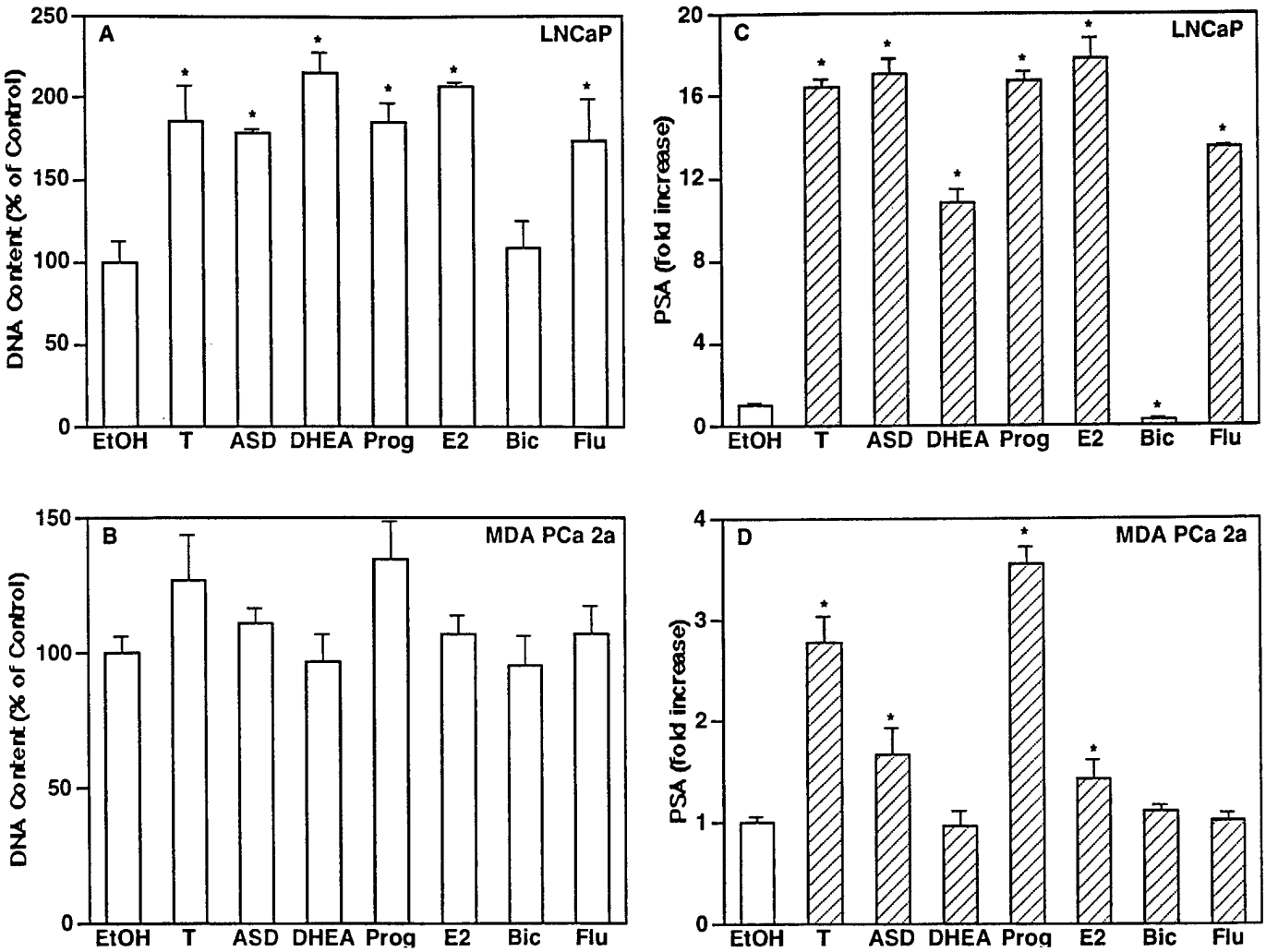

FIG. 5. Effect of various ligands on the growth (panels *A* and *B*) and secretion of PSA (panels *C* and *D*) by MDA PCa 2a and LNCaP cells after 6 day treatment. Data are shown as mean ± SEM of triplicate assays (n = 3). DNA contents are expressed as percent of control value (EtOH-treated). *A*, growth response of LNCaP cells to ethanol (EtOH) and 100 nM of the following agents: testosterone (T), androstenedione (ASD), DHEA, progesterone (Prog), estradiol (E2), bicalutamide (Bic), and hydroxyflutamide (Flu). *B*, growth response of MDA PCa 2a cells to same ligands. PSA levels are expressed as fold increase over control (ethanol-treated). *C*, PSA secretion by LNCaP cells in response to various ligands. PSA value of ethanol-treated control was 0.6 ng./μg. DNA. *D*, PSA secretion by MDA PCa 2a cells in response to the same ligands. The PSA value of ethanol-treated control was 14 ng./μg. DNA. *, $p < 0.05$ compared with ethanol-treated control.

mutation (L701H) in the AR of MDA PCa 2a cells is responsible for the decreased affinity for androgens and the reduced agonistic responses to non-androgens, as compared with LNCaP cells. Furthermore, both the elevated basal level of PSA in the absence of androgens and the decreased androgen responsiveness of MDA PCa 2a cells may contribute to the failure of androgen ablation therapy in this patient. We speculate that the double mutations in the AR gene may well result in the androgen-insensitive growth of the prostate cancer in this patient who had been treated with an orchiectomy. Therefore, MDA PCa 2a cells represent an excellent model for studying events in the progression of prostate cancer from an androgen-dependent to an androgen-independent phenotype. Further investigation of these cells may provide more information about the mechanisms responsible for the failure of androgen ablation therapy in patients with advanced prostate cancer.

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Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor

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The androgen receptor (AR) is involved in the development, growth and progression of prostate cancer[1] (CaP). CaP often progresses from an androgen-dependent to an androgen-independent tumor, making androgen ablation therapy ineffective. However, the mechanisms for the development of androgen-independent CaP are unclear. More than 80% of clinically androgen-independent prostate tumors show high levels of AR expression[1]. In some CaPs, AR levels are increased because of gene amplification[2] and/or overexpression, whereas in others, the AR is mutated[3–5]. Nonetheless, the involvement of the AR in the transition of CaP to androgen-independent growth and the subsequent failure of endocrine therapy are not fully understood. Here we show that in CaP cells from a patient who failed androgen ablation therapy, a doubly mutated AR functioned as a high-affinity cortisol/cortisone receptor (AR^{ccr}). Cortisol, the main circulating glucocorticoid, and its metabolite, cortisone, both equally stimulate the growth of these CaP cells and increase the secretion of prostate-specific antigen in the absence of androgens. The physiological concentrations of free cortisol and total cortisone in men[6,7] greatly exceed the binding affinity of the androgen and cortisol/cortisone receptor (AR^{ccr}) and would activate the receptor, promoting CaP cell proliferation. Our data demonstrate a previously unknown mechanism for the androgen-independent growth of advanced CaP. Understanding this mechanism and recognizing the presence of glucocorticoid-responsive AR mutants are important for the development of new forms of therapy for the treatment of this subset of CaP.

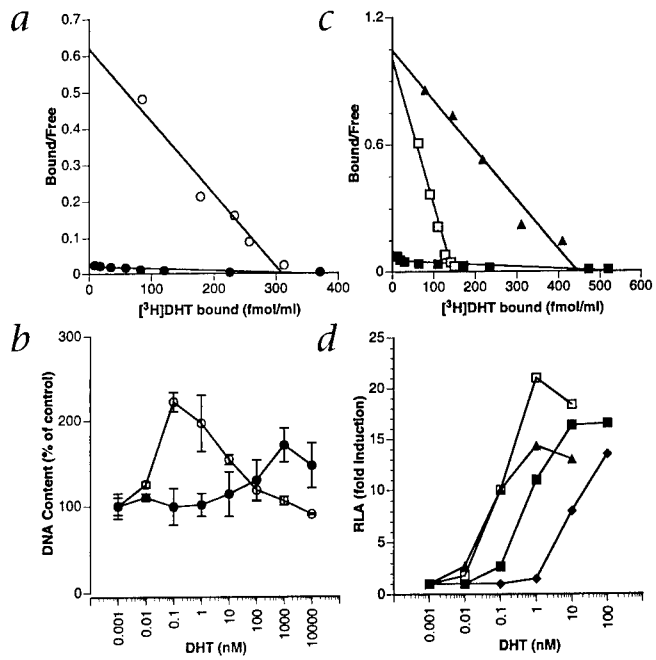
Two CaP cell lines with different karyotypes, MDA PCa 2a and 2b, recently established from a bone metastasis from a patient whose CaP showed androgen-independent growth, have been characterized[8,9]. Here, we investigated the mechanism of androgen-independent growth of the MDA PCa 2b cells. Initial experiments using radioligand binding assays with tritiated dihydrotestosterone (DHT), the main prostatic androgen, showed decreased binding by the androgen receptor (AR). Scatchard analyses of the binding of ^{3}H-DHT (Fig. 1*a*) showed that MDA PCa 2b cells expressed ARs at levels similar to those seen in LNCaP cells (a well-characterized, AR-expressing human CaP cell line derived from a lymph node (LN) metastasis[10]), but had a reduced affinity for DHT, to about 2% (dissociation constant (K_d) = 23.3 ± 3.3 nM (n = 3) for MDA PCa 2b; K_d = 0.5 nM for LNCaP). Correspondingly, MDA PCa 2b cells required higher concentrations of DHT for growth stimulation (Fig. 1*b*) than did LNCaP cells, which typically have a bi-phasic growth response to androgens[10]. DHT also induced secretion of prostate-specific antigen (PSA) in MDA PCa 2b cells, with an effective concentration for half-maximum response (EC_{50}) of 100 nM DHT (compared with 0.5 nM in LNCaP cells) (data not shown). These findings demonstrate that MDA PCa 2b cells express a low-affinity AR that is less responsive to DHT than the AR in LNCaP cells in promoting growth and secretion of PSA.

Sequencing of the entire coding region of the AR gene from MDA PCa 2b cells demonstrated two missense mutations in the ligand-binding domain that changed leucine at position 701 to histidine (L701H) and threonine at position 877 to alanine (T877A). As the AR gene is on the X chromosome and as these cells contain only a single X chromosome[9], the two mutations must be on the same allele. Consistently, sequencing analyses of RT–PCR products showed that these two mutations were in the same AR mRNA molecule. Thus, this case differs from a published case in which the two mutations were not found in the same tissue[11].

The AR T877A mutation has been identified in LNCaP cells[13] and in some advanced CaP cases[3–5]. The effects of this mutation on AR function have been well documented[13]. Although the AR L701H mutation has also been identified in metastatic CaP specimens[11,12], the biological consequences of this mutation, like those of most AR mutations discovered in metastatic CaP, have not been characterized mainly because of a limited supply of tumor tissue and the difficulty in establishing CaP cell lines.

To determine the individual and combined effects of these mutations on AR function, we recreated the singly mutated AR cDNAs L701H and T877A and AR cDNA with both mutations, L701H&T877A. We expressed the wild-type and mutant AR proteins in COS-7 and CV-1 cells and assessed their ^{3}H-DHT-binding and transactivation properties. The T877A mutant bound DHT with high affinity (K_d = 0.38 ± 0.04 nM), similar to the wild-type AR (K_d = 0.20 ± 0.07 nM) (Fig. 1*c*). In contrast, the L701H mutant failed to show substantial DHT binding. The L701H&T877A double mutant, however, had an affinity for DHT of 2% (K_d =11.80 ± 2.00 nM) that of wild-type AR. In transactivation assays using the androgen-responsive luciferase (Luc) reporter pMMTV–Luc, the T877A mutant had an EC_{50} of 0.04 nM DHT, similar to that of the wild-type AR (Fig. 1*d*), whereas the L701H mutant had an EC_{50} of 10 nM, and the L701H&T877A mutant had an EC_{50} of 0.4 nM. Western blot analyses showed that the expression levels of each mutant AR in transfected cells were similar (data not shown). The luciferase assay (Fig. 1*d*) is more sensitive than the radioligand binding assay (Fig. 1*a* and *c*). Both assays indicate that the L701H mutation decreases the ability of AR to bind and respond to DHT,

Fig. 1 The doubly mutated AR shows decreased androgen binding and responsiveness. ***a***, Scatchard analysis of ^{3}H-DHT binding in LNCaP cells (○; K_d = 0.5) and MDA PCa 2b cells (●; K_d = 23). ***b***, Growth response to DHT. Control levels (100%) correspond to 8.25 μg and 2.96 μg DNA per well for LNCaP cells (○) and MDA PCa 2b cells (●), respectively. Data represent means ± s.e.m. (n = 3). ***c***, Scatchard analysis of ^{3}H-DHT binding to wild-type AR (□), AR T877A (▲) and AR L701H&T877A (■) expressed in COS-7 cells. ***d***, Transactivation assays. CV-1 cells were transiently transfected with pMMTV–Luc and AR expression vectors, as well as pRL–SV40 (Renilla luciferase) to normalize for transfection efficiency. Cells were then treated with DHT and relative luciferase activity (RLA) was determined. □, wild-type; ▲, T877A; ◆, L701H; ■, L701H&T877A. Data represent 'fold induction' over control (no added ligand). The s.e.m. did not exceed 10% for each treatment.



and that ligand binding and androgen responsiveness can be partially restored by the acquisition of the T877A mutation.

As the T877A mutant has a much broader ligand binding profile than the wild-type AR (ref. 13), we examined the transactivation response of each mutant AR to ligands of the class I nuclear receptors, including DHT, progesterone, 17β-estradiol, aldosterone and hydrocortisone (cortisol), as well as an antiandrogen hydroxyflutamide. As predicted from the earlier studies[13], the T877A mutant had substantial transactivation responses to progesterone, 17β-estradiol and hydroxyflutamide (Fig. 2*a*). The L701H mutant responded to DHT and, unexpectedly, to cortisol. The L701H&T877A mutant, however, was activated by all of the ligands except aldosterone. Correspondingly, aldosterone had low binding affinity for the L701H&T877A mutant in competition binding assays. The maximum transactivation response was with cortisol, and the response of the L701H&T877A mutant to cortisol was much greater (300%) than that of the L701H mutant. Thus, the L701H mutation confers cortisol responsiveness to the AR, and the unique profile of hormone response by the L701H&T877A mutant reflects the combined effects of the two mutations.

