

Apoptosis Induction by $1\alpha,25$ -Dihydroxyvitamin D_3 in Prostate Cancer¹

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Abstract

Calcitriol [$1\alpha,25$ -dihydroxyvitamin D_3] is the natural ligand of the vitamin D receptor (VDR). Using cultured prostate cancer (PC) cell lines, LN-CaP and ALVA-31, we studied the effects of $1\alpha,25(OH)_2$ -Vitamin D_3 (VD_3) on expression of several apoptosis-regulating proteins including: (a) Bcl-2 family proteins (Bcl-2, Bcl- X_L , Mcl-1, Bax, and Bak); (b) the heat shock protein 70-binding protein BAG1L; and (c) IAP family proteins (XIAP, cIAP1, and cIAP2). VD_3 induced decreases in levels of antiapoptotic proteins Bcl-2, Bcl- X_L , and Mcl-1, BAG1L, XIAP, cIAP1, and cIAP2 (without altering proapoptotic Bax and Bak) in association with increases in apoptosis. In contrast to VDR-expressing LN-CaP and ALVA-31 cells, VDR-deficient prostate cancer line Du-145 demonstrated no changes in apoptosis protein expression after treatment with VD_3 . In sensitive PC cell lines, VD_3 activates downstream effector protease, caspase-3, and upstream initiator protease caspase-9, the apical protease in the mitochondrial (“intrinsic”) pathway for apoptosis, but not caspase-8, an initiator caspase linked to an alternative (“extrinsic”) apoptosis pathway triggered by cytokine receptors. VD_3 induced declines in antiapoptotic proteins and also stimulated cytochrome c release from mitochondria by a caspase-independent mechanism. Moreover, apoptosis induction by VD_3 was suppressed by overexpressing Bcl-2, a known blocker of cytochrome c release, whereas the caspase-8 suppressor CrmA afforded little protection. Thus, VD_3 is capable of inhibiting expression of multiple antiapoptotic proteins in VDR-expressing prostate cancer cells, leading to activation of the mitochondrial pathway for apoptosis.

Introduction

Prostate cancer is the most common lethal form of malignancy in North American men and is responsible currently for >200,000 new cancer cases and >31,000 deaths annually in the United States alone. Although adenocarcinoma of the prostate is generally a slow-growing malignancy, morbidity

from the disease is nonetheless considerably high because of urological impairment and painful bone metastases. Because prostate cancer incidence increases with advancing age, it is expected that this malignancy will become an increasingly greater problem as worldwide life expectancy improves. The elderly and often debilitated status of many patients with metastatic prostate cancer has raised the need for well-tolerated palliative regimens that slow progression and improve quality of life. Biological response modifiers represent a class of agents with potential utility in this clinical context.

Calcitriol [$1\alpha,25(OH)_2$ -Vitamin D_3] is a member of a steroid hormone family that regulates calcium homeostasis and bone formation but which also has been shown to have significant antiproliferative activity when administered to many cancer cell lines *in vitro* (reviewed in Refs. 1, 2). The effects of VD_3 ³ are largely mediated via interaction with a specific nuclear VDR. The VDR is a ligand-dependent transcriptional regulator, belonging to the nuclear receptor superfamily (reviewed in Refs. 3, 4). VDR primarily interacts with specific DNA sequences composed of a hexanucleotide direct repeat, binding as either a homodimer or as heterodimer with RXR. Known target genes of VDR include the cell cycle inhibitors p21^{Waf1} and p27^{Kip1}, perhaps accounting in part for the antiproliferative effects on VDR ligands on some type of cells.

VDR ligands can also promote apoptosis of some types of tumor cell lines, including the prostate cancer line LN-CaP (5). The target genes relevant to the proapoptotic actions of VDR ligands are largely unknown, but Bcl-2 expression is reportedly down-regulated by treatment of LN-CaP cells with VD_3 . Moreover, enforcing Bcl-2 expression by gene transfer sustains survival of these cells in the face of VD_3 , implying an important role for this antiapoptotic protein that is known to be overexpressed in many advanced prostate cancers (6).

Bcl-2 is a member of a large family of apoptosis-regulating proteins that target mitochondrial membranes. This protein family includes both antiapoptotic members, such as Bcl-2, Bcl- X_L , and Mcl-1, and proapoptotic members such as Bax and Bak (reviewed in Ref. 7). These proteins govern mitochondrial membrane permeability, either promoting or suppressing release of apoptogenic proteins from these or-

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³ The abbreviations used are: VD_3 , $1\alpha,25(OH)_2$ -vitamin D_3 ; VDR, vitamin D receptor; RXR, retinoid X receptor; Cyt-c, cytochrome c; PARP, poly(ADP-ribose) polymerase; IAP, inhibitor of apoptosis protein; cRA, 9-*cis*-retinoic acid; DAPI, 4',6-diamino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; XTT, sodium 3'-(1-(phenylamino)-carbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro); Hsp, heat shock protein; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone; Ac-DEVD-AFC, benzoyloxycarbonyl-Asp-Glu-Val-Asp-amino-4-trifluoro-methyl-coumarin; Ac-LEHD-AFC, *N*-acetyl-Leu-Glu-His-Asp-AFC; Ac-IETD-AFC, *N*-acetyl-Ile-Glu-Thr-Asp-AFC.

ganelles. Among the mitochondrial proteins released into the cytosol during apoptosis is Cyt-c, which binds and activates Apaf-1, an oligomeric protein that activates the cell death protease, pro-caspase-9. This event triggers a cascade of proteolytic events, with caspase-9 cleaving and activating downstream caspases, which then cleave a variety of substrates responsible for apoptotic demise of the cell.

Additional nonmitochondrial pathways for apoptosis also exist, such as the pathway activated by tumor necrosis factor family death receptors. Upon ligand binding, these death receptors cluster at the plasma membrane, recruiting caspase-binding adapter proteins to their cytosolic domains and triggering protease activation by the induced proximity mechanism (reviewed in Ref. 8). The apical protease in the death receptor pathway is caspase-8, which in turn cleaves and activates directly or indirectly various downstream effector proteases, caspase-3, caspase-6, and caspase-7. Cleavage of specific substrates by effector caspases during apoptosis promotes the degradation of key structural proteins, including PARP and endonuclease regulator ICAD, causing chromatin condensation, DNA fragmentation, and other events typically associated with apoptosis.

The IAPs are a family of antiapoptotic proteins that regulate both the mitochondrial ("intrinsic") and death receptor ("extrinsic") pathways for apoptosis. The antiapoptotic activity of IAPs has been attributed to the conserved baculovirus IAP repeat domain, which is found in all members of this protein family (9). Some of the human IAPs, including XIAPs, c-IAP1, and c-IAP2, have been shown to directly bind pro-caspase-9 and prevent its activation in response to Cyt-c (10) and to also directly suppress the protease activity of the effector proteases, caspase-3 and caspase-7 (11). Thus, IAPs serve as endogenous antagonists of certain caspases, including downstream proteases that operate at the convergence of the intrinsic and extrinsic pathways for apoptosis. Overexpression of certain IAPs has been documented in cancers, often correlating with adverse clinical outcome (12).

In this report, we characterized the effects on apoptosis gene expression of calcitriol (VD₃) in the VDR-expressing prostate cancer cell lines LN-CaP and ALVA-31. Comparisons were also made with the RXR ligand, cRA, alone and in combination with VD₃. Our findings reveal that VD₃ down-regulates the expression not only of Bcl-2 but also several other antiapoptotic proteins, including Bcl-2 family members, Bcl-X_L and Mcl-1, as well as IAP-family proteins, XIAP, cIAP1, and cIAP2. Dissection of the apoptosis mechanism suggests that VD₃ primarily triggers the mitochondrial pathway for cell death. The findings suggest that VDR ligands could be useful for inducing apoptosis of prostate cancer cells directly or for lowering barriers to apoptosis in prostate cancers before treatment with cytotoxic anticancer drugs or radiation therapy.

