Calcitriol-Induced Apoptosis in LNCaP Cells Is Blocked By Overexpression of Bcl-2*

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ABSTRACT

While the role of vitamin D in bone and mineral metabolism has been investigated extensively, the role of the vitamin D receptor in other tissues is less well understood. 1,25-dihydroxyvitamin D3 (calcitriol) can act as a differentiating agent in normal tissues and can inhibit the growth of many cancer cell lines including LNCaP prostate cancer cells. We have shown previously that calcitriol causes LNCaP cell accumulation in the G0/G1 phase of the cell cycle. In this study, we demonstrate that calcitriol also induces apoptosis of LNCaP cells.

The calcitriol-induced apoptosis is accompanied by a down-regulation of Bcl-2 and Bcl-XL proteins, both of which protect cells from undergoing apoptosis. Other proteins important in apoptotic control, Bax, Mcl-1, and Bcl-XL, are unaffected by calcitriol treatment. We find that overexpression of Bcl-2 blocks calcitriol-induced apoptosis and reduces, but does not eliminate, calcitriol-induced growth inhibition. We conclude that both regulation of cell cycle and the apoptotic pathway are involved in calcitriol action in prostate cancer cells. (Endocrinology 141: 10–17, 2000)

PROSTATE CANCER incidence has been steadily increasing and is currently the second most common cause of death from cancer in American males (1, 2). Although androgen ablation therapy is useful initially in controlling tumor progression, prostate tumors eventually become resistant and alternative means of slowing or stopping tumor growth are being sought. One possible avenue for controlling tumor growth is through the use of differentiation agents. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (calcitriol) can regulate growth and differentiation of both normal and cancerous cells (3). The actions of calcitriol are mediated by the vitamin D receptor [NR1I1 according to the recently published nomenclature for steroid receptors (4)], a member of the steroid/thyroid receptor superfamily of ligand activated transcription factors (5, 6). Several human prostate cancer cell lines are growth inhibited by calcitriol (7, 8). Although the vitamin D receptor is clearly required for calcitriol-mediated growth inhibition of prostate cancer cells (9, 10), VDR expression levels and transcriptional activity are not good predictors of response to calcitriol (7, 11). This suggests an additional requirement for calcitriol-induced growth inhibition.

Studies in a number of cell lines have shown that calcitriol can cause cancer cells to accumulate in the G1 phase of the cell cycle (12), accumulate in G2 (13), or undergo apoptosis (programmed cell death) (14–16), all three of which can significantly alter cell growth patterns. Studies of breast cancer cell lines have shown that calcitriol or calcitriol analogs cause extensive apoptosis in some lines (17) but not in others (18), indicating that apoptosis is not a universal response to calcitriol treatment. In prostate cancer cells, there have been conflicting reports on the induction of apoptosis by calcitriol (19–21) and the relative contributions of the various responses (apoptosis and cell cycle alterations) to the overall growth inhibition have not been evaluated.

Although there are multiple pathways leading to apoptosis, most pathways are in some way ultimately regulated by the Bcl-2 family of proteins (22). The Bcl-2 family of proteins is divided into two subclasses that either promote (Bcl-XL, Bax) or suppress (Bcl-2, Bcl-XL, Mcl-1) apoptosis (22, 23). These proteins can form hetero- and homodimers and the ratio of apoptosis promoters to apoptosis suppressors is one determinant of cellular response (22). Increased Bcl-2 expression has been observed in a number of cancers including prostate, lung, and breast (24–26) and is often associated with advanced stages of the disease. Because this protein is critical in preventing the cell from initiating apoptosis, methods to reduce this protein have been sought in hopes that they will provide potential anti-cancer therapies by making cancer cells more susceptible to apoptosis-inducing agents.

To study the relationship between calcitriol and apoptosis in prostate cancer cells, we have chosen the LNCaP human prostate cancer cell line as a model because the cells most closely resemble typical human prostate cancer. These cells express androgen receptor, prostate specific antigen, and retain functional p53 and retinoblastoma protein (Rb) (27–30), all of which are typical of a majority of prostate cancers (31). In these studies, we demonstrate that calcitriol induces apoptosis of LNCaP cells, identify Bcl-2 family members regulated by calcitriol in LNCaP cells, and assess the con-
tribution of apoptosis to the overall response to calcitriol using an LNCaP cell line stably overexpressing Bcl-2.