As the L701H&T877A mutant showed a greater response to cortisol than to DHT in the transactivation assays (Fig. 2*a*), we reexamined the binding properties of this mutant using ^{3}H-cortisol as the ligand. The L701H&T877A mutant had a specific, saturable and high-affinity binding site for cortisol with a K_d of 4.8 ± 0.1 nM (Fig. 2*b*). This binding affinity was 1,000% greater than the affinity showed by the human glucocorticoid receptor α for cortisol (K_d = 50 nM), which we assayed at the same time. Our value is consistent with the K_d reported before[14]. In transactivation assays using the pMMTV–Luc reporter (Fig. 2*c*), the L701H&T877A mutant had an EC_{50} of about 1 nM cortisol, and the L701H mutant had a substantial response to cortisol at a concentration of 10–50 nM. Neither the T877A mutant nor the wild-type AR was responsive to cortisol.

In competition binding assays using ^{3}H-cortisol as the ligand,

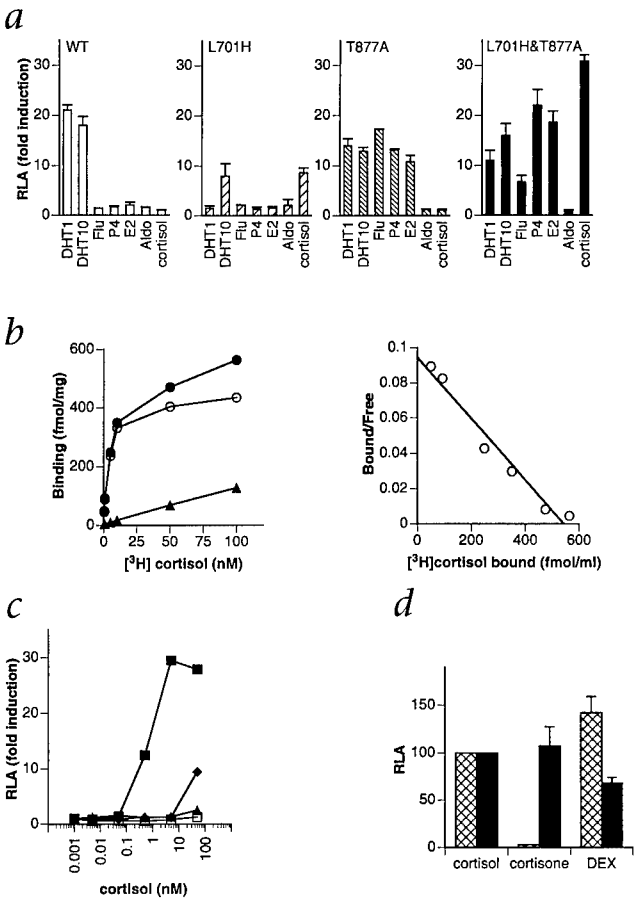


Fig. 2 The recreated AR mutants show broadened ligand specificity. ***a***, CV-1 cells were transfected with AR expression vectors (above graphs), the pMMTV–Luc reporter and pRL–SV40 (Renilla luciferase), and were treated with 1 nM or 10 nM DHT (DHT1 and DHT10), or 10 nM hydroxyflutamide (Flu), progesterone (P4), 17β-estradiol (E2), aldosterone (Aldo) or cortisol. Relative luciferase activity (RLA) was assayed. WT, wild-type. ***b***, Specific ^{3}H-cortisol binding (left) and Scatchard analysis (right) of the L701H&T877A mutant expressed in COS-7 cells, showing total binding (●), specific binding (open circles) and nonspecific binding (▲). The calculated K_d is 4.7 nM (r^2 = 0.969). ***c***, Transfected CV-1 cells were treated with cortisol, and relative luciferase activity (RLA) was determined. □, wild-type; ▲, T877A; ◆, L701H; ■, L701H&T877A. ***d***, CV-1 cells were co-transfected with pMMTV–Luc and expression vectors for human glucocorticoid receptor α pSG5-GRα (cross-hatched bars) or the L701H&T877A mutant AR (■) along with pRL-SV40. Cells were then treated with 10 nM cortisol, cortisone or dexamethasone (DEX). Data represent relative luciferase activity (RLA; means ± s.e.m.; n = 3) due to cortisone or DEX, as a percent of the cortisol-induced activity (set as 100%).

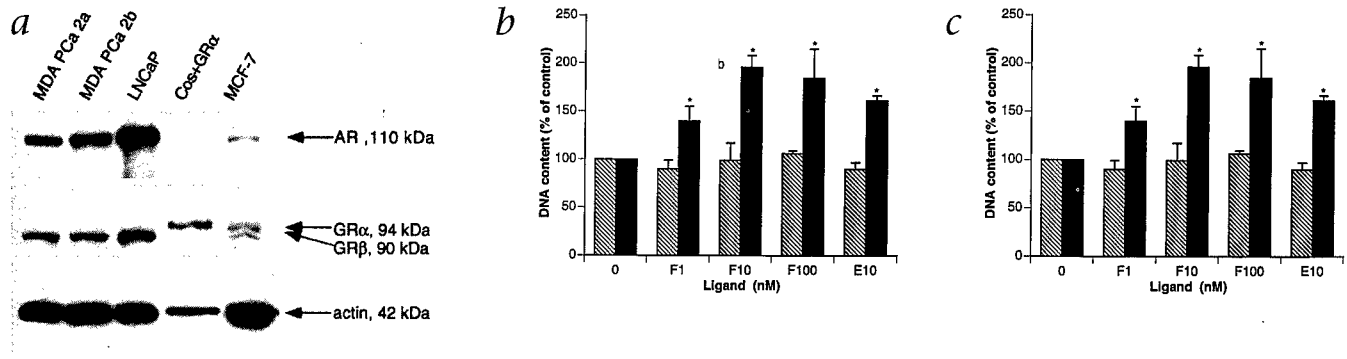


Fig. 3 Glucocorticoids promote growth and secretion of PSA in MDA PCa 2b cells. ***a***, Western blot analyses. MCF-7 cells express both glucocorticoid receptors α and β (ref. 20). Above blot, cell lines. Right margin, expected proteins and sizes. GR, glucocorticoid receptor. ***b*** and ***c***, Cells were grown in the presence of 0, 1, 10 or 100 nM cortisol (0, F1, F10 and F100) or 10 nM cortisone (E10) in media 'stripped' of endogenous steroids. ▨, LNCaP; ■, MDA PCa 2b. *b*, Growth, measured by determining the DNA content in each sample. *c*, PSA levels in the conditioned media. Data represent means ± s.e.m. (n = 3). *, $P < 0.05$, compared with control.

the L701H&T877A mutant had the highest affinity for cortisol (100%) and cortisone (100%), followed by R1881 (a synthetic androgen; 65%), DHT (41%), dexamethasone (18%), hydroxyflutamide (16%), 17β-estradiol (11%), progesterone (8%), aldosterone (less than1%) and bicalutamide (an anti-androgen; less than1%). Its binding profile for glucocorticoids differs from that of the human glucocorticoid receptor α in that the latter had a higher affinity for dexamethasone, a synthetic glucocorticoid (K_d = 2.2 nM) than cortisol, the natural glucocorticoid. The L701H&T877A mutant also responded to many C_{19} and C_{21} steroids that circulate in the human bloodstream (data not shown), which further distinguishes it from other nuclear hormone receptors. Cortisone, which is a natural metabolite of cortisol and is inactive for human glucocorticoid receptor α, activated the L701H&T877A mutant as efficiently as cortisol (Fig. 2*d*). This experiment used CV-1 cells, which are deficient in 11β-hydroxysteroid dehydrogenase[15], the enzyme that converts cortisone to cortisol. Thus, the combination of the L701H and T877A mutations effectively transforms the AR into a cortisol/cortisone receptor (AR^{ccr}), allowing glucocorticoids to activate androgen-responsive genes in CaP.

To test the effects of cortisol and cortisone on MDA PCa 2b cells, we first determined whether these cells express glucocorticoid receptor, by western blot analysis. Glucocorticoid receptor α was undetectable in MDA PCa 2b cells (Fig. 3*a*); however, these cells did express glucocorticoid receptor β (Fig. 3*a*), a truncated form of glucocorticoid receptor that does not bind glucocorticoids or mediate transactivation[16]. In growth studies using androgen-free media (Fig. 3*b*), both cortisol and cortisone stimulated MDA PCa 2b cell proliferation in a dose-dependent manner. In contrast, neither hormone had any growth-stimulatory effect on LNCaP cells (Fig. 3*b*), which do not express glucocorticoid receptor α and do not respond to dexamethasone[17]. Furthermore, secretion of PSA, a marker of androgen action, was increased by both cortisol and cortisone in MDA PCa 2b cells but not in LNCaP cells (Fig. 3*c*). MDA PCa 2a cells also responded to cortisol and cortisone stimulation (data not shown). Our results show that in these CaP cells, cortisol and cortisone, acting through the doubly mutated AR^{ccr}, promote androgen-independent growth and PSA secretion.

Glucocorticoid activation of a mutated AR may be one of many potential pathways by which CaP cells escape androgen dependence. The mutations described here, L701H by itself or in combination with T877A, convert the AR into a receptor that responds to glucocorticoids by stimulating cell growth and activating androgen-responsive genes, including PSA. PSA is an androgen-dependent marker of CaP progression, and serum PSA levels positively correlate with tumor burden in patients[18]. Our findings indicate the possibility that PSA secretion in some androgen-independent CaPs may reflect glucocorticoid or other hormone stimulation instead of androgen stimulation of the AR. Glucocorticoids circulate at high levels with most cortisol bound to transcortin. In men, the circulating levels of free cortisol (15–45 nM) and total cortisone (39–63 nM) do not decline with age[6,7]. These concentrations exceed the K_d (4.8 nM) of the AR^{ccr} receptor and are high enough to sufficiently activate both the L701H mutant and the double-mutant AR^{ccr} in transactivation assays. Therefore, these circulating glucocorticoids could substantially activate mutant ARs *in vivo* and promote androgen-independent growth of CaP. We are now testing this using animal models.

To appreciate the frequency with which glucocorticoids promote androgen-independent CaP growth in patients, the prevalence of these AR mutations in metastatic CaP needs to be determined. Although the cells in distant metastases, after androgen ablation fails, often contain AR mutations[1–5], metastatic tissues are not routinely biopsied, and therefore are not readily available.

In conclusion, we have characterized mutations in the AR that may account for a previously unknown mechanism for androgen-independent growth of CaP. Although AR mutants with a variety of 'promiscuities' exist, the recognition of these glucocorticoid-responsive ARs (L701H or L701H&T877A) is an important step in the development of new forms of therapy for the treatment of this subset of androgen-independent CaP.

Methods

Cell culture, cell proliferation and PSA assays. LNCaP and MDA PCa 2b cells were maintained as described[8,9]. Cell proliferation assays were done 6 d after 5×10^4 cells were seeded per 35-mm well and cultured in RPMI-1640 medium supplemented with 5% charcoal-stripped fetal bovine serum and the steroids being studied. Determination of DNA content/well was used as an index of cell proliferation, as described[8]. PSA levels in the conditioned

media were determined by the TOSOH assay, an automated immunoenzymometric assay system (TOSOH Medics, Foster City, California).

Sequencing of genomic DNA. Genomic DNA was isolated from MDA PCa 2b cells, and each exon of the AR gene was amplified by PCR using intronic primers[19]. The PCR products were cloned and DNA was sequenced by the core facility at Stanford University. The data were analyzed using the GCG software (GCG, Madison, Wisconsin). Three independent clones from two preparations of DNA were tested, and the same mutations were found each time.

Sequencing of cDNA after RT–PCR. Total RNA was isolated from MDA PCa 2b cells and cDNA was made using the MuLV reverse transcriptase and an oligo-dT_{16} primer (Roche Molecular Systems, Branchburg, New Jersey). Two gene-specific primers (AR2401, 5′–ACTCTGGGAGCCCGGAAGCTG–3′; and AR3294, 5′–AATGCTTCACTGGGTGTGGAA–3′) were used to amplify an intact AR ligand binding domain (exons D–H) by PCR. The RT–PCR product was an 893-base-pair fragment encompassing the AR coding sequence, nucleotides 2401–3294. The RT–PCR products were inserted into a cloning vector, TA-vector (Invitrogen, San Diego, California). A total of 36 clones were screened, and 15 had the 893-base-pair insert. Each positive clone was sequenced in both directions. Every clone contained both L701H and T877A mutations.

Site-directed mutagenesis. Mutations were recreated in the AR cDNA in pSG5-AR (from Z. Culig). pSG5–GR was provided by P. Kushner. The mutants were generated using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). The mutagenic oligonucleotides (Operon Technologies, Alameda, California) used were 5′–GCAGCCTTGCACTCTAGCCTC–3′ for L701H and 5′–GCATCAGTTCGCTTTTGACCT–3′ for T877A (mutated bases are underlined). Final constructs were sequenced to confirm the mutations.

Transfection and luciferase assay. Plasmids were transfected into CV-1 (for transactivation) or COS-7 (for ligand binding) monkey kidney cells using lipofectamine (Life Technologies). After this transfection, the CV-1 cells were treated with various ligands for 24 h and luciferase activity was measured using the dual-luciferase assay system (Promega). The experiment was done three times using triplicate wells for each treatment. Triplicate wells contained 1.25 μg pMMTV–Luc (from R. Evans), 0.625 μg expression vectors for AR or glucocorticoid receptor α, and 5 ng pRL-SV40 (as a control for transfection efficiency).

Radioligand binding, Scatchard analysis and competition binding assays. The recreated AR L701H&T877A mutant was expressed in COS-7 cells. Binding assays using ^{3}H-DHT, ^{3}H-cortisol and ^{3}H-dexamethasone, as well as Scatchard analyses using these steroids, were done as described[8]. High-salt extracts (200 μl at a concentration of 0.5–1 mg protein/ml) were incubated with 0–100 nM labeled ligand for 16–20 h at 0 °C. Bound and free hormones were separated by hydroxylapatite. Specific binding was calculated by subtracting nonspecific binding obtained in the presence of a 250-fold excess of unlabeled ligands from the total binding measured in the absence of unlabeled ligands. Competition binding analyses of the double-mutant AR were done in the presence of 20 nM ^{3}H-cortisol with unlabeled ligands at an excess of 1-fold to 100-fold. The relative ability of various compounds to inhibit 50% of ^{3}H-cortisol binding is expressed as the relative binding affinity value, with cortisol set at 100%.