Materials and Methods

VDR and RXR Ligands. VD₃ was obtained from Fluka, (Sigma Chemical Co., St. Louis, MO). cRA was obtained from Sigma. These VDR and RXR ligands were prepared as 10⁻³ M stock solutions in ethanol and stored at -20°C. Stock solution concentrations were confirmed by spectroscopy

(Spectra Max 190; Molecular Devices), using an extinction coefficient at 220–290 nm of ϵ 18300 for 1 α ,25(OH)₂D₃ (13) and 280–388 nm of ϵ 39750 for cRA (14).

Cell Culture and Apoptosis Assays. Human prostate cancer cell lines, Du-145 and LN-CaP, were obtained from American Type Culture Collection (Rockville, MD). The ALVA-31 human prostate cancer cell line was generously provided by Dr. G. Miller (University of Colorado, Denver, CO; Ref. 15). Cells were maintained in humidified atmosphere with 5% CO₂ in RPMI 1640, supplemented with 10% FCS, 1 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Irvine Scientific, Santa Ana, CA). ALVA-31 and LN-CaP cells were cultured with VD₃, cRA, or both of these agents, for up to 4 days. The medium was changed every 2 days, and new hormones were added to the cultures. In some cases, cells were treated with 100 ng/ml of anti-Fas antibody CH-11 (Naka-ku, Nagoya, Japan).

For apoptosis assays, both floating and adherent cells were recovered, and fixed in 3.7% paraformaldehyde in PBS (pH 7.4), and nuclear apoptotic morphology was determined by staining with 2 μ g/ml DAPI (mean \pm SD; n = 3) with examination by UV microscopy, as described (16). Alternatively, the percentage of cells with fragmented DNA was assayed by the TUNEL method, as described below. In some cases, results were confirmed by labeling of freshly recovered cells with Annexin V-FITC (Biovision, Palo Alto, CA), followed by analysis by flow cytometry (Becton-Dickinson FACScan, San Jose, CA) in the presence of propidium iodide, as described (17). Alternatively, to assess the percentage of cells with fragmented DNA and to monitor cell cycle progression, DNA content analysis was performed by fixing and permeabilizing cells, followed by treatment with RNase and staining with propidium iodide, analyzing cells by flow cytometry essentially as described previously (18). The relative proportion of cells with DNA content indicative of apoptosis (<2N), diploid G₀-G₁ cells (2N), S-phase (>2N but <4N), and G₂-M-phase (4N) was determined.

For cell viability assessments, the trypan blue dye exclusion method was used, counting a minimum of 200 cells/assay and expressing data as a percentage of viable (dye-excluding) cells. Alternatively, relative numbers of viable cells were determined by XTT (Polysciences, Warrington, PA) dye-reduction assays (19). Briefly, cells were seeded at a density of ~2000 cells/well in 96-well, flat-bottomed tissue culture plates (Corning Inc., Corning, NY) in 200 μ l of culture medium. The cells were allowed to attach for 24 h, and the medium was replaced with fresh medium containing either ethanol diluent (control), various concentrations of VD₃, cRA, or a combination of these agents. The medium containing vehicle or test compounds was renewed every 2 days during the course of experiments. After incubation, cells were processed by replacing the medium with fresh RPMI 1640 containing XTT reagent (50 μ l of 0.025 mM PMS-XTT reagent/well). Plates were incubated 37°C in a humidified atmosphere of 5% CO₂ for ~4 h. Absorbance at 450 nm was read using an automated plate reader (Power Wave 340; Biotechnology Institute, Winooski, VT) using the KC4 program. Pilot assays demonstrated linear production of XTT substrate product for cell densities up to 40,000 cells/well,

which was empirically determined to be within the linear range of the assay under these culture conditions. All experiments were performed in triplicate.

TUNEL Assays. Floating and adherent cells were harvested by trypsinization of cells into culture medium, pelleted by centrifugation, and fixed in 1% formaldehyde in PBS (Sigma) for 15 min on ice. Cells were then recentrifuged at $\sim 200 \times g$ for 5 min, washed with PBS, and treated with 70% ethyl alcohol. Then, 5×10^5 cells/sample were centrifuged for 5 min, washed in PBS, and resuspended in 50 μ l of a DNA deoxynucleotidyl-transferase (terminal transferase) reaction mixture (Roche Molecular Biochem, Indianapolis, IN), using the manufacturer's protocol for labeling DNA ends with Bio-16-dUTP. After incubation at 37°C for 30 min, 1 ml of PBS was added, followed by centrifugation as before. The cell pellet was suspended in 100 μ l of an avidin-FITC buffer consisting of 2.8 μ g/ml Avidin-FITC (Sigma), $4 \times$ SSC ($20 \times$ SSC: 3 M NaCl, 0.3 M sodium citrate (pH 7.0), 0.1% Triton X-100, 5% nonfat dried milk (prepared in 0.02% NaN_3 ; Refs. 20, 21). After incubation at room temperature for 30 min in the dark, the cells were washed once in 1 ml of PBS and then resuspended in 1% formaldehyde/PBS, and propidium iodide was added to a final concentration of 5 μ g/ml (Becton-Dickinson) before analysis of samples using a fluorescence-activated cell sorter (Becton-Dickinson FACStar-Plus), using the Cell Quest program data analysis. All experiments were repeated three times.

Antibodies and Immunoblotting. Cell lysates were prepared using RIPA buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA], normalized for total protein content (25 μ g of protein), and subjected to SDS-PAGE using 12% gels, followed by electrotransfer to 0.45 μ m nitrocellulose transfer membranes (Bio-Rad, Hercules, CA). Blots were incubated as described (22) with primary antibodies, including 1:250 (v/v) mouse antihuman p21 monoclonal antibody (IgG1; PharMingen, San Diego, CA), 1:1000 (v/v) mouse anti-PARP monoclonal antibody (PharMingen), 1:1000 (v/v) mouse anti-caspase-8 monoclonal antibody (Alexis Corp., San Diego, CA), 2 μ g/ml purified anti-cleaved-caspase-3 rabbit polyclonal antibody (Alexis Corp.), 1:1000 (v/v) anticleft-caspase-3 (Asp 175) rabbit polyclonal antibody (Cell Signaling, Beverly, MA), 0.05 μ g/ml mouse anti-Hsp-60 monoclonal antibody (Stressgen, San Diego, CA), 1 μ g/ml purified mouse anti- β -tubulin monoclonal antibody, 1 μ g/ml purified mouse anti-Cyt-c monoclonal antibody (PharMingen), 1:2000 (v/v) of rabbit polyclonal antisera recognizing Bcl-2, Bax, or Mcl-1 (23–25), 1:1000 (v/v) of mouse monoclonal (IgG1) specific for human BAG1 (KS6C8; Ref. 26), 1:5000 (v/v) monoclonal anti β -actin clone AC-15 mouse ascites fluid (Sigma), 1:1000 (v/v) GFP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:200 (v/v) cIAP1 rabbit polyclonal antibody (Santa Cruz Biotechnology), 1:250 (v/v) hILP (XIAP) mouse monoclonal antibody, and 1.5 μ g/ml affinity-purified rabbit antihuman/mouse cIAP2 (R&D Systems, Minneapolis, MN). Immunodetection was accomplished using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA) and an enhanced chemiluminescence detection method

(ECL; Amersham/Pharmacia Biotechnology) with exposure to X-ray film (XAR; Eastman Kodak Co., Rochester, NY). Protein bands were quantified by scanning densitometry (low light imaging system, Chemilmager 4000; 4000×4.04 ; Alpha Innotech Corp., San Leandro, CA), and the intensity of each band was calculated as a percentage relative to the band intensity for control (untreated) samples.