Materials and Methods

1α,25-dihydroxyvitamin D₃ (calcitriol) was obtained from Solvay DuPhar (Weesp, The Netherlands). Phorbol 12-myristate 13-acetate (TPA) was obtained from Sigma (St. Louis, MO). All compounds were dissolved in ethanol and stored at −20 °C protected from light. All other chemicals are reagent grade unless otherwise stated. Tissue culture supplies were obtained from Fisher Scientific (Pittsburgh, PA).

Cell lines

The LNCaP cell line was obtained from the American Type Culture Collection (Manassas, VA). The Bcl-2 overexpressing line, described previously (32, 33), was derived from the parental cell line under G418 selection after transfection with a Splenic Focus Forming Virus containing the human full length Bcl-2 complementary DNA (cDNA). Cells were grown as a monolayer in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% Rehautin PBS (Intergen, Co., Purchase, NY) at 37 °C in a humidified atmosphere of 5% CO₂. Cells were treated for 6 days with the indicated concentrations of hormones with a change of medium and addition of fresh hormone on the third day. DU145, PC3, and HeLa cells were also obtained from the American Type Culture Collection and were grown as monolayers in MEM, DMEM/F12, and DMEM (Life Technologies, Inc.) respectively, supplemented with 10% Rehautin PBS and kept under the same conditions as LNCaP cells.

Terminal transferase labeling

Floating and adherent cells were harvested by scraping the adherent cells into culture medium, followed by centrifugation and fixation in 1% formaldehyde (Transmission Electron Microscopy grade, Toussaints Research Corp., Rockville, MD) in 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄; pH 7.4) for 15 min on ice. Cells were collected by centrifugation at 3000 rpm for 5 min, washed in 1× PBS and fixed overnight in 70% ethanol. After fixation, 1×10⁷ cells were centrifuged in a microcentrifuge for 4 min at room temperature, washed in 1× PBS and resuspended in 50 µl of a DNA deoxynucleotidyltransferase (terminal transferase) reaction mixture (Roche Molecular Biochemicals) consisting of 10 µl 5× reaction buffer (supplied with the enzyme), 5 µl 25 mM CoCl₂, 1.5 µl terminal transferase (25 U/µl) or 1× PBS as a negative control, and 0.5 µl biotin-16-DUTP (1 mM stock, Roche Molecular Biochemicals, Indianapolis, IN). Samples were incubated at 37 °C for 1 h. 1 µl of 1× PBS was added followed by centrifugation as before. The cell pellet was resuspended in 100 µl of Avidin-FITC Buffer consisting of 2.5 µg/µl Avidin DCS FITC (Vector Laboratories, Inc., Burlingame, CA), 4× SSC (20× SSC: 3 mM NaCl, 0.3 M sodium citrate), 0.1% Triton X-100, and 5% (wt/vol) nonfat Carnation dry milk and incubated at room temperature for 30 min in the dark. One µl of 1× PBS containing 0.1% Triton X-100 was added, and cells were collected as before. The cell pellet was resuspended in propidium iodide solution (1× PBS, 5 µg/µl propidium iodide, 0.1% RNase A both from Sigma). The sample was analyzed by flow cytometry to quantify both green and red fluorescence. These experiments were done at least three times, and a representative example is shown.

Cell cycle analysis

LNCaP and LNCaP-Bcl-2 cells were plated and treated with hormone as above. Cells were pulsed with 10 µM bromodeoxyuridine (Sigma) and prepared for flow cytometry as described in Blutt et al. (12). Each experiment was done a minimum of three times. An experiment in which both cell lines were processed simultaneously is shown.

Flow cytometry

Samples were analyzed using a Profile I Flow Cytometer (Coulter Electronics, Hialeah, FL). At least 5000 forward scatter gated events were collected per sample. Propidium iodide (PI) fluorescence was collected using linear amplification with doublet discrimination engaged and FITC fluorescence was collected using logarithmic amplification. The emissions were split using a 550 long pass dichroic filter. FITC emissions were collected after a 525-band pass filter and PI emissions were collected after a 575-band pass filter. Controls for the terminal transferase labeling included cell populations incubated in the absence of terminal transferase and stained only with PI to adjust for spectral overlap.