Western blot analysis. Cell extracts containing 50 μg of protein were separated by 4–12% gradient SDS–PAGE and transferred to nitrocellulose membranes. The blots were probed with polyclonal antibodies against AR, glucocorticoid receptors (Santa Cruz Biotechnology, Santa Cruz, California), and actin (Sigma). Peroxidase-conjugated goat antibody against rabbit IgG (Zymed, South San Francisco, California) was used as secondary antibody. The signal was detected using enhanced chemiluminescence (ECL; Amersham).

Statistical analysis. For Fig. 3*b* and *c*, the ANOVA Scheffe's F test was used to assess statistical significance of differences between the treated group and the untreated controls, using the StatView 4.5 program (Abacus Concepts, Berkeley, California). $P < 0.05$ was considered statistically significant.

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1α,25-Dihydroxyvitamin D_3 Inhibits Prostate Cancer Cell Growth by Androgen-Dependent and Androgen-Independent Mechanisms*

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ABSTRACT

We recently reported that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits the growth of the LNCaP human prostate cancer cell line by an androgen-dependent mechanism. In the present study we examined the actions and interactions of 1,25-$(OH)_2D_3$ and the androgen 5α-dihydrotestosterone (DHT) on two new human prostate cancer cell lines (MDA), MDA PCa 2a and MDA PCa 2b. Scatchard analyses revealed that both cell lines express high affinity vitamin D receptors (VDRs) with a binding affinity (K_d) for [^{3}H]1,25-$(OH)_2D_3$ of 0.1 nM. However, the MDA cell lines contain low affinity androgen receptors (ARs) with a K_d of 25 nM for [^{3}H]DHT binding. This is 50-fold lower than the AR in LNCaP cells (K_d = 0.5 nM). Their response to DHT is greatly reduced; 2a cells do not respond to 100 nM DHT, and 2b cells show a modest response at that high concentration. 1,25-$(OH)_2D_3$ causes significant growth inhibition in both MDA cell lines, greater (for 2b cells) or lesser (for 2a cells) than that in the LNCaP cell line. Moreover, 1,25-$(OH)_2D_3$ significantly up-regulates AR messenger RNA in all three cell lines, as shown by Northern blot analysis. The growth inhibitory effect of 1,25-$(OH)_2D_3$ on LNCaP cells is blocked by the pure antiandrogen, Casodex, as we previously reported. However, Casodex (at 1 μM) did not block the antiproliferative activity of 1,25-$(OH)_2D_3$ in MDA cells. In conclusion, the growth inhibitory action of 1,25-$(OH)_2D_3$ in the MDA cell lines appears to be androgen independent, whereas the actions of 1,25-$(OH)_2D_3$ in LNCaP cells are androgen dependent. Most importantly, the MDA cell lines, derived from a bone metastasis of human prostate carcinoma, remain sensitive to 1,25-$(OH)_2D_3$, a finding relevant to the therapeutic application of vitamin D and its low calcemic analogs in the treatment of advanced prostate cancer. (*Endocrinology* **141:** 2548–2556, 2000)

D1α,25-DIHYDROXYVITAMIN D_3 [1,25-$(OH)_2D_3$], the hormonal form of vitamin D, modulates cellular proliferation and differentiation in a broad range of cell types (1–3), in addition to its classical role of maintaining mineral homeostasis (4, 5). The hormone exerts its actions via a specific nuclear vitamin D receptor (VDR), a ligand-inducible transcription factor (6). Recently, the presence of VDR has been demonstrated in prostate epithelial cells (7–9). Moreover, we (8–14) and others (15–24) have shown that 1,25-$(OH)_2D_3$ and its analogs significantly inhibit the growth of primary cultures derived from human prostatic tissues as well as several established human prostate cancer cell lines.

Among the commonly used cell lines, the LNCaP cell line exhibits the greatest sensitivity to growth inhibition by 1,25-$(OH)_2D_3$ (8, 15). LNCaP cells express the androgen receptor (AR) and respond to androgen stimulation (25). However, androgen action in LNCaP cells is biphasic, in that low concentrations of androgen stimulate cell growth, whereas high concentrations of androgen lead to inhibition of cell proliferation (26). We showed that 1,25-$(OH)_2D_3$ increases AR abundance and enhances cellular responses to androgen in these cells (10). Growth inhibition of LNCaP cells by 1,25-$(OH)_2D_3$ is mediated by an androgen-dependent mechanism and is preceded by the induction of AR gene expression (12). Furthermore, growth inhibition by androgens has been reported in LNCaP sublines that express high basal levels of AR and in other AR-containing cells (27–29).

To study the interactions of 1,25-$(OH)_2D_3$ with androgens in other prostate cancer cells beside the LNCaP model, we used two new AR-positive cell lines, MDA PCa 2a and MDA PCa 2b (30). Both cell lines were derived from a single bone metastasis of prostate carcinoma in a patient who failed androgen ablation therapy. These two cell lines have different genetic features (karyotype) and different phenotypes (morphology and growth rate), reflecting the genetic heterogeneity of the tumor (30). These cells retain two important characteristics of cells of prostate origin: the expression of both AR and prostate-specific antigen (PSA). We recently identified two mutations in the ligand-binding domain (L701H and T877A) of the AR in the MDA PCa 2a cell line (13). Both mutations were also found in the AR gene of MDA PCa 2b cells (30a). The single T877A mutation is present in the AR gene of the LNCaP cell line (31).

In the current study we first characterized the VDR and AR in MDA PCa 2a and MDA PCa 2b cell lines. We then examined the actions and interactions of vitamin D and the androgen 5α-dihydrotestosterone (DHT) in these cells and compared the results to those in LNCaP cells. We found that 1,25-$(OH)_2D_3$ induced AR gene expression in all three cell lines. DHT had small effects on cell growth and PSA secretion when MDA cells were cultured in FBS-containing me-

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dium. MDA PCa 2a and MDA PCa 2b cells, in contrast to LNCaP, maintained a response to 1,25-$(OH)_2D_3$ in the presence of the pure antiandrogen, Casodex. Thus, 1,25-$(OH)_2D_3$ inhibits the growth of these cells by an androgen-independent mechanism, whereas growth inhibition of LNCaP cells by 1,25-$(OH)_2D_3$ is mediated by an androgen-dependent mechanism.

Materials and Methods

Materials

1,25-$(OH)_2D_3$ and Casodex (bicalutamide or ICI 17,334) were gifts from Dr. M. Uskokovic (Hoffmann-La Roche Co., Nutley, NJ) and Zeneca Pharmaceuticals (Macclesfield, UK), respectively. [^{3}H]1,25-$(OH)_2D_3$ (SA, 102 Ci/mmol) and 5α-dihydro-[1α,2α-^{3}H]testosterone (SA, 40–70 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Nonradioactive DHT was obtained from Steraloids, Inc. (Wilton, NH). The human AR complementary DNA (cDNA) was a gift from Dr. M. McPhaul (University of Texas Southwestern Medical Center, Dallas, TX). Tissue culture medium RPMI 1640 and BRFF-HPC-1 were obtained from Mediatech (Herndon, VA) and Biological Research Faculty Facility, Inc. (Ijamsville, MD), respectively. FBS was obtained from Life Technologies, Inc. (Gaithersburg, MD). Charcoal-stripped FBS was purchased from HyClone Laboratories, Inc. (Logan, UT). Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents, except where indicated, were purchased from Sigma (St. Louis, MO).

Cell culture and hormone treatment

The LNCaP human prostate carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD). LNCaP cells were routinely cultured in RPMI 1640 medium supplemented with 5% FBS and antibiotics at 37 C in a humidified atmosphere of 5% CO_2. The human prostate cancer cell lines MDA PCa 2a and 2b (30) were maintained in BRFF-HPC-1 medium, supplemented with 20% FBS and gentamicin.

Hormone stocks [1,25-$(OH)_2D_3$, DHT, and Casodex] were prepared in 100% ethanol at a concentration 1000-fold higher than the working concentrations. Fresh culture media were premixed with hormone stock and then added to triplicate wells. Medium and hormone were replenished every 3 days. Controls received ethanol vehicle at a concentration equal to that in hormone-treated cells.

Steroid receptor ligand binding and Scatchard analysis

Cell monolayers were harvested, and high salt nuclear extracts were made as previously described (10–13). The protein concentration of the extract was determined by the method of Bradford (32). In a typical binding assay, 200 μl soluble extract (0.5–1 mg protein/ml) were incubated with the indicated concentrations of [^{3}H]DHT or [^{3}H]1,25-$(OH)_2D_3$ for 16–20 h at 0 C. Bound and free hormones were separated by hydroxylapatite. Specific binding was calculated by subtracting nonspecific binding obtained in the presence of a 250-fold excess of radioinert steroid from total binding measured in the absence of radioinert steroid. Data were expressed as femtomoles of [^{3}H]DHT or [^{3}H]1,25-$(OH)_2D_3$ bound per mg protein.

Assay of cell proliferation

Cell proliferation was assessed by measurement of attained cell mass using an assay of DNA content. As previously described (10), cells were seeded in six-well tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ) at a density of 50,000–200,000 cells/well in 3 ml medium containing 5% FBS. After incubation for 24 h, the medium was replaced with fresh medium containing 5% FBS. Cells were treated with vehicle (ethanol; final concentration, 0.1%), 1,25-$(OH)_2D_3$, DHT, or Casodex. On the sixth day, cell monolayers were processed for DNA assay using the method of Burton (33). The DNA content of each treatment was derived from the mean value of triplicate wells in an experiment. Each experiment was repeated three times.

Assay of PSA secretion

The conditioned medium collected in cell proliferation assays was subjected to a low speed centrifugation to remove cell debris. PSA values in the supernatant were determined by the TOSOH assay, an automated immunoenzymometric assay system (TOSOH Medics, Inc., Foster City, CA), as previously described (10). Results were expressed as nanograms of PSA per μg DNA.

Northern blot analysis

The method has been described previously (12). In brief, semiconfluent monolayer cells were treated with 1,25-$(OH)_2D_3$ in RPMI medium containing 5% charcoal-stripped FBS or 5% FBS for 24 h before isolation of total RNA. Ten micrograms of total RNA were denatured, fractionated by electrophoresis, and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech). The bound RNA was immobilized and hybridized with a random primed ^{32}P-labeled 1.1-kb *Hin*dIII-*Eco*RI fragment of the human AR cDNA at 60 C (34). To control for RNA sample loading and transfer, Northern blots were also hybridized with a ^{32}P-labeled 0.9-kb *Eco*RI fragment of the human cDNA for the ribosomal protein gene L7 (7). The silver grain pixel intensity of each AR and L7 band was scanned by a densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 messenger RNA (mRNA).

Statistical analysis

ANOVA was used to assess the statistical significance of difference. $P < 0.05$ was considered significant.

Results

Analysis of VDR by ligand binding

The hormonal action of 1,25-$(OH)_2D_3$ is mediated by its receptor, the VDR. In our first studies, we characterized the VDR in the two new human prostate cancer cell lines, MDA PCa 2a and 2b. The data from equilibrium binding experiments using a range of concentrations of [^{3}H]1,25-$(OH)_2D_3$

(0.03–1 nM) are shown in Fig. 1. The *inset* illustrates dose-response saturation plots obtained after correction for nonspecific binding. Linear regression analysis of Scatchard plots revealed a single class of specific and high affinity receptors with an apparent dissociation constant (K_d) of 0.1 ± 0.02 (n = 3) and 0.18 ± 0.03 (n = 3) nM for MDA PCa 2a and 2b cells, respectively. These binding affinities are similar to values in LNCaP cells (7) (K_d = 0.14 nM) and other classical vitamin D target tissue (35). The VDR content was higher in MDA PCa 2a (51 ± 3 fmol/mg protein; n = 3) than in 2b cells (33 ± 2 fmol/mg protein; n = 3) or LNCaP cells (31 fmol/mg protein) (7), and this difference is statistically significant ($P < 0.05$). Thus, these two new MDA cell lines express VDR with affinity and abundance similar to those of other established vitamin D target cells.

Analysis of AR by ligand binding

We then examined the AR in MDA PCa 2a and 2b cells. Ligand binding experiments were performed initially using the usual concentrations of [^{3}H]DHT (0.03–1 nM), and we could not achieve saturation of binding sites. We then used high concentrations of [^{3}H]DHT (2–100 nM) for binding experiments. Scatchard analyses of [^{3}H]DHT binding (Fig. 2A) revealed low affinity binding receptors with apparent K_d values of 25 ± 4 (n = 3) and 23 ± 3 (n = 3) nM for MDA PCa 2a and 2b cells, respectively. These binding affinities were

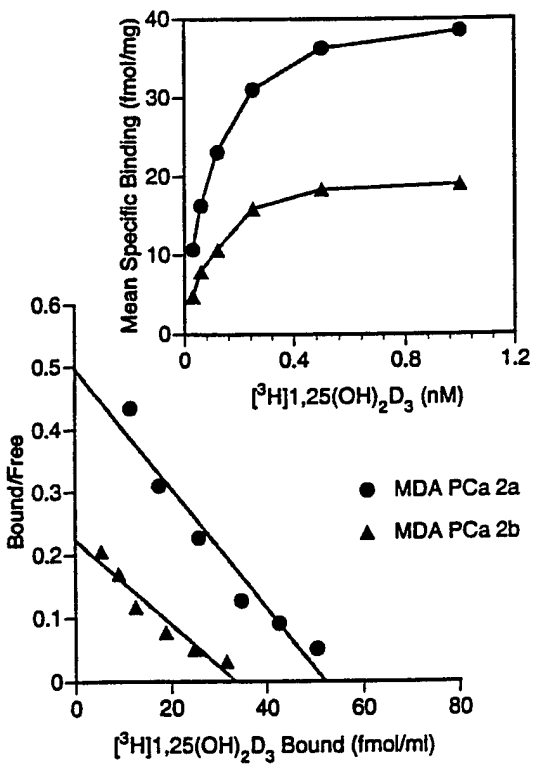

FIG. 1. Analysis of VDR by specific [^{3}H]1,25-(OH)$_2$D$_3$ binding in the human prostate cancer cell lines. Soluble extracts were prepared from MDA PCa 2a (*circle*) and MDA PCa 2b (*triangle*) cells and incubated for 16–20 h at 4 C in the presence of increasing concentration of [^{3}H]1,25-(OH)$_2$D$_3$. Specific binding was measured by subtracting the binding in the presence of a 250-fold excess of radioinert hormone from total binding. *Inset*, Saturation plot of specific binding data. A Scatchard plot of the binding data is shown.