Subcellular Fractionation. For Cyt-c release assays, ALVA-31 cells (1.2×10^6 cells/10-cm dish) were treated with 0.1 μ M VD_3 , with or without 50 μ M zVAD-fmk (Biomol, Plymouth Meeting, PA). At various times thereafter, cells were collected for subcellular fractionation analysis, as described previously (27), with slight modifications. Briefly, cells were recovered from cultures by centrifugation, washed with ice-cold PBS, resuspended in $5 \times$ volume of buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl_2 , 1 mM sodium EDTA, and 300 mM sucrose] containing protease inhibitors, and cells were lysed with 30 strokes of a Teflon homogenizer. Nuclei were discarded by centrifugation at $1,200 \times g$ for 10 min at 4°C, and the resulting postnuclear lysates were then centrifuged at $7000 \times g$ for 25 min at 4°C to obtain a membrane and organelle-enriched fraction (pellet) containing mitochondria. The cytosolic (supernatant) fraction was further clarified by centrifugation at $143,000 \times g$ for 60 min at 4°C before analysis. Fractions were normalized for cell equivalents and analyzed by immunoblotting, using antibodies specific for Cyt-c (PharMingen), mitochondrial Hsp-60 (Stressgen), and cytosolic β -tubulin (PharMingen).

Caspase Activity Assays. ALVA-31 cells were stimulated with VD_3 , cRA, or the combination of these agents, in the presence or absence of 50 μ M zVAD-fmk. At various times thereafter, the cells were lysed on ice in caspase buffer [10 mM Tris (pH 7.3), 25 mM NaCl, 0.25% Triton X-100, and 1 mM EDTA]. Caspase activity in the resulting cell lysates was determined using fluorogenic peptide substrates, including Ac-DEVD-AFC and Ac-LEHD-AFC (Enzyme Systems, Livermore, CA), which had been prepared as stock solutions at 10 mM in DMSO and diluted into caspase buffer immediately before assays. Cell lysates (25 μ g protein) were incubated at 30°C with 100 μ M LEDH-AFC for caspase-9 activity or DEVD-AFC for caspase-3/caspase-7 activity measurements. For caspase-8 activity assays, 40 μ M Ac-IETD-AFC (Biomol, Plymouth Meeting, PA) was used in caspase buffer [50 mM HEPES (pH 7.4), 10% sucrose, 1 mM EDTA, 0.1% 3-[[3-(cholamidopropyl)dimethylammonio-1-propanesulfonate], 100 mM NaCl, and 10 mM DTT], measuring substrate cleavage at 37°C. Initial rates of substrate hydrolysis were determined using a Tecan spectroFluor fluorimeter in kinetic mode, with excitation at 400 nm and emission at 500 nm (slit width, 30 nm).

Transfections. ALVA-31 and LN-CaP cells were transiently transfected with various plasmids using LipofectAMINE reagent (Carlsbad, CA). Briefly, cells at $\sim 50\%$ confluence ($\sim 2 \times 10^5$ cells) in 6-well (9.4-cm²) plates (Corning, Inc., Corning, NY) in 2 ml/well of steroid-depleted medium were incubated with 1–2.2 μ g of plasmid DNA (1 μ g pEGFP *versus* pEGFP-Crm A; Refs. 28, 29) or 2.2 μ g pRc-CMV *versus* pRcCMV-Bcl-2 (30) combined with 6 μ l of LipofectAMINE in a total volume of 375 μ l of Opti-MEM medium (Life Technologies, Inc., Grand Island, NY) and

incubated for ~0.5 h. Adherent cells were washed twice with serum-free, prewarmed Opti-MEM, and then DNA/LipofectAMINE mixtures were applied in 750- μ l total volume of Opti-MEM. After culturing at 37°C and 5% CO₂ for 3 h, 1.5 ml of 20% charcoal-stripped FCS-containing medium was added per well. After 36 h, medium was changed to fresh RPMI 1640-deficient medium supplied with charcoal-stripped FBS. 0.1 μ M VD₃, CH-11 (100 ng/ml), or VP-16 (100 μ M) was added, and cells were cultured for various times before performing apoptosis or immunoblot assays. Transfection efficiencies were routinely >70% based on transfecting or cotransfecting a GFP-encoding plasmid. Both floating and adherent cells (after trypsinization) were collected 4 days after addition of VD₃, 1.5 days after VP-16, and 16 h after CH-11 and analyzed by DAPI staining for assessing nuclear morphology.

Statistical Methods and Analysis. All experiments were performed in triplicate, unless otherwise indicated, and mean values were presented as \pm SE. A one-way ANOVA was used to statistically analyze the recorded data for XTT assays. When ANOVA indicated significant differences between treatment groups with respect to one of the data sets analyzed, the Dunnett Multiple Comparisons Test was used on that data set to ascertain where the differences occurred. Data on trypan blue exclusion and Annexin V were analyzed by Mann-Whitney *U* Test (31). The software used for these analyses was Graph Pad InStat, v2.02. For all statistical tests, *P* < 0.05 was considered to be significant.

Results

Effects of VD₃ and cRA on Prostate Cancer Cell Growth.

The effects of VD₃, cRA, and the combination of these biological response modifiers were tested on the growth of ALVA-31 prostate cancer cells, using an XTT assay for monitoring changes in the relative numbers of viable cells over time in culture. Treatment with concentrations of VD₃ ranging from 1 to 100 nM suppressed the growth of ALVA-31 cells, with ~50% reductions occurring at 6 days after adding VD₃ to cultures (Fig. 1A). cRA also suppressed ALVA-31 cell growth but was less potent than VD₃ when tested at similar concentrations. At some concentrations (Fig. 1A, right panel), the combination of cRA and VD₃ was superior to VD₃ alone at suppressing growth of ALVA-31 prostate cancer cells *in vitro*. Similar results were obtained either using LN-CaP cells instead of ALVA-31 or and when using propidium iodide staining for DNA content analysis as an alternative to XTT assays, providing evidence of G₁ arrest.

The suppression of cell growth seen in cultures of prostate cancer cells treated with VD₃ and cRA was associated with induction of p21^{Waf1}, an endogenous inhibitor of cyclin-dependent kinases (Fig. 1B). As determined by immunoblotting, levels of p21^{Waf1} increased over time, with higher levels of this cell cycle inhibitor induced in cells treated with VD₃ than cRA, consistent with the differential potency in the growth-suppressing activity of these agents. Loading of equivalent amounts of protein for all samples was confirmed by incubating the same blot with anti- β -tubulin antibody.

The XTT method does not distinguish between suppression of cell growth as a result of proliferation *versus* apo-