Western blots

Unless indicated otherwise, cells were treated for 6 days with the indicated concentrations of hormones, collected by scraping into medium, and pelleted by centrifugation. Cells were washed once in 1× PBS and lysed in 1% Triton X-100, 150 mM NaCl, 25 mM Tris pH 7.4, containing 1 µg/ml leupeptin, antipain, apronitin, benzamidine HCl, chymostatin, and pepstatin, and 2 mM phenylmethylsulfonyl fluoride. Ly-sates were incubated at 4 °C for 1 h and spun in a microcentrifuge at 12,000 rpm for 10 min. Total protein was determined by Bradford Assay (34). Equal amounts of total protein were electrophoresed on a 15% SDS-PAGE gel and transferred to nitrocellulose using liquid transfer. The Bcl-2 blot was blocked for 15 min in 1× Tris-buffered saline (25 mM Tris, 125 mM NaCl, pH 7.5) with 1% BSA and 0.1% Tween-20. Mem-1, Bcl-X₁, Bcl-X₂, and Bax bands were blocked for 15 min in 1× PBS, 1% Carnation Instant dry milk, and 0.1% Tween-20. All blots were incubated with antibodies overnight at 4 °C at concentrations recommended by the manufacturers. Mem-1 Bcl-X₁ and Bax antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bcl-X₂ antibody was from Calbiochem (Cambridge, MA) and the Bcl-2 antibody was from DAKO Corp. (Carpinteria, CA). Proteins were detected using ECL reagents from Amershams Pharmacia Biotech. Each experiment was done at least three times.

Cell growth assays

LNCaP and LNCaP-Bcl-2 cells were seeded at 13,000 cells per well in 3 ml medium in six-well culture plates. After allowing the cells to attach for 24 h, cells were treated with vehicle (ethanol, final concentration 0.1%) or the indicated concentrations of hormones. The medium containing vehicle and/or hormones was changed every 3 days. At the indicated time points, cells were washed with HBSS without calcium or magnesium, removed from the plate by incubation with 0.5 ml of 0.05% trypsin-EDTA, and the reaction stopped with an equal volume of medium containing serum. A total of 0.3 ml of cell suspension was diluted in 10 ml of isoton (Coulter Corp., Miami, FL), treated with three drops of Zapoglobin II (Coulter Corp.) to lyse the cells and each sample was counted twice in a Coulter Counter (Coulter Cytometry, Hialeah, FL). All samples were done in triplicate and statistical significance was determined using a one-way ANOVA using the SigmaStat program (Jandel Scientific, San Rafael, CA). Each experiment was done a minimum of three times.

Ki67 immunofluorescence

Cells were seeded on coverslips at 15,000 cells per well in a 6 well plate and treated with either vehicle (ethanol) or 100 nM calcitriol for 6 days. Cells were then fixed in 100% ethanol for 5 min at −20 °C and then stored in 70% ethanol for up to 1 week. Cells were washed in PBS for 5 min, followed by a wash in PBS containing 0.1% Triton X-100, and then another wash in PBS. Staining was performed using a mouse monoclo-nal antibody to Ki67 nuclear antigen (Coulter Immunotech, Miami, FL) for 1 h at room temperature. Coverslips were then washed as stated above, except washes were performed for 10 min on a shaker. Staining to detect Ki67 expression was performed in the dark using a fluorescein-labeled goat antimouse antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:50 in PBS containing 0.1% BSA (Sigma). Next, coverslips were washed as stated above for 10 min on a shaker in the dark, except that the last wash contained 1 µg/ml propidium iodide (Sigma). Finally, the coverslips were washed in water for 5 min and mounted for viewing on a Carl Zeiss Axiophot fluorescence microscope.
Results
Calcitriol-treated LNCaP cells are refractory to recovery of growth

We and others have previously shown that LNCaP cells are growth inhibited by 10–100 nM calcitriol (7, 12). To determine whether the changes induced by calcitriol are long term, we asked whether growth inhibition of LNCaP cells by calcitriol can be reversed by removal of hormone. Cells were plated at a low confluence and treated with 100 nM calcitriol for 6 days. On day six, hormone was removed and replaced with fresh medium, and vehicle and cells were allowed to grow for an additional 9 days. Figure 1 demonstrates that there was no significant growth in cells that were continuously treated with calcitriol. Moreover, no significant growth was detected in populations that had been treated for 6 days with calcitriol and subsequently allowed to grow in the absence of hormone for 6 days. By day nine of recovery, a small but significant increase in growth was observed (Fig. 1). When we examined cell cycle distribution after calcitriol treatment using propidium iodide staining, we found that in addition to the large numbers of cells in G1, a portion of the cells contained less than a G1 complement of DNA, a characteristic of cells undergoing apoptosis (Fig. 2).