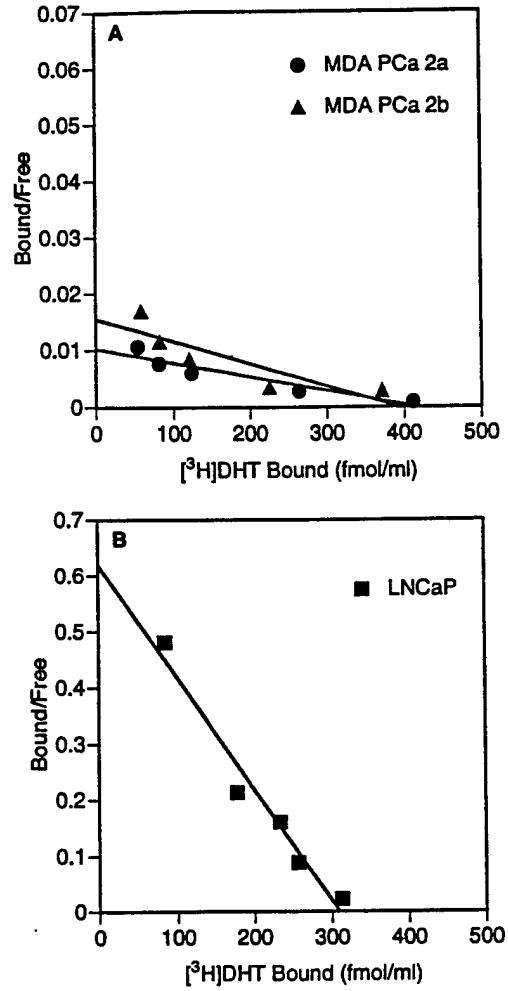

FIG. 2. Analysis of AR by specific [^{3}H]DHT binding in the human prostate cancer cell lines. Soluble extracts were prepared from MDA PCa 2a (*circle*), MDA PCa 2b (*triangle*), and LNCaP (*square*) cells and were incubated for 16–20 h at 4 C in the presence of increasing concentrations of [^{3}H]DHT with or without a 250-fold excess of radioinert hormone. A, Scatchard plot of the binding data for MDA PCa 2a and 2b cells. B, Scatchard plot of the binding data for LNCaP cells.

significantly lower (higher K_d) than the AR in LNCaP cells (K_d = 0.5 nM; Fig. 2B). MDA cells expressed a higher abundance of AR [685 ± 149 fmol/mg protein for 2a (n = 6) and 840 ± 32 fmol/mg protein for 2b (n = 3)] than LNCaP cells (457 fmol/mg protein). Therefore, these two new cell lines express AR with low affinities (23–25 nM), and their AR contents were in the range of 600–900 fmol/mg protein.

Effect of 1,25-(OH)$_2$D$_3$ and androgen on cell growth

We next examined the effect of 1,25-(OH)$_2$D$_3$ and DHT on the growth of both new cell lines and compared their responses to LNCaP cells. The doubling times for LNCaP and MDA PCa 2a and 2b cells are 36, 84, and 42 h, respectively. Consistent with our previous report (10), the growth of LNCaP cells in FBS-containing medium was inhibited by either 1,25-(OH)$_2$D$_3$ or DHT in a dose-dependent manner. Also, both hormones caused a greater inhibition in combination than when given alone, as shown in Fig. 3A. Both

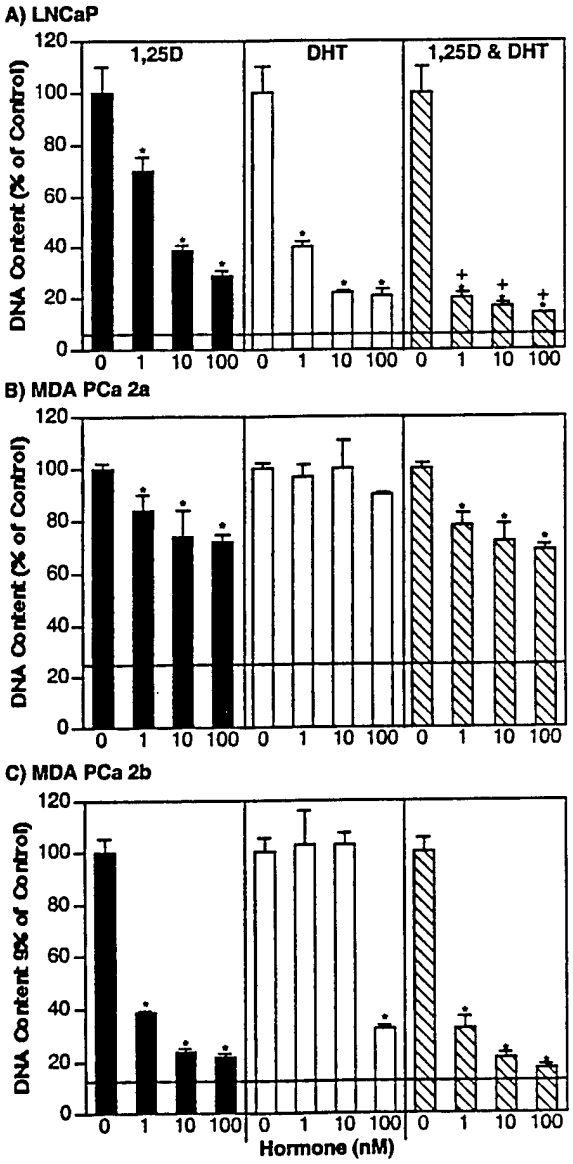

FIG. 3. Dose-response effect of 1,25-$(OH)_2D_3$, DHT, or their combination on cell growth. LNCaP (A), MDA PCa 2a (B), and MDA PCa 2b (C) cells were treated with 1,25-$(OH)_2D_3$ or DHT individually or in combination for 6 days. Cellular DNA contents were determined. The data are expressed as the mean ± SEM (n = 3). The untreated control sample on day 6 is set at 100%. The baseline value (the amount of DNA at the beginning of treatment time zero) is indicated by a *solid line*. *, $P < 0.05$ compared with the untreated control group. +, $P < 0.05$ compared with either the 1,25-$(OH)_2D_3$-treated or DHT-treated group.

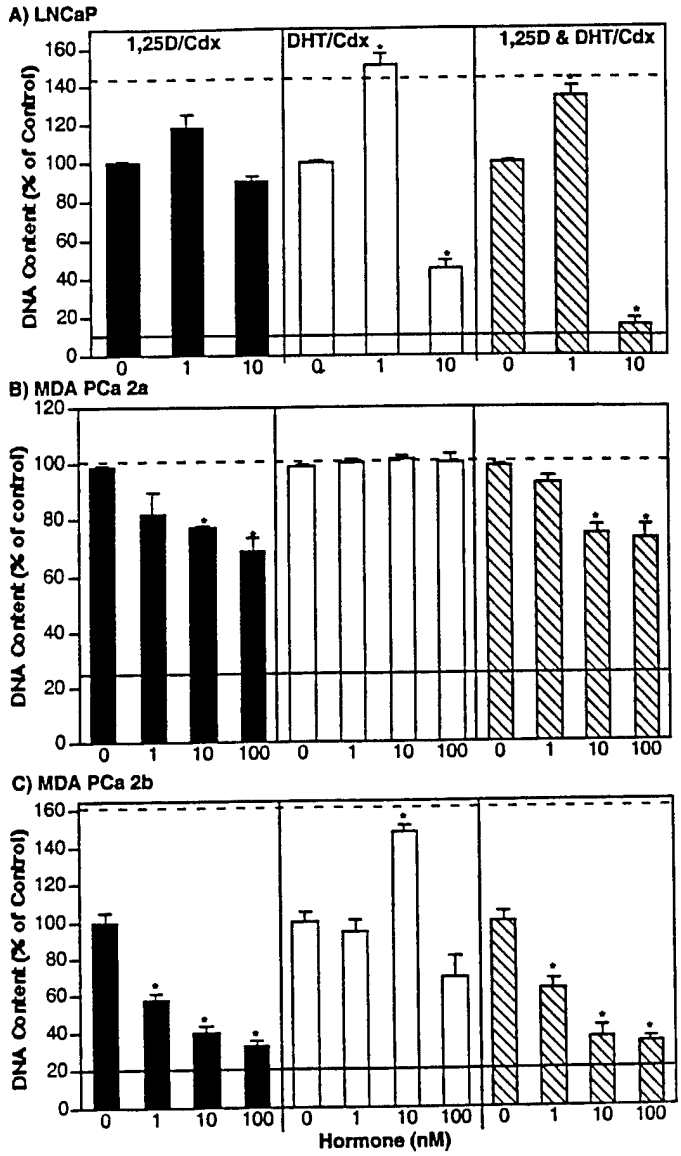

FIG. 4. Dose-response effect of 1,25-$(OH)_2D_3$, DHT, or their combination on cell growth in the presence of the antiandrogen Casodex. Cells were treated with 1,25-$(OH)_2D_3$ or DHT individually or in combination in the presence of 1 μM Casodex, and after 6 days, cellular DNA contents were determined. The data are expressed as the mean ± SEM (n = 3). The Casodex-treated sample on day 6 is set at 100%. The baseline value (the amount of DNA at the beginning of treatment time zero) is indicated by a *solid line*. The amount of DNA in the untreated control sample on day 6 is indicated by a *dotted line*. The differences between the *dotted lines* and the 100% value (Casodex-treated) indicate 31%, 0.8%, and 38% of growth inhibition caused by Casodex alone in LNCaP (A), MDA PCa 2a (B), and MDA PCa 2b (C) cells, respectively. *, $P < 0.05$ compared with the untreated control group in the presence of Casodex.

MDA PCa 2a and 2b cells were similar to LNCaP cells in their response to 1,25-$(OH)_2D_3$ treatment (Fig. 3, B and C).

In contrast to LNCaP cells, MDA PCa 2a and 2b cells did not respond to ordinary concentrations of DHT (Fig. 3, B and C). A higher concentration of DHT (100 nM) did not affect the growth of MDA PCa 2a cells, but caused growth inhibition (60–70%) in MDA PCa 2b cells. Combination treatment with 1,25-$(OH)_2D_3$ and DHT did not result in a greater inhibition of the growth of these cells than treatment with 1,25-$(OH)_2D_3$ alone. These data suggest that MDA PCa 2a and 2b cells differ from LNCaP cells in exhibiting reduced sensitivity to androgens. Moreover, the enhanced response to the 1,25-$(OH)_2D_3$/androgen combination as seen in LNCaP cells was not detected in these cells.

Effect of 1,25-$(OH)_2D_3$ and androgen on cell growth in the presence of Casodex

We previously demonstrated that 1,25-$(OH)_2D_3$ elicits its antiproliferative effect on LNCaP cells by an androgen-

dependent mechanism (10, 12). Here we investigated the mechanism of the 1,25-$(OH)_2D_3$/androgen interaction in MDA PCa 2a and 2b cell lines using the antiandrogen, Casodex. Casodex alone has an inhibitory effect on the growth of LNCaP and MDA PCa 2b cells, but not on MDA PCa 2a cells. Treatment of these cells with 1 μM Casodex in FBS-containing medium for 6 days resulted in 31%, 38%, and 0.8% growth inhibition for LNCaP, 2b, and 2a cells, respectively.

As shown in Fig. 4A, compared with Fig. 3A, Casodex completely blocked the inhibition of LNCaP cell growth by 1,25-$(OH)_2D_3$. Casodex at a concentration of 1 μM reversed the effect of 1 nM DHT on LNCaP cell growth, but not the effect of 10 nM DHT. Furthermore, in the presence of 1 μM Casodex, no growth inhibition was seen with the combined treatment of both hormones at 1 nM. A high concentration of DHT (10 nM) overcame the blockade by 1 μM Casodex. Therefore, Casodex antagonized the inhibitory effect of 1,25-$(OH)_2D_3$ or a low dose of DHT on LNCaP cell growth.

In contrast, the growth inhibitory effect of 1,25-$(OH)_2D_3$ on MDA PCa 2a (Fig. 4B) and MDA PCa 2b cells (Fig. 4C) was unaffected by 1 μM Casodex. These results suggest that the antiproliferative actions of 1,25-$(OH)_2D_3$ in these cells are androgen independent. Interestingly, MDA PCa 2a cells remained unresponsive to DHT in the presence of Casodex (Fig. 4B), whereas MDA PCa 2b cells showed a response to DHT at 10 nM (Fig. 4C). DHT at 10 nM and Casodex at 1 μM produced a 50% increase in cell growth of MDA PCa 2b cells (Fig. 4C), similar to the effect of DHT at 1 nM and Casodex at 1 μM on LNCaP (Fig. 4A). This finding is consistent with the data in Fig. 3 showing that MDA PCa 2b cells responded to 100 nM DHT in the absence of Casodex (60–70% growth inhibition). Hence, MDA PCa 2b cells are more responsive to DHT than MDA PCa 2a cells, but both are less responsive than LNCaP cells.

Effect of 1,25-$(OH)_2D_3$ and androgens on PSA secretion

All three cell lines express PSA (10, 30). As shown in Fig. 5, treatment with 1,25-$(OH)_2D_3$ resulted in the dose-dependent stimulation of PSA secretion by LNCaP cells as well as by MDA PCa 2a and 2b cells. Because cell number changed with hormonal treatment (Fig. 3), PSA levels are expressed as nanograms of PSA per μg DNA.

As shown in Fig. 5A, both 1,25-$(OH)_2D_3$ and DHT alone enhanced the secretion of PSA by LNCaP cells in a dose-dependent manner. 1,25-$(OH)_2D_3$-treated cells showed a 5-fold increase in PSA secretion (from 5 to 27 ng PSA/μg DNA), similar to the increase seen in the DHT-treated cells. In combination, 1,25-$(OH)_2D_3$ and DHT synergistically augmented PSA secretion up to 22-fold. These data indicate that 1,25-$(OH)_2D_3$ and DHT interact cooperatively in regulating PSA secretion by LNCaP cells, as shown previously (10).