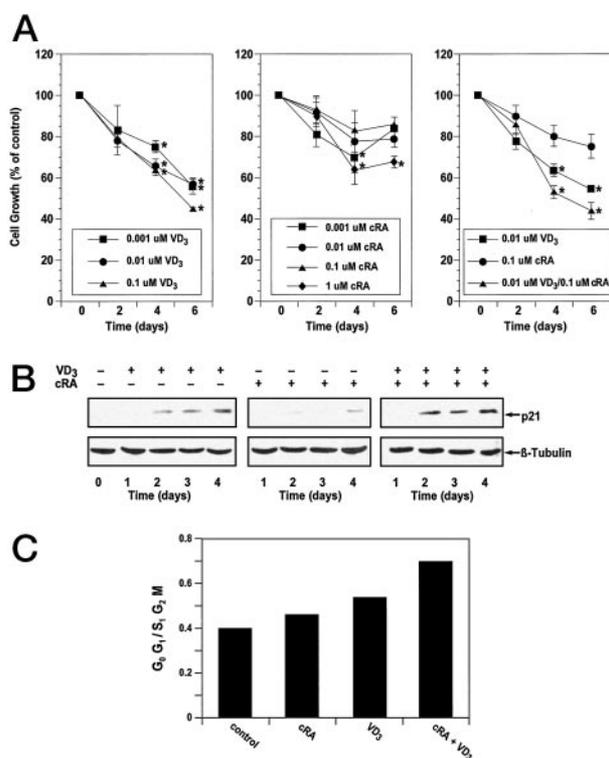
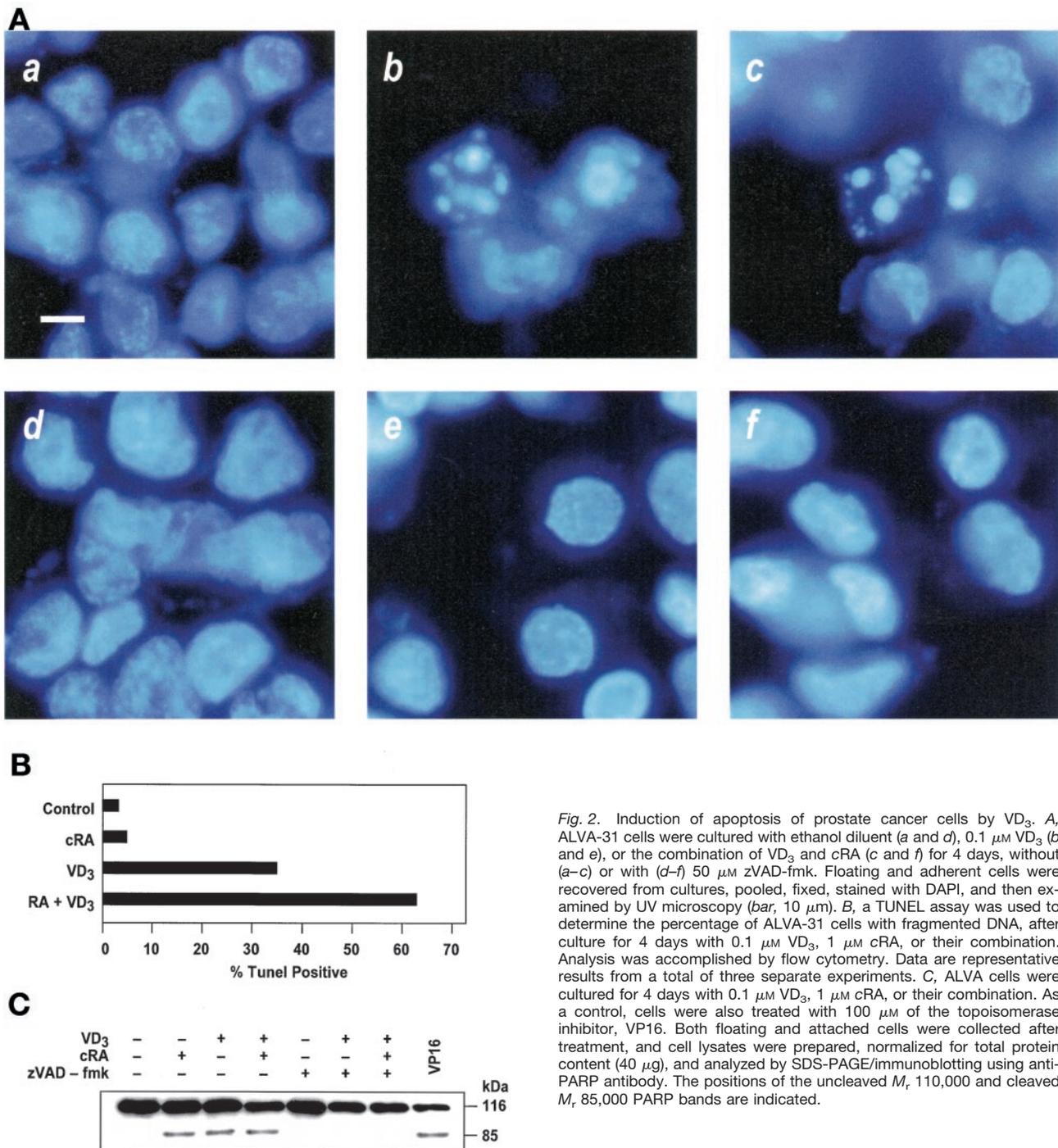


Fig. 1. VD₃ and cRA suppress growth of ALVA-31 prostate cancer cells. **A**, ALVA-31 cells were plated at 2000 cells/well in 96-well plates in 200 μ l of medium and cultured for up to 6 days with the indicated concentrations of VD₃ (left), cRA (middle), or the combination (right) of these agents. Medium was changed every 2 days and replaced with fresh medium containing VD₃ and cRA. Relative numbers of viable cells were measured by XTT assay, expressing data as a percentage relative to control (diluent only) treated cells (means; bars, SD; *n* = 3). *, statistically significant differences (*P* < 0.05). **B**, ALVA-31 cells were plated at 10⁶ cells/10-cm dish in 10 ml of medium with 0.1 μ M VD₃, 1 μ M cRA, or their combinations. Cell lysates were prepared after 0, 1, 2, 3, or 4 days of culture, normalized for total protein content (25 μ g/lane), and subjected to SDS-PAGE immunoblot assay using anti-p21^{Waf1}-specific antibody. The same blots were also incubated with an antibody recognizing β -tubulin. **C**, ALVA31 cells were cultured for 6 days with or without 5 nM VD₃, 1 μ M cRA, or both of these reagents, as indicated. Cells were recovered from cultured, fixed, permeabilized, treated with RNase, and then stained with propidium iodide. DNA content was analyzed by flow cytometry, and the relative proportion of cells with DNA contents indicative of G₀-G₁ phase was determined relative to cells with DNA content indicative of S or G₂-M-phase cells. A representative experiment is shown.

ptosis. Thus, to examine the effects of VD₃ and cRA on cell proliferation, we performed cell cycle analysis where cellular DNA content was measured in propidium iodide-stained cells by flow cytometry. As shown in Fig. 1C, even after 6 days of culture, either VD₃ or cRA had only a modest effect on prostate cancer cell cycling. In the experiment shown, for example, the ratio of cells with DNA content indicative of G₀-G₁ phase relative to cells in S and G₂-M phases was increased by <15% cRA treatment and by <35% by VD₃. In contrast, the combination of cRA and VD₃ was considerably more potent at inducing G₀-G₁ arrest, causing a >60% increase in the ratio of G₀-G₁:S/G₂-M cells (Fig. 1C).

Effects of VD₃ and cRA on Apoptosis of Prostate Cancer Cell Lines. In addition to suppressing proliferation of prostate cancer cells, we explored whether VD₃, cRA, or the



combination of these agents induces apoptosis of prostate cancer cells. Examination of DAPI-stained cells by UV microscopy showed clear evidence of increased percentages of cells with typical apoptotic morphology in cultures of ALVA-31 and LN-CaP cells treated with VD₃ and to a lesser extent with cRA. Fig. 2 presents some representative results for ALVA-31 cells treated for 4 days with VD₃, cRA, or the combination of these agents. Although precise time-course

Fig. 2. Induction of apoptosis of prostate cancer cells by VD₃. **A**, ALVA-31 cells were cultured with ethanol diluent (*a* and *d*), 0.1 μM VD₃ (*b* and *e*), or the combination of VD₃ and cRA (*c* and *f*) for 4 days, without (*a-c*) or with (*d-f*) 50 μM zVAD-fmk. Floating and adherent cells were recovered from cultures, pooled, fixed, stained with DAPI, and then examined by UV microscopy (*bar*, 10 μm). **B**, a TUNEL assay was used to determine the percentage of ALVA-31 cells with fragmented DNA, after culture for 4 days with 0.1 μM VD₃, 1 μM cRA, or their combination. Analysis was accomplished by flow cytometry. Data are representative results from a total of three separate experiments. **C**, ALVA cells were cultured for 4 days with 0.1 μM VD₃, 1 μM cRA, or their combination. As a control, cells were also treated with 100 μM of the topoisomerase inhibitor, VP16. Both floating and attached cells were collected after treatment, and cell lysates were prepared, normalized for total protein content (40 μg), and analyzed by SDS-PAGE/immunoblotting using anti-PARP antibody. The positions of the uncleaved *M_r* 110,000 and cleaved *M_r* 85,000 PARP bands are indicated.

analysis was not performed, the onset of apoptosis and cell cycle arrest occurred with similar kinetics in cultures of prostate cancer cells treated with VD₃ alone or in combination with cRA.