MDA PCa 2a and 2b cells express high basal levels of PSA, as previously reported (30). The baseline PSA levels in these cells range between 50–60 ng/μg DNA, which are 10-fold higher than values in LNCaP cells. DHT had a minimal effect on PSA secretion by the MDA cell lines (Fig. 5, B and C). 1,25-$(OH)_2D_3$-treated cells showed 2- and

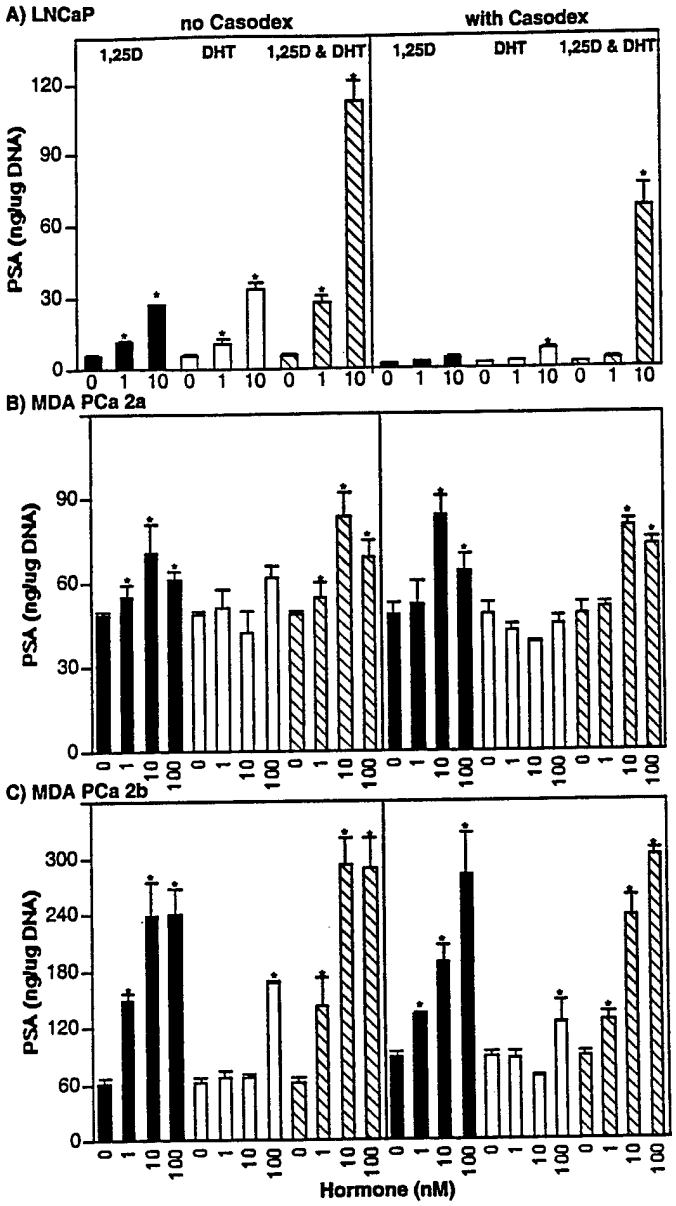

FIG. 5. Dose-response effect of 1,25-$(OH)_2D_3$, DHT, or their combination on PSA secretion in the presence and absence of the antiandrogen Casodex. The conditioned media from the cell proliferation assays (Figs. 3 and 4) were collected, and the PSA concentrations were determined. The data are expressed as nanograms of PSA per μg DNA/well (mean ± SEM; n = 3).

4-fold increases in PSA secretion in MDA PCa 2a and 2b cells, respectively. In combination, DHT did not significantly enhance the effect of 1,25-$(OH)_2D_3$ on PSA secretion. Therefore, 1,25-$(OH)_2D_3$ and DHT did not interact cooperatively in regulating PSA secretion by these cells, in contrast to LNCaP cells.

Effect of 1,25-$(OH)_2D_3$ and androgen on PSA secretion in the presence of Casodex

1,25-$(OH)_2D_3$ at 1 or 10 nM in the presence of 1 μM Casodex no longer induced PSA in LNCaP cells, as shown in Fig. 5A. Casodex completely blocked the action of DHT at a low

concentration (1 nM). The induction of PSA by DHT at 10 nM was also partially inhibited by Casodex. Moreover, in the presence of Casodex, the combined treatment with 1,25-$(OH)_2D_3$ and DHT at 1 nM did not increase the PSA level. Administration of both hormones (10 nM each) to LNCaP cells appeared to overcome the blockade by Casodex (from 1.7–67 ng PSA/μg DNA). These data suggest that the antiandrogen blocked the 1,25-$(OH)_2D_3$ action to stimulate PSA in LNCaP cells. In contrast, MDA PCa 2a and 2b cells responded to 1,25-$(OH)_2D_3$ in the presence of Casodex (Fig. 5, B and C). The high baseline PSA levels in these cell lines were unaffected by Casodex. Moreover, the antiandrogen did not modify the effect of 1,25-$(OH)_2D_3$, DHT, or both hormones on these cells, in contrast to the result seen with LNCaP cells (Fig. 5A).

1,25-$(OH)_2D_3$ up-regulation of AR in three prostate cancer cell lines

We previously reported that 1,25-$(OH)_2D_3$ increased AR gene expression in LNCaP cells (12). Here we examined 1,25-$(OH)_2D_3$ regulation of AR in MDA PCa 2a and 2b cells. Cells were treated with 1,25-$(OH)_2D_3$ for 24 h, and the effect on steady state AR mRNA levels was assessed by Northern blot analysis. As shown in Fig. 6, AR mRNA transcripts were increased by 1,25-$(OH)_2D_3$ in all three cell lines in both charcoal-stripped FBS-containing medium (Fig. 6A) and FBS-containing medium (Fig. 6C). The levels of AR mRNA were quantitatively determined by densitometric scanning of the autoradiographs, with correction for the L7 mRNA signal (Fig. 6, B and D). At 10 nM 1,25-$(OH)_2D_3$, AR mRNA was up-regulated 8-fold in LNCaP, 5-fold in MDA PCa 2b, and 20-fold in MDA PCa 2a cells when cells were cultured in charcoal-stripped FBS-containing medium (Fig. 6A). Similarly, in FBS-containing medium, 25 nM 1,25-$(OH)_2D_3$ increased AR mRNA 2-fold in LNCaP, 3-fold in MDA PCa 2b, and 2-fold in MDA PCa 2a cells (Fig. 6C). The positive effect of 1,25-$(OH)_2D_3$ on AR mRNA was greater (5-fold over the control untreated value) when MDA cells were treated with 1,25-$(OH)_2D_3$ for 48 h than for 24 h in FBS-containing medium, consistent with our previous report on LNCaP cells (12). Therefore, 1,25-$(OH)_2D_3$-mediated up-regulation of AR is a general phenomenon among the AR-positive prostate cancer cells that we tested.

Discussion

In this report we analyzed the vitamin D regulation of cell growth and gene expression in two newly established human

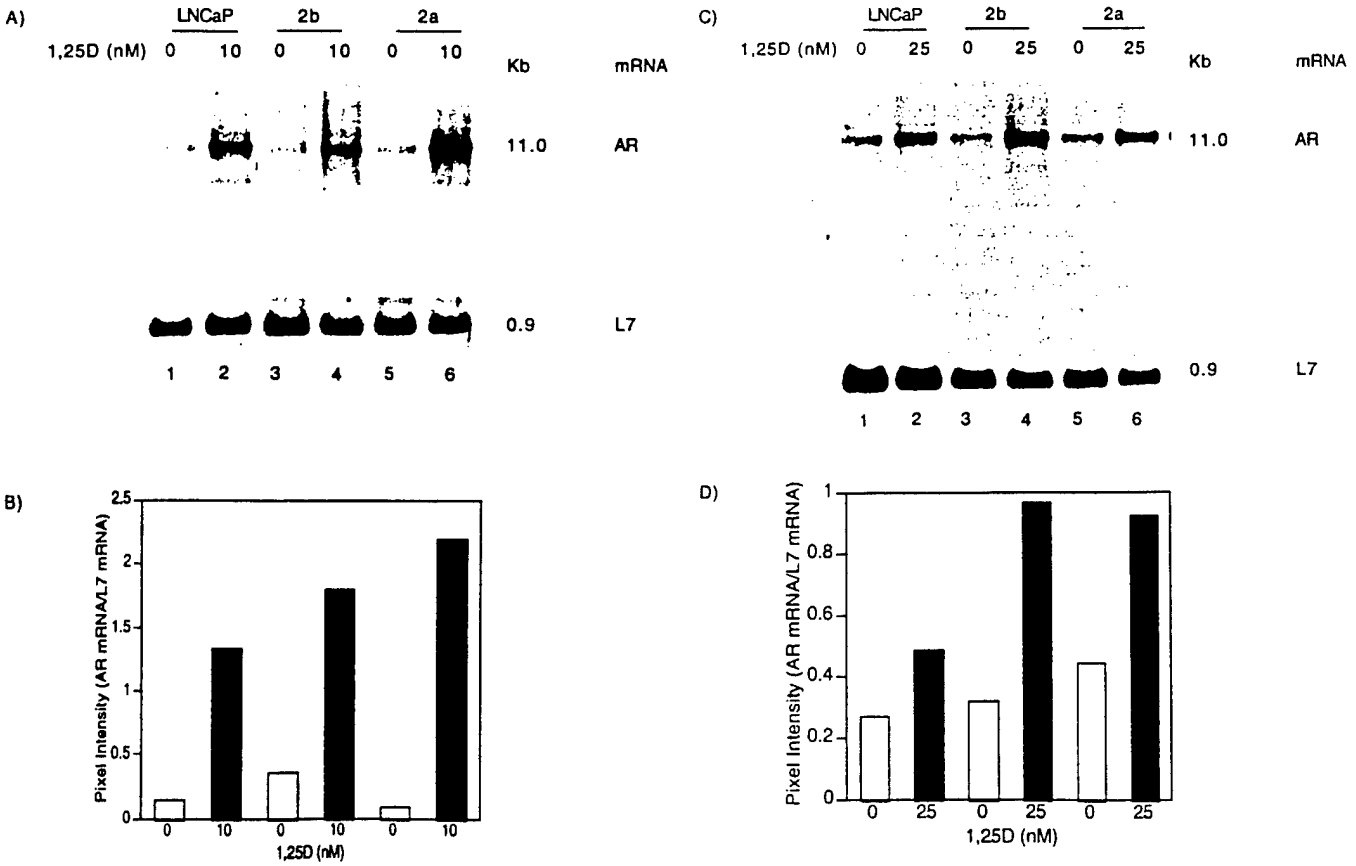

FIG. 6. 1,25-$(OH)_2D_3$ up-regulation of AR mRNA in LNCaP and MDA PCa 2a and 2b cells. A, Northern blot analysis. Cells were treated with 1,25-$(OH)_2D_3$ at 10 nM for 24 h in RPMI medium containing 5% charcoal-stripped FBS. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P-labeled 712-bp *Hin*dIII-*Eco*RI fragment of the human AR cDNA at 60 C. The blot was simultaneously probed for expression of the L7 ribosomal protein gene as a control for sample loading and transfer. B, The pixel intensity of each AR band in A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA. C, Northern blot analysis. Cells were treated with 1,25-$(OH)_2D_3$ at 25 nM for 24 h in RPMI medium containing 5% FBS. D, The pixel intensity of each AR band was indexed to the corresponding L7 band in C.

prostate cancer cell lines, MDA PCa 2a and 2b (30). We first characterized the VDR, and then examined two functional parameters for 1,25-$(OH)_2D_3$ action: growth inhibition and AR gene regulation. We also evaluated the AR protein and androgen responses in these new cell lines. We found that these cell lines exhibited reduced androgen sensitivity that differs significantly from the well established LNCaP cell line when either cell growth or PSA secretion was compared. Our comparative analysis revealed that the antiproliferative action of 1,25-$(OH)_2D_3$ was mediated by an androgen-dependent mechanism in LNCaP cells and by an androgen-independent mechanism in MDA cells.

The limited number of established human prostate cancer cell lines available for investigation has hindered prostate cancer research. The three commonly used cell lines representing progressively more transformed phenotypes are LNCaP (least transformed), PC-3 (intermediate transformation), and DU 145 (most transformed). They were derived from different metastases of prostate carcinoma: LNCaP from a lymph node metastasis (25), PC-3 from a bone metastasis (36), and DU 145 from a brain metastasis (37). The newly established human prostate cancer cell lines MDA PCa 2a and 2b were derived from a bone metastasis (30), similar to PC-3 cells. However, unlike PC-3 cells, which express extremely low or undetectable level of AR, these cells, like LNCaP cells, express abundant AR as well as inducible PSA, features typical of prostate cancer cells (13, 30). The MDA PCa 2a and 2b cells represent new cell line models for advanced prostate cancer. We were therefore interested in exploring the hormonal responses of these cells.

Regulation of cell growth and gene expression by steroid hormones depends upon the presence of functional receptors. We first characterized the VDR and AR in these cells and then compared them with those in the LNCaP cell line. The affinities for [^{3}H]1,25-$(OH)_2D_3$ and abundance of VDRs are similar in all three cell lines: LNCaP, MDA PCa 2a, and MDA PCa 2b. Correspondingly, their responses to 1,25-$(OH)_2D_3$ are qualitatively similar, but the antiproliferative effect is greater in MDA PCa 2b than in 2a or LNCaP cells or PC-3 cells (data not shown), although PC-3 cells have twice as many VDRs as MDA PCa 2b or LNCaP cells (8). This finding supports the idea that 1,25-$(OH)_2D_3$ responses require VDR, but the VDR content does not necessarily correlate with the magnitude of the hormonal response (8, 24). The growth inhibitory effect of 1,25-$(OH)_2D_3$ on all of the cell lines that we tested can be ranked in the following sequence: 2b>LNCaP>2a>PC-3≫DU 145. Other factors besides the VDR content contribute to the amplitude of the 1,25-$(OH)_2D_3$ response. For example, inhibition of 24-hydroxylase, the 1,25-$(OH)_2D_3$-inducible enzyme that initiates the 1,25-$(OH)_2D_3$ inactivation pathway, increases the sensitivity of DU145 cells to 1,25-$(OH)_2D_3$-induced growth inhibition (14).

We observed that the ARs in MDA PCa 2a and 2b cells exhibited low affinity for [^{3}H]DHT. This is probably responsible for the decreased androgen sensitivity observed in these cells. We recently demonstrated that the AR genes in both 2a and 2b cell lines contain double mutations (L701H and T877A) (13). One of the mutations, T877A, is present in the AR gene of LNCaP cells (31). The second mutation L701, or possibly the interaction of the two mutations, may be responsible for differences in androgen responsiveness between these cells and LNCaP cells. The androgen responses of the cell lines that we tested can be ranked in the following sequence: LNCaP≫2b>2a. As the AR affinity for DHT binding is similar in 2a and 2b cells, other factors must contribute to the differences in androgen sensitivity.