To confirm that the morphological changes were attributable to apoptosis, the broad-spectrum caspase inhibitor, zVAD-fmk, was included in cultures along with VD₃ and cRA. As shown in Fig. 2A (*panels d-f*), 50 μM zVAD-fmk prevented

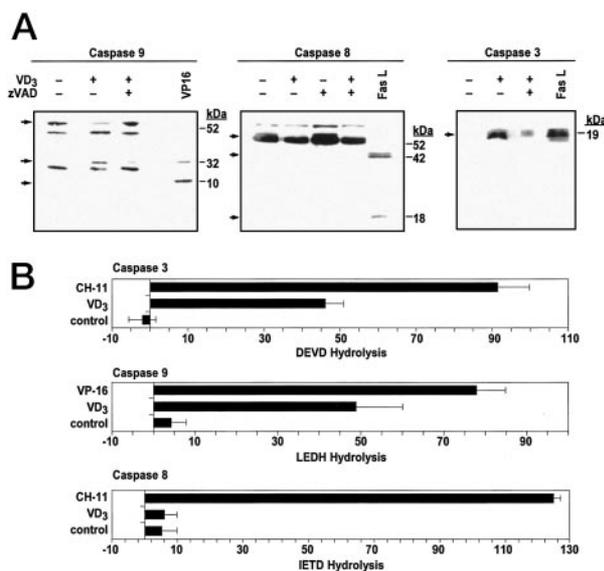


Fig. 3. VD₃ induces caspase-9 processing and activation in prostate cancer cells. **A**, immunoblot analysis was performed using lysates from ALVA-31 cells cultured with 0.1 μ M VD₃, 50 μ M zVAD-fmk, or a combination of these reagents for 4 days. Alternatively, cells were treated with 100 μ M VP16 for 2 days or with 100 ng/ml CH-11 antibody for 16 h before preparing lysates. After normalizing for total protein content (25 μ g), lysates were analyzed by SDS-PAGE/immunoblotting using antibodies specific for caspase-9 (left), caspase-8 (middle), or activate (cleaved) caspase-3 (right). Arrows, the positions of: left, full-length and cleaved large and small subunits of caspase-9; middle, full-length pro-caspase-8, a partially processed form of caspase-8, and the fully processed large subunit; and right, the processed large subunit of caspase-3. Molecular weight markers are indicated in thousands. **B**, ALVA-31 cells were cultured with 0.1 μ M VD₃, 1 μ M cRA, or both of these agents, in the absence or presence of 50 μ M zVAD-fmk. Cell lysates were prepared, normalized for total protein content, and analyzed for activity of various caspase-family proteases using the AFC-based fluorogenic substrates: top, Ac-DEVD-AFC (caspase-3); middle, Ac-LEHD-AFC; and below, Ac-IETD-AFC (caspase 8). Data represent relative fluorescent units/min (means; bars, SD; $n = 3$).

apoptosis, inhibiting chromatin condensation and nuclear fragmentation. Consistent with apoptosis induction, VD₃ also caused DNA fragmentation in cultured ALVA-31 and LN-CaP cells, as determined by TUNEL assays (Fig. 2B and data not shown). cRA, in contrast, was not effective at inducing significant increases in TUNEL-positive cells, although the combination of cRA and VD₃ was slightly more potent than either agent individually (Fig. 2B). Similar conclusions were reached by using an Annexin V/propidium iodide staining method to measure percentages of apoptotic cells, as well as based on DNA content analysis where the proportion of cells with subdiploid amounts of DNA were measured (not shown).

During apoptosis, activated caspases cleave various substrate proteins, including the nuclear protein PARP (32). We therefore assessed the effects of VD₃ and cRA on PARP cleavage by immunoblot analysis. VD₃, cRA, and the combination of these agents induced cleavage of $M_r \sim 110,000$ PARP, producing the $M_r \sim 85,000$ proteolytic product typical of caspase digestion (Fig. 2C). PARP cleavage was blocked by culturing ALVA-31 cells with caspase inhibitor, zVAD-fmk.

Assessment of Caspase Cleavage Patterns in VD₃-treated Prostate Cancer Cells. To preliminarily assess which caspase activation pathway might be responsible for

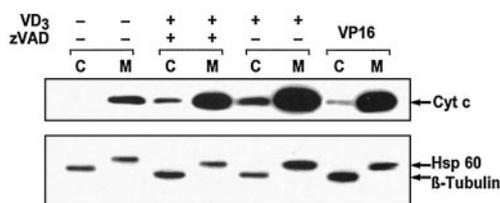


Fig. 4. VD₃ induces caspase-independent release of Cyt-c from mitochondria. ALVA-31 cells were cultured with 0.1 μ M VD₃ alone or together with 50 μ M caspase inhibitor zVAD-fmk, where zVAD-fmk was added to cultures 12 h before VD₃. Medium was changed every 2 days, replenishing VD₃ and zVAD-fmk. The cells were lysed in isotonic, detergent-free solution 4 days after addition of VD₃. Mitochondria-containing membrane (M) and cytosolic (C) fractions were prepared, normalized for cell equivalents, and analyzed by SDS-PAGE/immunoblotting using anti-Cyt-c antibody, as well as antibodies recognizing mitochondrial Hsp60 and cytosolic β -tubulin which served as loading controls, and verifying proper fractionation.

VD₃-mediated apoptosis, we analyzed lysates from ALVA-31 cells by immunoblotting using antibodies recognizing either caspase-8 or caspase-9, the apical proteases in the extrinsic and intrinsic pathways, respectively. As shown in Fig. 3A, VD₃ induced cleavage of pro-caspase-9, as ascertained by reduced levels of the $M_r \sim 50,000$ proform and appearance of a $M_r \sim 35,000$ band corresponding to the large subunit of processed caspase-9. The cleavage of pro-caspase-9 was partially blocked by zVAD-fmk, demonstrating the specificity of these results. Treatment of cells with the DNA-damaging agent VP16 served as a control for an apoptotic stimulus known to activate the intrinsic pathway. In contrast to caspase-9, treatment of prostate cancer cells with VD₃ failed to induce proteolytic cleavage of pro-caspase-8. As a control, culturing cells with anti-Fas antibody, CH-11, a known inducer of caspase-8 processing (Fig. 3A), stimulated caspase-8 cleavage. In addition to caspase-9, VD₃ also induced cleavage of pro-caspase-3, a downstream effector protease. Active caspase-3 was detected using an antibody that specifically recognizes the cleaved large subunit of the processed protease (Fig. 3A).

Caspase activity in VD₃-treated prostate cancer cells was also assessed by enzyme assays, measuring hydrolysis of fluorogenic peptide substrates in cell lysates. As shown in Fig. 3B, VD₃ induced increases in protease activity cleaving the caspase-9 substrate Ac-LEHD-AFC and the caspase-3 substrate Ac-DEVD-AFC but not the caspase-8 substrate Ac-IETD-AFC. Treatment of prostate cancer cells with anti-Fas antibody (CH-11) or VP16 served as positive controls for activation of caspase-8 and caspase-9, respectively (Fig. 3B).

VD₃ Induces Caspase-independent Release of Cyt-c from Mitochondria.

Because Caspase-9 activation commonly results from release of mitochondrial Cyt-c (33), we explored the effects of VD₃ on Cyt-c using subcellular fractionation methods. For these experiments, mitochondria-containing membrane (M) and soluble cytosolic (C) fractions were compared by immunoblotting using anti-Cyt-c antibody. As shown in Fig. 4, nearly all of the Cyt-c was found in M fractions before VD₃ treatment. After culturing prostate cancer cells with VD₃, marked increases in cytosolic Cyt-c

were detected. The release of Cyt-c into the cytosol was not inhibited by zVAD-fmk, indicating a caspase-independent process. Incubating the same blots with antibodies to mitochondrial Hsp60 and cytosolic β -tubulin confirmed successful fractionation of the relevant cellular compartments (Fig. 4). Taken together, these data suggest that VD_3 directly induces release of Cyt-c from mitochondria without requirement for caspases.

Enforced Overexpression of Bcl-2 Protects Prostate Cancer Cells from VD_3 -induced Apoptosis. To further explore the whether VD_3 induces apoptosis primarily through the intrinsic pathway, we transiently transfected ALVA-31 cells with a plasmid encoding Bcl-2 protein, an inhibitor of Cyt-c release and suppressor of the intrinsic pathway for caspase activation. In addition, transfectants were generated that express the CrmA protein, a potent inhibitor of caspase-8, which suppresses the extrinsic pathway for cell death (34). Expression of plasmid-derived Bcl-2 and CrmA proteins was verified by immunoblotting. Comparisons were made with control-transfected ALVA-31 cells that received the same plasmid lacking a cDNA insert (Fig. 5, A and B). Expression efficiency was similar for all transfectants, as determined by cotransfection with a GFP encoding plasmid.

ALVA-31 cells overexpressing Bcl-2 displayed marked resistance to apoptosis induced either by VD_3 or by the DNA-damaging agent VP16 (Fig. 5C), which was included as a positive control (35). In contrast, CrmA expression in ALVA-31 cells had comparatively little effect on VD_3 -induced apoptosis (Fig. 5D). Although reducing the percentage of apoptotic cells, CrmA-mediated protection from VD_3 failed to reach statistical significance, suggesting a relatively minor role for the extrinsic pathway in VD_3 -induced apoptosis. In contrast, CrmA potently protected ALVA-31 cells from apoptosis induced by anti-Fas antibody CH11 (Fig. 5D), confirming the functionality of this antiapoptotic protein. Altogether, these data lend further support to the contention that VD_3 induces apoptosis predominantly through the intrinsic pathway.

VD_3 Down-Regulates Expression of Antiapoptotic Bcl-2 Family Proteins in Prostate Cancer Cells. Because Bcl-2 family members are critical regulators of mitochondrial release of Cyt-c (8), we assessed the effects of VD_3 , cRA, and the combination of these agents on the expression of the Bcl-2 family proteins Bcl-2, Bcl- X_L , Mcl-1, Bax, and Bak by immunoblotting. For these experiments, ALVA-31 cells were cultured for 1–4 days with 0.1 μM VD_3 , 1 μM cRA, or the combination of these reagents (note that the combination of 0.1 μM VD_3 and 1 μM cRA showed similar cell growth suppression and apoptotic effects compared with 0.05 μM VD_3 + 0.5 μM cRA; data not shown). Lysates were prepared on sequential days (1–4 days) and analyzed by immunoblotting, using antibodies specific for these Bcl-2-family proteins. At 0.1 μM , a pharmacologically relevant concentration, VD_3 induced time-dependent declines in the steady-state levels of antiapoptotic proteins Bcl-2, Bcl- X_L , and Mcl-1, without substantially changing the levels of proapoptotic proteins, Bax and Bak (Fig. 6). On the basis of quantification of the data by scanning densitometry, reductions in the steady-state levels of Bcl-2, Bcl- X_L , and Mcl-1 began within 1 day of treatment

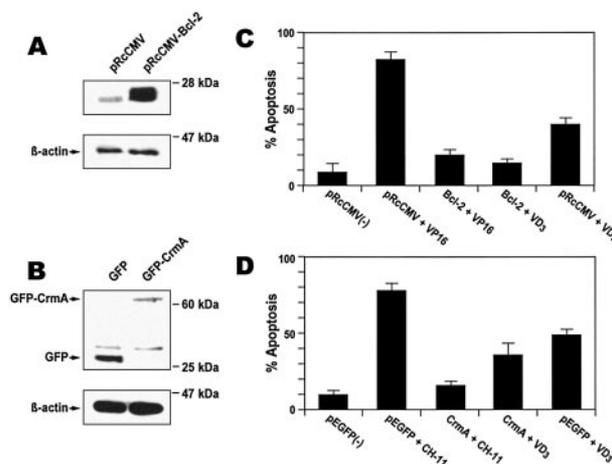


Fig. 5. Overexpression of Bcl-2 suppresses VD_3 -induced apoptosis. ALVA-31 cells were transiently transfected with pRCCMV control or pRCCMV-Bcl-2 plasmids (A) or with pEGFP ("GFP") or pEGFP-CrmA (B). Lysates were prepared from transiently transfected cells treated with vehicle and apoptosis-inducing agents (VD_3 and VP-16) normalized for total protein content (25 μg /lane) and analyzed by SDS-PAGE/immunoblotting using antibodies specific for Bcl-2 (A) and GFP (B). Blots were also incubated with anti- β -tubulin antibody as a control for loading. C and D, transfectants of ALVA-31 cells were cultured with or without 0.1 μM VD_3 for 4 days, 100 μM VP16 for 1.5 days, or 100 ng/ml CH11 antibody for 16 h, as indicated, and the percentage of apoptotic cells was determined by DAPI staining (means; bars, SD; $n = 3$).

with VD_3 , declining to ~20–25% of control by 2–3 days (Fig. 6C). cRA (1 μM) was comparatively less potent than VD_3 (0.1 μM) at inducing decreases in the levels of Bcl-2 and Bcl- X_L , although its effects on Mcl-1 expression were similar to VD_3 at the doses tested. The combination of cRA and VD_3 was only slightly more effective than VD_3 alone at reducing expression of antiapoptotic Bcl-2 family proteins, with the clearest difference observed for Bcl- X_L (Fig. 6C). These effects of VD_3 and cRA on expression of Bcl-2 family proteins were caspase-independent, as determined by experiments in which ALVA-31 cells were pretreated with zVAD-fmk, demonstrating that VD_3 and cRA reduce levels of these proteins even in the presence of a broad-spectrum caspase inhibitor (data not shown).

VD_3 Reduces Levels of Bag1L Protein. Although VD_3 was found to suppress the expression of several antiapoptotic Bcl-2 family proteins, we explored the possibility that this steroid hormone might additionally have effects on other apoptosis regulators. The human *BAG1* gene encodes at least two Hsp70-binding proteins, including M_r ~50,000 nuclear and M_r ~36,000 cytosolic proteins, which have been implicated in apoptosis suppression and other functions (26, 36). We examined the effects of VD_3 , cRA, and the combination of these agents on levels of p36 Bag1 and p50 Bag1L in ALVA-31 prostate cancer cells by immunoblotting, as described above. When applied individually, VD_3 and cRA each profoundly down-regulated levels of p50 Bag1L, having less inhibitory influence on p36 Bag1 (Fig. 6B). The combination of VD_3 and cRA resulted in more rapid decline in p50 Bag1L levels compared with treatment of ALVA-31 cells with either agent alone (Fig. 6C). Thus, in addition to Bcl-2 family pro-