When comparing LNCaP with MDA PCa 2b cells, two lines of evidence demonstrated that MDA PCa 2b cells have decreased sensitivity to DHT. First, in FBS-containing medium, both LNCaP and MDA PCa 2b cells were growth inhibited by DHT, at 1 nM for LNCaP and at 100 nM for 2b cells. Second, in the presence of Casodex (1 μM) and FBS, the response of LNCaP cells to 1 nM DHT was identical to the response of MDA PCa 2b cells to 10 nM DHT.

As the AR in MDA cells has a 50-fold lower affinity for DHT binding than the AR in LNCaP cells, it is not surprising that the low affinity AR of MDA cells has a decreased affinity for Casodex. We tried competitive binding analysis to evaluate the ligand specificity of the mutant AR in MDA cells using [^{3}H]DHT as a ligand and various agents, including Casodex as cold competitors. Unfortunately, we were unable to obtain useful data due to the very low affinity of the mutant receptor for [^{3}H]DHT and the high levels of nonspecific binding. As Casodex usually requires a 1000-fold excess concentration to inhibit DHT binding, this approach was not successful. We are in the process of recreating the mutant AR *in vitro* that will be used to test the antagonist activity of Casodex in more sensitive assays than the competitive binding analysis.

The high basal level of PSA seen in MDA PCa 2a and 2b cells is worth noting. It is not clear whether the mutated AR or other mechanisms unrelated to the AR are responsible for the constitutive production of PSA in these cells. Furthermore, this high basal PSA was unaffected by Casodex, whereas in LNCaP cells, the low basal level of PSA was decreased by Casodex.

DHT has a minimal effect on PSA secretion by MDA PCa 2a and 2b cells in FBS-containing medium. In contrast, 1,25-$(OH)_2D_3$ increased PSA in these cells. We have previously shown that 1,25-$(OH)_2D_3$ increases PSA by AR signaling in LNCaP cells (10). It is possible that 1,25-$(OH)_2D_3$ regulates PSA in MDA PCa 2a and 2b cells by other mechanisms. For example, 1,25-$(OH)_2D_3$ may induce a more differentiated phenotype that secretes more PSA per cell. Evidence that 1,25-$(OH)_2D_3$ stimulates prostate cell differentiation includes increased expression of both PSA and E-cadherin (22), a cell adhesion protein that may act as a putative tumor suppressor, in LNCaP cells. E-Cadherin is also increased by 1,25-$(OH)_2D_3$ in PC-3 cells (22). In accord with mechanisms for 1,25-$(OH)_2D_3$-induced PSA, transforming growth factor-β1, a known differentiating factor, up-regulates PSA production in MDA PCa 2a cells (38).

AR up-regulation by 1,25-$(OH)_2D_3$ appears to be common in all three AR-positive cell lines that we tested. Hence, 1,25-$(OH)_2D_3$ as a differentiating agent may alter the androgen sensitivity of prostate cancer cells. Interestingly, 1,25-$(OH)_2D_3$ does not regulate AR gene expression in the human breast cancer cell line T47D (data not shown), indicating cell type specificity.

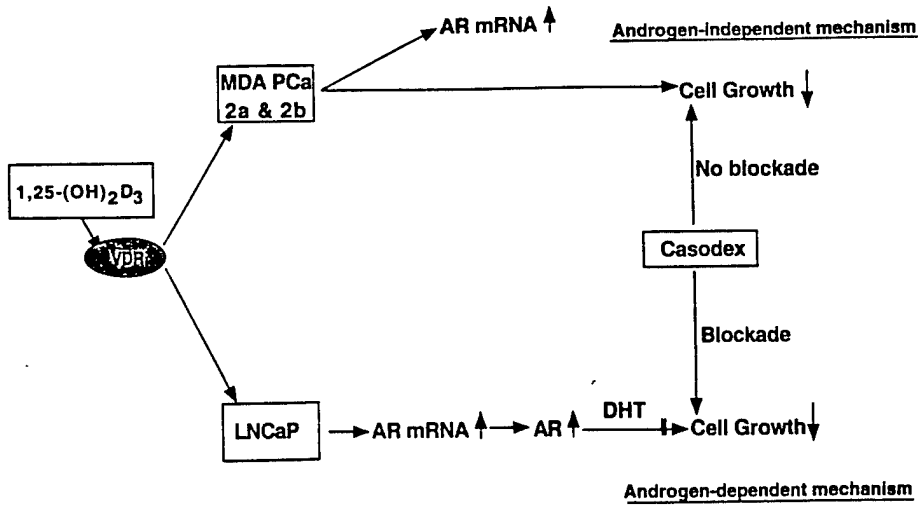

FIG. 7. A tentative model of 1,25-$(OH)_2D_3$ action on three VDR^+AR^+ cell lines. In LNCaP cells, 1,25-$(OH)_2D_3$ increases AR mRNA expression. The increased AR mRNA leads to an increase in AR protein levels. AR protein mediates androgen action on cell proliferation. The pure antiandrogen, Casodex, blocks AR action, and, in turn, it blocks the growth inhibitory action of 1,25-$(OH)_2D_3$. Hence, the growth inhibitory action of 1,25-$(OH)_2D_3$ in LNCaP cells is androgen dependent. In MDA PCa 2a and 2b cells, Casodex does not block the 1,25-$(OH)_2D_3$ action to inhibit cell growth, although 1,25-$(OH)_2D_3$ increases AR mRNA expression. Hence, the growth inhibitory action of 1,25-$(OH)_2D_3$ in these cells is androgen independent.

Collectively, our data indicate that 1,25-$(OH)_2D_3$ causes prostate cell growth inhibition by two different pathways: an androgen-dependent and an androgen-independent mechanism (Fig. 7). The androgen-dependent mechanism of 1,25-$(OH)_2D_3$ action has been demonstrated in LNCaP cells (10). Androgens are powerful regulators of prostate cell growth and gene expression. When both AR and VDR signaling pathways coexist, as in LNCaP cells, the 1,25-$(OH)_2D_3$ antiproliferative actions are AR dependent. On the other hand, examples of the androgen-independent mechanism of vitamin D action include MDA PCa 2a and MDA PCa 2b cells (low affinity ARs due to mutations), PC-3 cells (low or undetectable levels of AR), and primary cultures of prostate epithelial cells (lack of AR expression), as well as AR-negative DU 145 cells cotreated with liarozole (14). Two findings, 1) the minimal response of MDA cells to DHT and 2) the lack of Casodex inhibition of 1,25-$(OH)_2D_3$ antiproliferative action, both suggest that 1,25-$(OH)_2D_3$ acts by an androgen-independent mechanism in these cells. Hence, when the AR signaling pathway is absent or negligible, 1,25-$(OH)_2D_3$ acts via an androgen-independent pathway.

In summary, we have demonstrated that 1,25-$(OH)_2D_3$ is a potent inhibitor of cell proliferation in the new cell lines MDA PCa 2a and 2b. These cell lines are a relevant model for advanced prostate cancer. Our findings that 1,25-$(OH)_2D_3$, acting via an androgen-independent mechanism, inhibits the growth of these cells suggest that 1,25-$(OH)_2D_3$ may have clinical usefulness in the treatment of advanced prostate cancer after its progression into an androgen-independent state.

Acknowledgments

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INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 MEDIATES 1α,25-DIHYDROXYVITAMIN D_3 GROWTH INHIBITION IN THE LNCaP PROSTATE CANCER CELL LINE THROUGH P21/WAF1

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ABSTRACT

Purpose: We determined that insulin-like growth factor binding protein 3 (IGFBP-3) induction by 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is a necessary component of 1,25-$(OH)_2D_3$ mediated growth inhibition of the LNCaP human prostate cancer cell line. In addition, induction of the cyclin dependent kinase inhibitory protein p21/WAF/CIP1 by 1,25-$(OH)_2D_3$ is mediated by IGFBP-3.

Materials and Methods: Induction of IGFBP-3 by 1,25-$(OH)_2D_3$ was determined by enzyme-linked immunosorbent assay for IGFBP-3 protein and by Northern blot analysis for IGFBP-3 messenger (m) RNA. Growth assays for LNCaP cells were determined by measuring DNA content. The contribution of IGFBP-3 toward 1,25-$(OH)_2D_3$ mediated growth inhibition was determined by adding either antisense oligonucleotides or immuno-neutralizing antibodies media of culture to growth assays. Regulation of p21/WAF/CIP1 was determined by Western blot analysis.

Results: Adding 1,25-$(OH)_2D_3$ to LNCaP prostate cancer cells demonstrated that 1,25-$(OH)_2D_3$ significantly up-regulated IGFBP-3 at the mRNA and protein levels in these cells approximately 3-fold over control levels. Also, adding IGFBP-3 protein to LNCaP cell growth medium inhibited LNCaP cell growth. Interestingly adding IGFBP-3 antisense oligonucleotides or antibodies directed toward IGFBP-3 abolished the growth inhibitory actions of 1,25-$(OH)_2D_3$, indicating that this effect is IGFBP-3 dependent. Furthermore, to connect the mechanisms of IGFBP-3 and 1,25-$(OH)_2D_3$ mediated growth inhibition we demonstrated that IGFBP-3 up-regulates the expression of p21/WAF1 protein to approximately 2-fold over the control level. Adding an IGFBP-3 immuno-neutralizing antibody completely prevented the 1,25-$(OH)_2D_3$ induced up-regulation of p21/WAF1.

Conclusions: 1,25-$(OH)_2D_3$ up-regulates IGFBP-3 in the LNCaP cell line at the mRNA and protein levels. The growth inhibitory action of 1,25-$(OH)_2D_3$ on LNCaP cells depends on active IGFBP-3, as evidenced by the loss of growth inhibition induced by IGFBP-3 antisense oligonucleotide and immuno-neutralization experiments. A possible connection between IGFBP-3 and 1,25-$(OH)_2D_3$ lies in the cyclin dependent kinase inhibitory protein p21/WAF1 since IGFBP-3 and 1,25-$(OH)_2D_3$ each up-regulate this protein and both inhibit LNCaP cell growth. Therefore, we hypothesize that the mechanism of action by which IGFBP-3 and 1,25-$(OH)_2D_3$ induce growth inhibition is the induction of p21/WAF1 because IGFPB-3 immuno-neutralizing antibodies completely abrogate the 1,25-$(OH)_2D_3$ mediated up-regulation of p21/WAF1 and growth inhibition.

KEY WORDS: prostate, prostatic cancer, vitamin D, insulin-like growth factor binding protein 3, cyclin-dependent kinases

The hormonally active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), is a primary regulator of calcium homeostasis through actions on the intestine, bone, kidney and parathyroid glands.[1] However, more recently it has been shown that these processes describe only part of the action of 1,25-$(OH)_2D_3$. Evidence has revealed that 1,25-$(OH)_2D_3$ has antiproliferative and pro-differentiating characteristics with the potential for treating various types of cancer, including prostate cancer.[2–4]

1,25-$(OH)_2D_3$ is believed to act by binding to its nuclear receptor, the vitamin D receptor, which belongs to the superfamily of steroid-thyroid-retinoic acid receptors. Previous study has demonstrated that 1,25-$(OH)_2D_3$ mediates the transcription of target genes by initiating vitamin D receptor heterodimerization with the retinoid X receptor after ligand binding and this complex binds to vitamin D response element sequences in target genes.[5]

It has been demonstrated that 1,25-$(OH)_2D_3$ inhibits cell growth in prostate cancer cells, including the LNCaP human prostate cancer cell line.[6] Much research has been done to determine the precise mechanisms governing the antiproliferative actions of 1,25-$(OH)_2D_3$. Several antiproliferative mechanisms have been demonstrated, including arrest of the cell cycle in the G1/G0 phase.[7–9] In addition, 1,25-$(OH)_2D_3$ stimulates apoptosis and down-regulates the oncogenes bcl-2 and c-myc.[10] Importantly the cell cycle inhibitor cyclin-dependent kinase inhibitory protein p21/WAF1 has been shown to be stimulated after the addition of 1,25-$(OH)_2D_3$.[9,11] Recently others have postulated that a component of 1,25-$(OH)_2D_3$ induced growth inhibition may be the

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induction of the insulin-like growth factor binding protein-3 (IGFBP-3). They have demonstrated that 1,25-$(OH)_2D_3$ and its analogs may induce IGFBP-3 expression in breast and prostate cancer cells.[12,13] However, clarifying whether IGFBP-3 up-regulation is essential for 1,25-$(OH)_2D_3$ induced growth inhibitory activity is an important step that has not yet been established in these studies.

IGFBP-3 is a member of the IGFBP family of proteins, which has high affinity for insulin-like growth factors and partially acts through the regulation of insulin-like growth factor availability. IGFBP-3 is the insulin-like growth factor binding protein most prevalent in serum and it has been shown to bind insulin-like growth factors in a ternary complex with an acid-labile subunit.[14] In this manner a mechanism by which IGFBP-3 is thought to act is by limiting the amount of free and active insulin-like growth factors that are growth stimulatory in several tissues, including the prostate.[15] Another recently described mechanism through which IGFBP-3 acts is an insulin-like growth factor independent mechanism thought to be mediated by an IGFBP-3 receptor on the cell surface.[16,17] In addition, it has been shown that IGFBP-3 is a response gene for p53.[18]

We tested the hypotheses that 1,25-$(OH)_2D_3$ induces IGFBP-3 and IGFBP-3 is a critical mediator of antiproliferative actions of 1,25-$(OH_2)D_3$ in prostate cancer cells. Using the human prostate cancer cell line LNCaP as a model we show that 1,25-$(OH)_2D_3$ up-regulates IGFBP-3 secretion and expression at the mRNA and protein levels. Moreover, treatment of LNCaP cells with IGFBP-3 caused the inhibition of LNCaP cell growth comparable to treatment with 1,25-$(OH)_2D_3$. Most importantly we show that immunoneutralization of IGFBP-3, which blocks its activity, and antisense oligonucleotide treatment, which blocks IGFBP-3 production, abrogate the growth inhibitory action of 1,25-$(OH)_2D_3$. In addition, we demonstrate that p21/WAF1, a major mechanism through which 1,25-$(OH)_2D_3$ causes growth inhibition,[11] is also induced by IGFBP-3. Furthermore, through IGFBP immuno-neutralization we show that 1,25-$(OH)_2D_3$ induction of p21/WAF1 also depends on active IGFBP-3. Together these findings imply that IGFBP-3 is a principal and required component of the growth inhibitory activity of 1,25-$(OH)_2D_3$ in the LNCaP cell line.