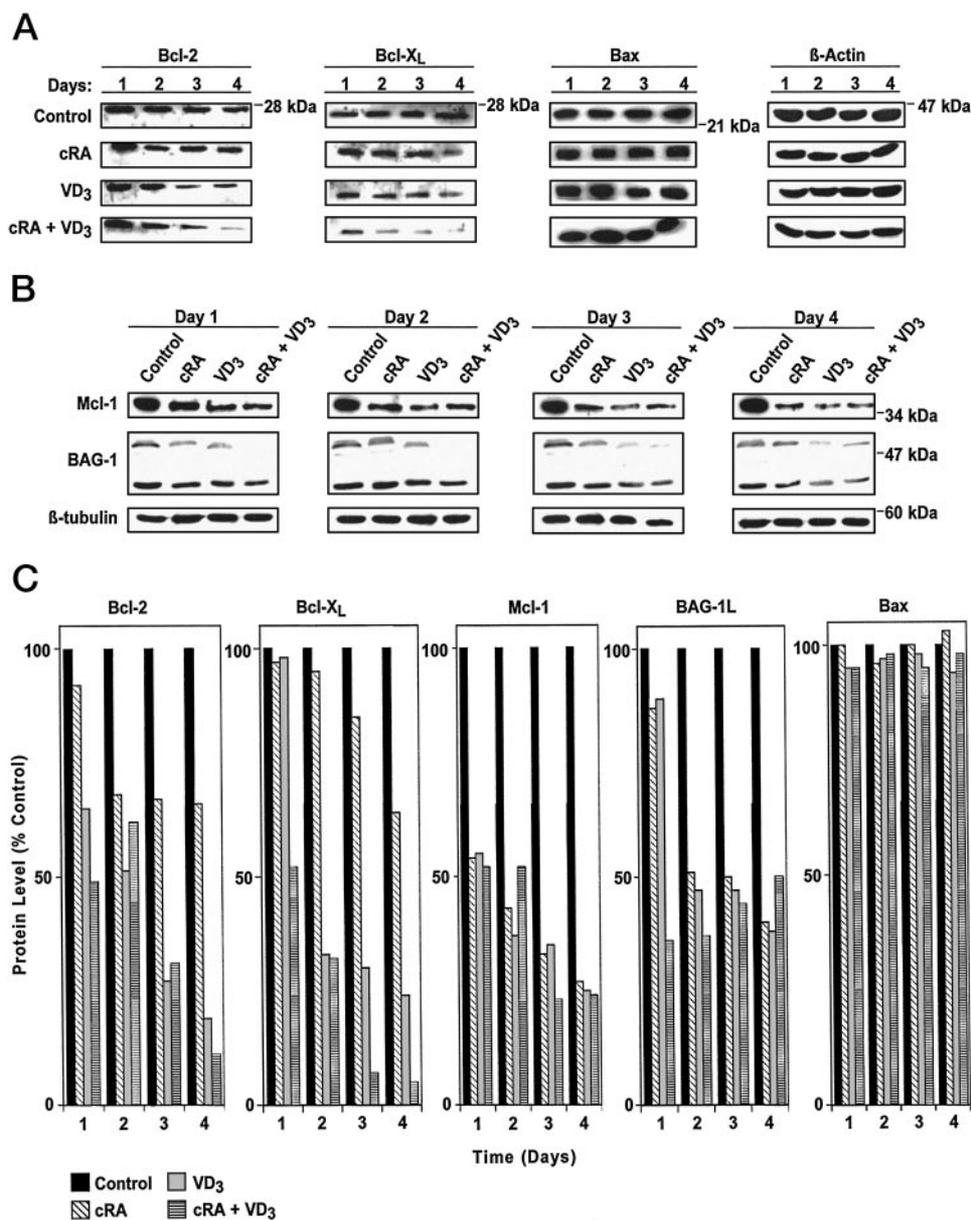


Fig. 6. VD₃ down-regulates expression of antiapoptotic proteins in prostate cancer cell lines. A, ALVA cells were cultured for 1–4 days with 0.1 μM VD₃, 1 μM cRA, or their combination. Cell lysates were prepared, normalized for total protein content (25 μg/lane), and subjected to SDS-PAGE/immunoblot assay (12% gels) using various antibodies. All data are representative of at least three experiments. A, the time course of changes in protein levels was examined for Bcl-2, Bcl-X_L, and Bax, using β-actin as a control. B, side-by-side comparisons of effects of VD₃, cRA, and the combination of VD₃ and cRA were performed at days 1–4, using antibodies recognizing Mcl-1, p50 Bag1L, and p36 Bag1, with β-tubulin serving as a control. C, immunoblot data were quantified by scanning densitometry, normalized relative to β-actin or β-tubulin loading controls, and expressed as a percentage relative to diluent-treated (control) cells. Representative data for Bcl-2, Bcl-X_L, Mcl-1, Bag1L, and Bax are presented.

teins, VD₃ also inhibits expression of Bag1L. The reductions in Bag1L levels induced by VD₃ and cRA were caspase-independent, as determined by experiments in which ALVA-31 cells were pretreated with zVAD-fmk, demonstrating that VD₃ and cRA reduce levels of Bag1L even in the presence of a broad-spectrum caspase inhibitor (data not shown).

VD₃ Reduces Levels of IAP-Family Proteins in VDR-expressing Prostate Cancer Cells. The human IAP-family members XIAP, cIAP1 and cIAP2 are antiapoptotic proteins, which have been demonstrated to directly bind and inhibit certain caspases (11, 37). We analyzed the effects of 0.1 μM VD₃, 1 μM cRA, and the combination of VD₃ and cRA on levels of XIAP (Fig. 7A), cIAP1 (Fig. 7B), and cIAP2 (Fig. 7C) proteins by immunoblotting in the VDR-expressing prostate

cancer cell lines ALVA-31 and LN-CaP. The VDR-insensitive prostate cancer line Du-145 was also tested, as a control. VD₃ reduced levels of XIAP and cIAP1 in both ALVA-31 and LN-CaP but not Du-145 cells. Levels of cIAP2 were also reduced in LN-CaP cells treated with VD₃ but not in the other cell lines tested. Pretreatment of cells with zVAD-fmk demonstrated that VD₃-induced reductions in levels of IAPs were largely, but not entirely, caspase-independent (Fig. 7).

cRA had more variable effects on IAP expression, slightly reducing XIAP levels in ALVA-31 and LN-CaP cells, partially depressing levels of cIAP1 in LN-CaP but not ALVA-31, and markedly reducing levels of cIAP2 in LN-CaP but not ALVA-31 cells. IAP expression was not affected by cRA treatment of Du-145 cells (Fig. 7). Treatment of ALVA-31 and LN-CaP cells with the combination of VD₃ and cRA resulted

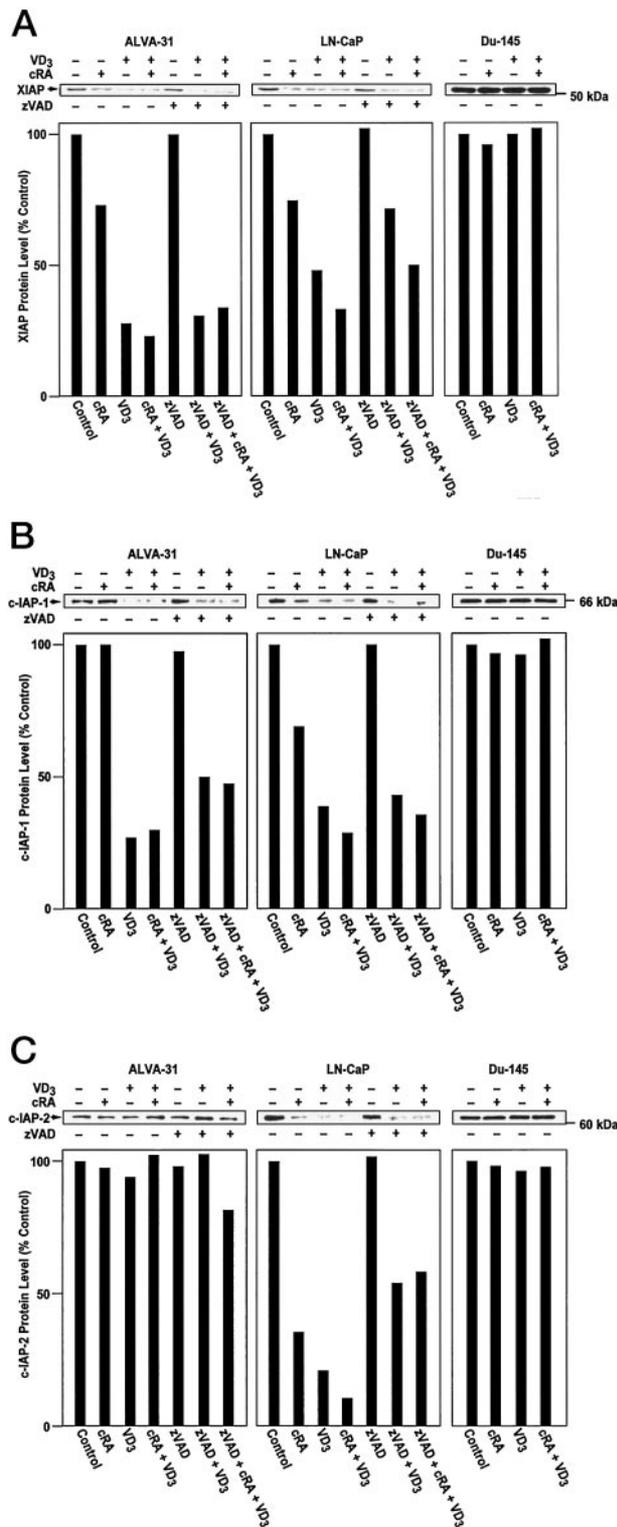


Fig. 7. VD₃ reduces levels of IAP-family proteins in prostate cancer cell lines. Prostate cancer cell lines ALVA-31 (left), LN-CaP (middle), and Du-145 (right) were cultured for 4 days with 0.1 μM VD₃, 1 μM cRA, or their combinations. In some cases, 50 μM caspase inhibitor zVAD-fmk was added to cultures 12 h before VD₃ and cRA. Medium was changed every 2 days, replenishing VD₃, cRA, and zVAD-fmk. Cell lysates were prepared, normalized for total protein content (25 μg/lane), and subjected to SDS-PAGE/immunoblot assay (12% gels) using antibodies recognizing XIAP

in suppression of IAP-family proteins, similar to VD₃ alone. Again, the suppression of IAP expression by the combination of cRA and VD₃ was partially caspase-independent.