MATERIALS AND METHODS

Sense and antisense oligonucleotides (Oligos Etc, Inc., Guilford, Connecticut) were directed at the 20 nucleotides encoding the N-terminus of human IGFBP-3 and used at a concentration of 8 μg./ml., as previously described.[17] IGFBP-3 immuno-neutralizing antibodies, which were affinity purified on an IGFBP-3 column, and recombinant human IGFBP-3 were provided by Diagnostics Systems Laboratories, Webster, Texas. Control affinity purified antigoat IgG was purchased from Pierce Biochemicals, Rockford, Illinois. Each polyclonal antibody was used at a concentration of 10 μg./ml., as previously described.[17] Mouse antihuman monoclonal p21/WAF1 antibody was purchased from BD Pharmingen, Franklin Lakes, New Jersey and used at a dilution of 1:250. The human prostate carcinoma cell line LNCaP was obtained from the American Type Culture Collection, Rockville, Maryland. LNCaP cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine solution and antibiotics at 37C in a humidified atmosphere of 5% CO_2.

For IGFBP-3 regulation assays cells were plated at 1×10^6 cells per 75 cm. flasks (NalgeNunc, Rochester, New York) overnight in regular growth medium. The following day cells were washed twice in serum-free OPTI-MEM (Life Technologies, Gaithersburg, Maryland) and then grown overnight in OPTI-MEM growth medium containing serum replacement supplement (Celox Laboratories, Inc., Hopkins, Minnesota) and 10 ng./ml. recombinant human epidermal growth factor (OPTI-MEM-GM) to support growth without exogenous insulin-like growth factors or insulin-like growth factor binding proteins from serum. The following day cells were treated with 1,25-$(OH)_2D_3$ or ethanol control for the indicated time points.

Cell growth was assessed by measuring attained cell mass using an assay of DNA content.[19] Cells were seeded in 96-well tissue culture plates (NuncNalge) at a density of 3,000 cells per well in 0.3 ml. RPMI-1640 containing 10% fetal bovine serum. After incubation for 24 hours the medium was replaced with OPTI-MEM-GM. Cells were subsequently grown for an additional 24 hours and the following day treatment compounds were added in triplicate wells for each experiment. After day 4 of treatment conditioned medium was collected for IGFBP-3 analysis and cell monolayers were processed for DNA assay. Cells were precipitated with ice-cold 5% trichloroacetic acid and washed with 100% ice-cold ethanol. The cell pellet was subsequently solubilized with 0.2 N NaOH and DNA content was determined using the diphenylamine assay of Burton.[19] The DNA content of each treatment was derived from the mean value of triplicate wells in 1 experiment. Each experiment was repeated 3 or more times and variations are presented as the standard error of mean (SEM).

For Northern blot analysis of messenger (m) RNA content total RNA was extracted, fractionated by agarose gel electrophoresis, transferred and immobilized on nylon membrane, as previously described.[20] Bound RNA was hybridized at 60C overnight with a random primed [^{32}P] labeled fragment of the human IGFBP-3 complementary DNA corresponding to exon 2. Exon 2 was used as a probe due to its specificity for IGFBP-3 and low similarity to other insulin growth factor-like binding proteins. To control for RNA loading and transfer Northern blots were also hybridized with a [^{32}P] labeled 0.9 kb EcoRI fragment of the human complementary DNA for the ribosomal protein gene L7.[21] The silver grain pixel intensity of each IGFBP-3 and L7 band was scanned by densitometry (Model 300A Densitometer, Molecular Dynamics, Sunnyvale, California). Data were integrated by scanner software (ImageQuant version 3.2, Molecular Dynamics) and indexed to the corresponding levels of L7 mRNA. For quantitating secreted IGFBP-3 protein, conditioned medium stored at −80C was defrosted at room temperature and IGFBP-3 was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Systems Laboratories) according to manufacturer instructions.

For Western blot analysis of p21/WAF1 LNCaP cell monolayers treated with control, 1,25-$(OH)_2D_3$, IGFBP-3, anti-IGFBP-3 or nonspecific IgG for 24 hours were harvested using a passive lysis buffer (Promega, Madison, Wisconsin) and stored at −80C for Western analysis. Lysate (50 μg.) from each flask was used in Western analysis, as previously described.[22] Anti-p21/WAF1 antibody and anti-actin (Sigma Chemical Co., St. Louis, Missouri) antibody were used as probes for Western blot analysis.

For immuno-neutralizing experiments 10 μg./ml. of antibodies directed toward IGFBP-3 or nonspecific IgG (Diagnostics Systems Laboratories) were added to cells in 1,25-$(OH)_2D_3$ growth assays, as aforementioned. Initially cells were plated at 5,000 cells per well in a 96-well plate. After 24 hours of initial growth in OPTI-MEM-GM antibodies or IgG was added to the OPTI-MEM-GM. Repeat administration of the antibodies or nonspecific IgG was done after 48 hours. At the end of 4 days cell growth was measured by assay for attained cell mass.[19]

In p21/WAF1 expression experiments antibodies or nonspecific IgG was added to cells in 75 mm.^{2} tissue culture flasks in OPTI-MEM-GM. Subsequently cells were treated with ethanol vehicle or 10 nM. 1,25-$(OH)_2D_3$ for 24 hours.

Monolayers were assayed for p21/WAF1 expression, as described for Western blot analysis.

For antisense experiments LNCaP cells were plated at a density of 5,000 cells per well for 24 hours. Subsequently cells were treated with antisense, sense or no oligonucleotides at a concentration of 8 μg./ml. for 4 days. After this period cell growth was measured by assay for attained cell mass and conditioned medium was collected for measuring IGFBP-3.[19]

RESULTS

To characterize the possible regulation of IGFBP-3 expression by 1,25-$(OH)_2D_3$ in LNCaP cells, protein was measured using the ELISA kit and mRNA was measured by Northern blot analysis. A range of concentrations of 1,25-$(OH)_2D_3$ (1, 10, 100 and 1,000 nM.) was added to LNCaP cell medium and after 48 hours conditioned medium was collected for IGFBP-3 protein quantitation. Cells treated in the same manner were collected for IGFBP-3 mRNA extraction at 24 hours. These 2 time points were selected due to previous determinations indicating maximal protein up-regulation at 48 hours and maximal mRNA up-regulation at 24.

Figure 1, *A* shows that adding a concentration range of 1 to 1,000 nM. of 1,25-$(OH)_2D_3$ for 48 hours to LNCaP cells caused the stimulation of IGFBP-3. Up-regulation of IGFBP-3 protein ranged from 1.8-fold for 1 nM. 1,25-$(OH)_2D_3$ to 2.9-fold for 1,000 nM. To account for possible differences in cell number at collection IGFBP-3 protein induction was standardized to the number of cells in each flask at medium collection.

Figure 1, *B* and *C* reveal that 1,25-$(OH)_2D_3$ also induces IGFBP-3 at the mRNA level. 1,25-$(OH)_2D_3$ (10 nM.) stimulated IGFBP-3 mRNA levels almost 2-fold, while maximal 3-fold stimulation was observed at a concentration of 100 nM. These results at the levels of protein and mRNA indicate that IGFBP-3 up-regulation is a mechanism through which LNCaP cells respond to 1,25-$(OH)_2D_3$ treatment. Therefore, it is plausible that this mechanism may be a pathway through which 1,25-$(OH)_2D_3$ exerts a portion of its biological activity in LNCaP cells.

To test the hypothesis that IGFBP-3 up-regulation is a mechanism by which 1,25-$(OH)_2D_3$ induces growth inhibition we examined whether IGFBP-3 inhibits LNCaP cell growth when added to cellular growth medium. LNCaP cells were grown in OPTI-MEM-GM and treated with active human IGFBP-3 for 4 days with a concentration range of 1 to 100 ng./ml.. Figure 2 shows that IGFBP-3 inhibited LNCaP cell growth, whereas 100 ng./ml. of control bovine serum albumin demonstrated no significant growth inhibition. These results indicate that IGFBP-3 added to the medium inhibits LNCaP growth, suggesting that 1,25-$(OH)_2D_3$ up-regulation of this protein is possibly a mechanism through which 1,25-$(OH)_2D_3$ induced growth inhibition occurs in these cells.

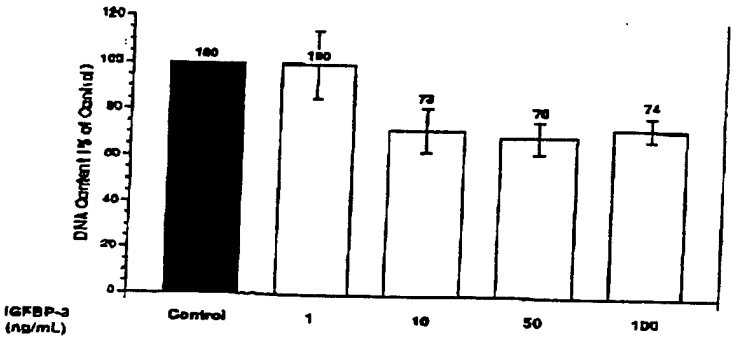

FIG. 2. IGFBP-3 inhibits growth of LNCaP cells. LNCaP cells were seeded and grown in 96-well plates in serum-free medium for 4 days. Dose range of 1 to 100 ng./ml. of recombinant human IGFBP-3 was added to growth medium. Growth is shown as percent of control with 100% equal to cells grown under 100 ng./ml. of bovine serum albumin control. Values are shown as mean plus or minus SEM of 3 experiments.

In addition, immuno-neutralizing antibodies to IGFBP-3 abrogate the growth inhibitory action of 1,25-$(OH)_2D_3$. To make a definitive connection between 1,25-$(OH)_2D_3$ and IGFBP-3 as a possible mechanism of its action we used immuno-neutralizing antibodies (Ab-IGFBP-3) in the growth medium to block IGFBP-3 action, while simultaneously treating cells with 10 nM. 1,25-$(OH)_2D_3$. Ab-IGFBP-3 (10 μg./ml.) was added to LNCaP cells grown in the presence and absence of 10 nM. 1,25-$(OH)_2D_3$. The control for the immuno-neutralizing antibody was nonspecific affinity purified anti-goat IgG. Figure 3 shows that the presence of Ab-IGFBP-3 eliminated the growth inhibitory action of 1,25-$(OH)_2D_3$, whereas control IgG did not significantly alter growth inhibition. To our knowledge this result shows for the first time that blocking IGFBP-3 also blocks 1,25-$(OH)_2D_3$ growth inhibition. This finding indicates that the growth inhibitory properties of 1,25-$(OH)_2D_3$ depend on the presence of IGFBP-3 in these cells.

To substantiate further the connection between 1,25-$(OH)_2D_3$, activity and IGFBP-3, antisense oligonucleotides directed toward the mRNA sequence encoding the N-terminal end of IGFBP-3 were used. It has previously been shown that these specific oligonucleotides are effective for

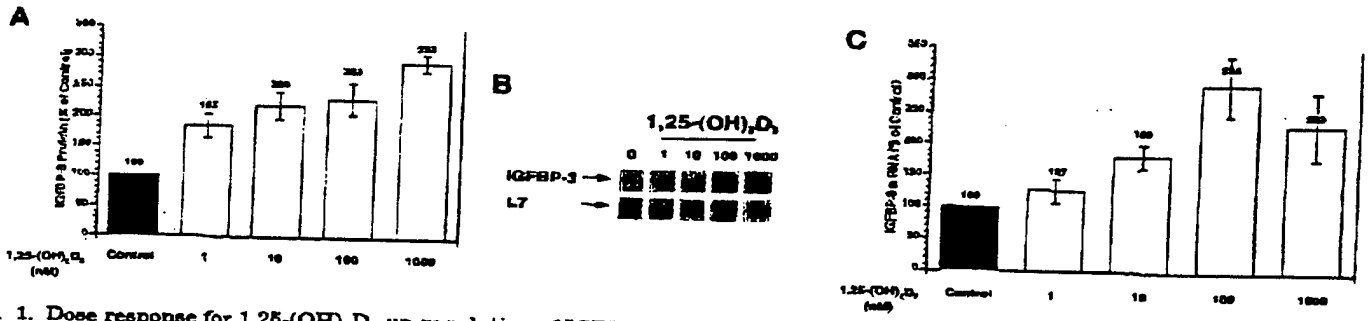

FIG. 1. Dose response for 1,25-$(OH)_2D_3$ up-regulation of IGFBP-3 protein and mRNA. LNCaP cells were plated at density of 1×10^6 cells per flask and grown in OPTI-MEM-GM for 24 hours. On following day cells were treated with vehicle, or 1, 10, 100, or 1,000 nM. 1,25-$(OH)_2D_3$ for additional 48 hours. Subsequently conditioned medium or cells were extracted for IGFBP-3 protein and mRNA quantitation, respectively. *A*, dose response of IGFBP-3 protein up-regulation by 1,25-$(OH)_2D_3$. Protein was measured by ELISA and normalized to number of cells per flask at collection. IGFBP-3 protein is presented relative to vehicle treated control as mean plus or minus SEM of 5 experiments. *B*, representative dose response Northern blot analysis shows IGFBP-3 (2.6 kb) and L7 (0.9 kb) bands. *C*, quantitation of Northern blots using densitometry analysis with each value expressed as percent of vehicle treated control. Values are shown as mean plus or minus SEM of 3 experiments.