Discussion

The discovery of VDR within the epithelial cells of the prostate almost a decade ago initiated numerous studies to explore the effects of VDR ligands on malignant cells originating from this gland, demonstrating that VD₃ is capable of inhibiting the growth of at least some prostate cancers (1). Recent investigations have provided evidence that this growth inhibition involves both reduced cell division and increased apoptosis (6). Here we confirmed the apoptosis-inducing effects of the naturally occurring VDR ligand, VD₃, on two VDR-expressing prostate cancer cell lines ALVA-31 and LN-CaP and explored the mechanisms involved.

Our data indicate that VD₃ activates the intrinsic pathway for apoptosis in prostate cancer cell lines. The evidence supporting this conclusion includes: (a) proteolytic processing of pro-caspase-9 but not pro-caspase-8; (b) induction of LEHD (caspase-9) but not IETD (caspase-8) protease activity; (c) caspase-independent release of Cyt-c from mitochondria; and (d) suppression of VD₃-induced apoptosis by overexpression of Bcl-2 but not CrmA. These findings thus confirm and extend previous studies of the action of VD₃ in human prostate cancer cell lines (6).

The mechanism by which VD₃ induces mitochondrial release of Cyt-c remains to be elucidated. One potential mechanism involves reducing expression of antiapoptotic Bcl-2 family proteins, Bcl-2, Bcl-X_L, and Mcl-1, without affecting levels of proapoptotic proteins, Bax and Bak. Bcl-2 family proteins are central regulators of Cyt-c release from mitochondria, with ratios of anti- and proapoptotic Bcl-2 family members dictating whether Cyt-c remains sequestered in these organelles or is released into the cytosol (reviewed in Ref. 8). Thus, VD₃-mediated down-regulation of the expression of these antiapoptotic Bcl-2 family proteins may precipitate Cyt-c release in prostate cancer cells. Future studies will determine whether VD₃ induces reductions in Bcl-2, Bcl-X_L, and Mcl-1 protein levels through direct transcriptional mechanisms *versus* alternative indirect mechanisms that may involve posttranscriptional steps in gene regulation. In this regard, the delayed time course with which VD₃ triggers reductions in Bcl-2-family proteins tends to suggest an indirect mechanism.

In addition to Bcl-2-family proteins, we observed that VD₃ also reduced levels of other types of proteins known to be involved in apoptosis suppression, including IAP-family members. Although it is possible that VD₃ directly reduces expression of IAP-family gene via VDR-mediated transcriptional mechanisms, the expression of XIAP, cIAP1, and cIAP2 has been reported to be controlled largely through

(A), cIAP1 (B), or cIAP2 (C), followed by antibody detection using an ECL method with exposure to X-ray film. Immunoblot data (top) were quantified by scanning densitometry, expressing results as a percentage relative to diluent-treated (control) cells (bottom). All data are representative of at least three experiments.

posttranscriptional mechanisms (38, 39). In this regard, these members of the IAP-family possess an E3-like function because of their ability to bind ubiquitin-conjugating enzymes, inducing their own proteasome-dependent degradation (40). Thus, VD₃ may reduce expression of IAPs by indirect mechanisms. Moreover, more than one mechanism may contribute to the reduced levels of IAPs seen in VD₃-treated prostate cancer cells, given that a caspase-inhibitory compound (zVAD-fmk) partially prevented VD₃-mediated down-regulation of some IAPs in some prostate cancer cell lines.

The caspases that are inhibited by XIAP, cIAP1, and cIAP2 operate in the intrinsic pathway (caspase-9) and at the convergence of the intrinsic and extrinsic pathways (caspase-3 and caspase-7). Thus, the ability of VD₃ to activate the intrinsic pathway could be partly attributable to its effects on IAP expression. However, IAP-family proteins operate downstream of Cyt-c release (11). Thus, although reduced levels of XIAP, cIAP1, and cIAP2 would be expected to lower the threshold of caspase activity needed to execute the apoptotic program, it would not be sufficient to cause Cyt-c release. Consequently, we favor the idea that effects of VD₃ on Bcl-2 family protein are more likely to explain the ability of this steroid hormone to trigger apoptosis of prostate cancer cell.

Interestingly, VD₃ reduced expression of Bag1L, a Hsp70-binding nuclear protein that has been shown to suppress apoptosis when overexpressed in breast cancer cell lines through an unknown mechanism (41). Bag1L has several potential functions, including modulating the transcriptional activity of steroid/retinoid family transcription factors. In this regard, we demonstrated previously that VD₃ induces association of VDR with Bag1L and provided evidence that Bag1L enhances the transcriptional activity of VDR in cells (42). The down-regulation of Bag1L protein levels seen in VD₃-treated cells thus suggests the possibility of a negative feedback mechanism in which reduced levels of Bag1L would be expected to render cells more resistant to VDR ligands. Consequently, the inhibitory effect of VD₃ on prostate cancer cells may be self-limited.

Because VDR binds its cognate response elements in target genes as either a homodimer (VDR/VDR) or heterodimer (VDR/RXR), we contrasted the effects of VD₃ alone and in combination with a RXR ligand, cRA. Although addition of cRA may reduce the amounts of VD₃ required to achieve reductions in antiapoptotic proteins, the net effect of using VD₃ in combination with cRA was not clearly superior to VD₃ alone. However, this issue deserves further investigation, particularly with respect to contrasting various synthetic ligands for VDR and RXR in an effort to identify synergistic combinations that might permit potent anticancer activity with reduced side effects. In this regard, combined treatment of some types of human tumor lines (*e.g.*, NCI-H82, HL-60, and MCF-7) with VDR and RXR ligands has been shown previously to provide superior growth-inhibitory activity compared with either agent individually (43–46). Also, it should be noted that although VD₃ acts through both nuclear VDR-dependent as well as through nongenomic mechanisms, for example causing rapid changes in uptake of Ca⁺² in the intestine (47), it has been shown clearly that

VDR is necessary for the growth-inhibitory actions of this ligand.

Taken together, the data reported here indicate that VD₃ reduces expression of multiple antiapoptotic proteins in sensitive prostate cancer cell lines and that it promotes induction of apoptosis via the mitochondrial (intrinsic) pathway. These findings suggest the possibility of using VDR ligands to reduce thresholds for apoptosis, thus sensitizing prostate cancer cells to the cytotoxic actions of conventional chemotherapeutic drugs and X-irradiation, which also generally induces apoptosis via the intrinsic pathway. An important consideration in applications of VD₃ for prostate cancer treatment is selecting patients whose tumors express VDR and in which VDR is transcriptionally competent, as revealed by the lack of efficacy of VD₃ in Du-145 prostate cancer cells. Thus, VD₃-based therapy for prostate cancer may be most applicable to early-stage tumors. Also, the ability of VD₃ to reduce expression of Bag1L suggests that VD₃ responses will be self-limiting, necessitating that careful consideration is given to the sequencing and timing of combination therapies using VD₃ together with chemotherapy or radiotherapy. These and other issues require further investigation if natural or synthetic ligands of VDR are to be optimally exploited for the treatment of prostate cancer.

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