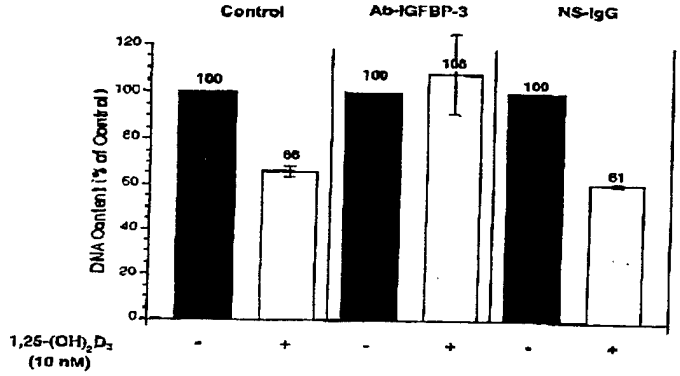

FIG. 3. Growth inhibitory action of 1,25-$(OH)_2D_3$ was blocked by immuno-neutralizing antibody to IGFBP-3. LNCaP cells were seeded and grown in 96-well plates for 4 days in serum-free medium at density of 5,000 cell per well. 1,25-$(OH)_2D_3$ (10 nM.) or ethanol vehicle were added with 10 μg./ml. IGFBP-3 immuno-neutralizing antibody (*Ab-IGFBP-3*) or nonspecific IgG control (*NS-IgG*). Values are expressed as per cent of corresponding control and as mean plus or minus SEM of 3 experiments.

blocking the production of IGFBP-3 in PC-3 prostate cancer cells.[17]

To ensure that the antisense oligonucleotides significantly inhibited IGFBP-3 production we analyzed IGFBP-3 levels in the conditioned medium in sense and antisense treated cells. Adding 8 μg./ml. of antisense oligonucleotides to the growth medium significantly decreased IGFBP-3 production by LNCaP cells by 63%. In contrast, sense oligonucleotides did not significantly decrease IGFBP-3 in the medium.

After determining that IGFBP-3 antisense oligonucleotides inhibit IGFBP-3 production in these cells we added it to the growth medium (8 μg./ml.) to assess its effect on 1,25-$(OH)_2D_3$ mediated growth inhibition. Figure 4 shows that antisense treatment blocks the effects of 1,25-$(OH)_2D_3$ on LNCaP growth, further demonstrating the dependence of 1,25-$(OH)_2D_3$ on IGFBP-3 production. This finding strongly F4

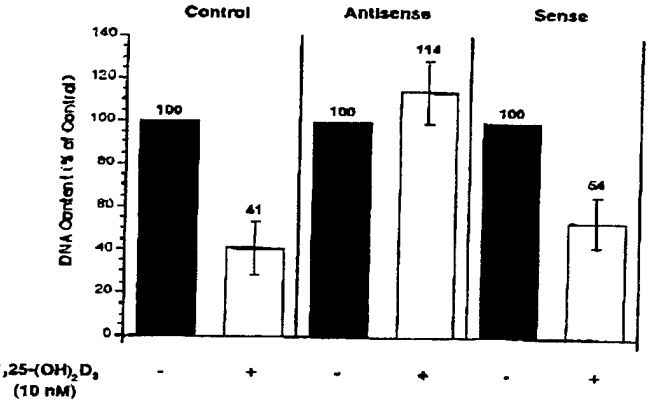

FIG. 4. LNCaP growth inhibition by 1,25-$(OH)_2D_3$ was abrogated by IGFBP-3 antisense oligonucleotides. Cells were seeded in 96-well plates and grown in serum-free growth medium for 4 days with 10 nM. 1,25-$(OH)_2D_3$ or vehicle plus 8 μg./ml. of antisense or sense IGFBP-3 oligonucleotides. DNA levels were determined at μg./ml. concentrations and each bar above was compared with corresponding control as percent of control. Values are shown as mean plus or minus SEM of 3 experiments.

indicates that 1,25-$(OH)_2D_3$ growth inhibitory activity depends on IGFBP-3 induction in the LNCaP cell line.

Moreover, we determined that IGFBP-3 up-regulates p21/WAF1 in LNCaP cells and p21/WAF1 induction by 1,25-$(OH)_2D_3$ is IGFBP-3 dependent. Because antisense oligonucleotides and immuno-neutralizing antibodies to IGFBP-3 eliminated the growth inhibitory effects of 1,25-$(OH)_2D_3$, we determined whether the cyclin dependent kinase inhibitor p21/WAF1 was induced by IGFBP-3. It has been established that the induction of p21/WAF1 is a mechanism by which 1,25-$(OH)_2D_3$ acts to inhibit cell growth.[11] Because blocking IGFBP-3 completely abrogated the effects of 1,25-$(OH)_2D_3$ in LNCaP cells, it was of interest to determine whether IGFBP-3 also stimulates p21/WAF1. Figure 5, *A* and *B* shows that like 1,25-$(OH)_2D_3$, IGFBP-3 up-regulates p21/WAF1 protein. To describe further this mechanism we added IGFBP-3 immuno-neutralizing antibodies to LNCaP cells in the presence of 1,25-$(OH)_2D_3$ to determine whether 1,25-$(OH)_2D_3$ induction of p21/WAF1 protein is IGFBP-3-dependent. Figure 6 shows that active IGFBP-3 is a requirement for the p21/WAF1 induction of 1,25-$(OH)_2D_3$. F5 F6

DISCUSSION

Although 1,25-$(OH)_2D_3$ is a promising new therapy for prostate cancer, a complete understanding of the mechanisms by which it inhibits prostate cancer growth does not currently exist.[4] A number of important pathways appear to be activated by 1,25-$(OH)_2D_3$ that contribute to antiproliferative activity, including cell cycle arrest, differentiation and in some cells apoptosis.[7, 8, 10, 23] Based on the studies described we propose that an additional important pathway through which 1,25-$(OH)_2D_3$ may inhibit prostate cancer cell growth is the induction of the IGFBP-3 gene, which subsequently decreases the growth of prostate cancer cells.

Using the LNCaP cell line model of human prostate cancer we have shown that 1,25-$(OH)_2D_3$ induces IGFBP-3. This induction has been demonstrated to occur at the level of mRNA and protein. There is significant up-regulation of IGFBP-3 at concentrations as low as 1 nM. 1,25-$(OH)_2D_3$ and up-regulation increases at higher doses, reaching a maximal plateau of 3-fold induction of the mRNA and protein levels.

After we established that IGFBP-3 was induced by 1,25-$(OH)_2D_3$ administration in LNCaP cells we determined whether IGFBP-3 itself may cause growth inhibition. This

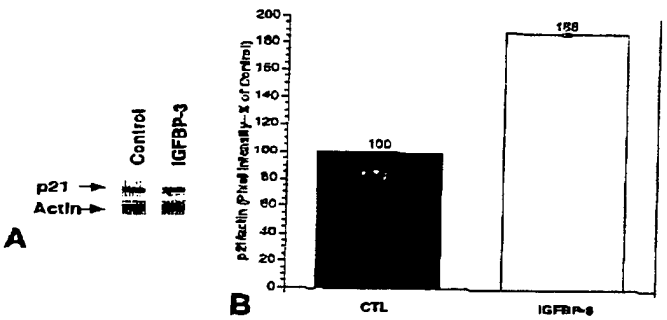

FIG. 5. IGFBP-3 up-regulated p21/WAF1 in LNCaP cells. LNCaP cells at density of 1×10^6 cells per flask were grown for 24 hours in OPTI-MEM-GM. Subsequently cells were treated for additional 24 hours with 100 ng./ml. IGFBP-3. After treatment cells were collected and proteins were analyzed by Western analysis. Mouse antihuman antibody to p21/WAF1 was used to detect p21/WAF1 in cell samples. All lanes were standardized for loading with anti-actin antibody. *A*, representative Western blot for p21/WAF1 (21 kDa.) and actin (42 kDa.). *B*, densitometry quantitation of the blot. Values are shown as percent of control (*CTL*), representing mean plus or minus range of 2 experiments.

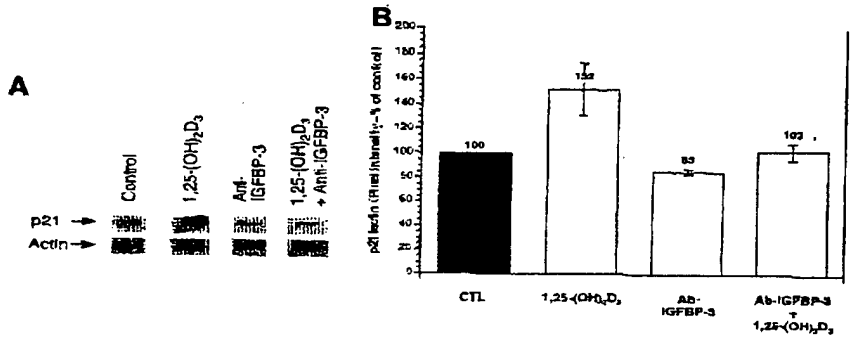

FIG. 6. p21/WAF1 induction by 1,25-$(OH)_2D_3$ depended on IGFBP-3. LNCaP cells at density of 1×10^6 cells per flask were grown for 24 hours in OPTI-MEM-GM. Subsequently cells were treated for additional 24 hours with 10 nM. 1,25-$(OH)_2D_3$ or ethanol vehicle plus 10 μg./ml. IGFBP-3 antibodies (*Ab-IGFBP-3*) or 10 μg./ml. nonspecific IgG. After treatment cells were collected and proteins were analyzed by Western analysis. Mouse antihuman antibody to p21/WAF1 was used to detect p21/WAF1 in cell samples. All lanes were standardized for loading with anti-actin antibody. *A*, representative Western blot for p21/WAF1 (21 kDa.) and actin (42 kDa.). *B*, densitometry quantitation of Western blot. Values are shown as percent of control (*CTL*), representing mean plus or minus range of 2 experiments.

point was important for supporting our hypothesis that 1,25-$(OH)_2D_3$ acts to induce growth arrest in these cells by inducing IGFBP-3. Others have reported that IGFBP-3 induces growth arrest in other cell types,[12,13,17] including prostate cancer, but to our knowledge it had not yet been investigated for LNCaP cells. We noted that IGFBP-3 added to the medium of LNCaP cells at a concentration as low as 10 ng./ml. caused significant growth inhibition with a maximal inhibition of up to 69% of the control level at 500 ng./ml.. This level of growth inhibition is similar to the effect of physiological doses of 1,25-$(OH)_2D_3$.[21] Therefore, the possibility of 1,25-$(OH)_2D_3$ induced growth inhibition functioning through IGFBP-3 regulation is plausible and concurs with previous studies of IGFBP-3 in other cell lines.

To this point our investigation had mostly confirmed and extended in LNCaP cells what had been described before in other cell types in regard to the 1,25-$(OH)_2D_3$ induction of IGFBP-3.[12,13] However, to our knowledge the degree to which 1,25-$(OH)_2D_3$ depended on IGFBP-3 induction to inhibit growth activity had not yet been clarified. Others have shown that in breast cancer cells IGFBP-3 is a requirement for growth inhibition by transforming growth factor-β and retinoic acid.[24,25] Therefore, the possibility existed that 1,25-$(OH)_2D_3$ growth inhibition would be mediated by IGFBP-3. To answer this question we first used an IGFBP-3 immuno-neutralizing antibody. When added to the growth medium of LNCaP cells, the ability of 1,25-$(OH)_2D_3$ to inhibit cell growth was clearly blocked by antibody binding of active IGFBP-3. Thus, we have shown for the first time that IGFBP-3 induction by 1,25-$(OH)_2D_3$ in LNCaP cells is required for 1,25-$(OH)_2D_3$ to inhibit growth.

Further supporting this finding are the antisense oligonucleotide data. Administering an oligonucleotide directed toward IGFBP-3 to inhibit its production in the LNCaP cells revealed that blocking production of IGFBP-3 also abrogated 1,25-$(OH)_2D_3$ induced growth inhibition. This finding, considered together with the IGFBP-3 immuno-neutralization antibody data, demonstrates that the induction of IGFBP-3 is a significant mechanism for 1,25-$(OH)_2D_3$ induced growth inhibition in LNCaP cells.

We were surprised that antisense oligonucleotides and immuno-neutralizing antibodies to IGFBP-3 each completely abrogated 1,25-$(OH)_2D_3$ growth inhibitory activity in LNCaP cells. As stated, the mechanism of action of 1,25-$(OH)_2D_3$ has been extensively investigated and several mechanisms have been shown to be induced by 1,25-$(OH)_2D_3$, including down-regulation of bcl-2 and c-myc, cell cycle arrest in the G1/G0 phase in several cancer cells and stimulation of the cell cycle inhibitor p21/WAF1.[7,8,10,11,26] Therefore, we expected that these other pathways would contribute some residual growth inhibiting activity when IGFBP-3 was blocked.

To explain the extent of the IGFBP-3 effect it should be noted that others reporting the mechanism of 1,25-$(OH)_2D_3$ action performed experiments in medium different from ours, often containing serum. To eliminate the effects of insulin-like growth factors and insulin-like growth factor binding proteins in serum, which was necessary in our IGFBP-3 antisense and antibody experiments, we used OPTI-MEM-GM, which supports LNCaP growth in the absence of insulin-like growth factors and insulin-like growth factor binding proteins. Thus, what occurred when we observed the complete blockade of 1,25-$(OH)_2D_3$ induced growth inhibition by IGFBP-3 antisense and antibody administration may have been an effect due to the type of growth medium used. Our results may be somewhat disproportionately dependent on IGFBP-3 because of the lack of other growth factors and steroids in our serum-free medium.

Another possible explanation for the complete growth inhibitory action of 1,25-$(OH)_2D_3$ by IGFBP-3 is that IGFBP-3 mediates other 1,25-$(OH)_2D_3$ induced mechanisms. Therefore, blocking its activity would also block these mechanisms. Our data showing that IGFBP-3 up-regulates p21/WAF1 protein in LNCaP cells provide some support for this hypothesis. Confirming previous reports on LNCaP cells,[9,11] 1,25-$(OH)_2D_3$ up-regulated p21/WAF1 protein on Western blot analysis. Interestingly, adding a growth inhibitory dose of IGFBP-3 revealed that p21/WAF1 is also induced by this factor. To our knowledge this report represents the first description of IGFBP-3 induction of p21/WAF1. Immuno-neutralization of IGFBP-3, which abrogates 1,25-$(OH)_2D_3$ induction of p21/WAF1, provided further support to our observation that IGFBP-3 induction is a required and integral process for 1,25-$(OH)_2D_3$ induced growth inhibition in LNCaP cells.

CONCLUSIONS

We believe that this study provides significant insight into the mechanism of action of 1,25-$(OH)_2D_3$ induced growth inhibition in prostate cancer cells by using the LNCaP cell line model. Our data show that IGFBP-3 is a 1,25-$(OH)_2D_3$ induced gene in these cells, has growth inhibitory activity and is an essential factor for mediating the growth inhibitory activity of 1,25-$(OH)_2D_3$. Since 1,25-$(OH)_2D_3$ and its analogs represent a promising therapy for prostate cancer, our study provides valuable insight into the mechanisms of action of 1,25-$(OH)_2D_3$ and its use in the treatment of prostate cancer.

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