Calcitriol-treated LNCaP cells undergo apoptosis

DNA fragmentation leaving free 3' OH ends is a characteristic feature of cells undergoing apoptosis and provides an easy avenue to quantify the amount of apoptosis in a given cell population (35). To quantify the amount of DNA fragmentation occurring in LNCaP cells after treatment with calcitriol, we used terminal deoxynucleotide transferase to label free 3' OH ends generated in apoptotic cells with biotin dUTP. This labeling was detected by flow cytometry using a FITC tagged antibody (see Materials and Methods). As shown in Table 1, 100 nM calcitriol caused a significant number of the cells to undergo DNA fragmentation as measured

![Image](https://academic.oup.com/endo/article-abstract/141/1/10/2987740)
by green fluorescence compared with control cells (>5 fold increase over control). Cells treated with 10 nM calcitriol also induced DNA fragmentation similar to cells treated with 100 nM calcitriol. As a positive control for apoptosis, cells were treated with TPA for 24 h, a treatment that has been reported previously to cause extensive apoptosis in LNCaP cells (36). Electron microscopy was used to confirm apoptosis in calcitriol-treated cells using ultrastructural criteria (data not shown).

**Bcl-2, Bax, Bcl-X<sub>s</sub>, Bcl-X<sub>L</sub>, and Mcl-1 expression**

Because Bcl-2 family members are critical regulators of apoptosis (22), we used Western blotting to measure Bcl-2, Bax, Bcl-X<sub>L</sub>, Bcl-X<sub>s</sub>, and Mcl-1 expression as a function of calcitriol treatment. As shown in Fig. 3A, levels of Bcl-2 (a protein that protects cells from undergoing apoptosis) were dramatically lower in extracts from 100 nM calcitriol-treated cells compared with vehicle treated cells consistent with induction of apoptosis. Extracts from cells treated with 10 nM calcitriol showed the same reduction in Bcl-2 protein (data not shown). Expression of Bcl-X<sub>L</sub>, which acts in a similar manner to Bcl-2, was also lower after calcitriol treatment. In contrast, expression of Mcl-1, which also acts to prevent apoptosis, was unchanged after treatment. Although reports have suggested that when Bcl-2 is down-regulated, expression of apoptosis promoters are frequently up-regulated, we observed no enhancement of Bcl-X<sub>s</sub> or Bax expression, both of which promote apoptosis. Decreases in both Bcl-2 and Bcl-X<sub>L</sub> protein levels were evident after 48 h of treatment (Fig. 3B). Typically, both Bcl-2 and Bcl-X<sub>L</sub> exhibit greater than a 5-fold reduction in protein levels after 6 days of treatment. We have also found that recovery of Bcl-2 expression after removal of calcitriol is slow. Western blots performed on cells treated identically to the cells in Fig. 1 demonstrate decreases in the protein and more than 6 days after the removal of calcitriol are required before Bcl-2 is reexpressed (Fig. 3C).

**Effect of calcitriol on Bcl-2 levels in other prostate cancer cell lines**

The finding that calcitriol reduces expression of Bcl-2 in LNCaP cells raises the question of whether this is a universal response to calcitriol treatment. We looked at the effect of calcitriol on Bcl-2 expression in several other cancer cell lines, all of which express functional VDR. Bcl-2 is decreased in PC3 cells (Fig. 4), a prostate cancer cell line that is growth inhibited by calcitriol (7). However, in the DU145 prostate cancer cell line and the human cervical carcinoma cell line HeLa (37), calcitriol fails to down-regulate Bcl-2 (Fig. 4) even though both cell lines contain a functional VDR (7, 37). Neither the DU145 (7) nor the HeLa cell line (data not shown) is significantly growth inhibited by calcitriol.

**Effects of Bcl-2 overexpression on the response of LNCaP cells to calcitriol**

Enhanced Bcl-2 expression protects cells against some, but not all, inducers of apoptosis (38, 39). Because calcitriol treatment decreased Bcl-2 levels in LNCaP cells, we tested whether artificial overexpression of Bcl-2 was sufficient to inhibit calcitriol induced apoptosis in LNCaP cells. The LNCaP-Bcl-2 cell line expresses much higher levels of Bcl-2 protein compared with the LNCaP cell line as demonstrated by Western blotting in Fig. 5A. As was expected, we also

![Fig. 3](https://academic.oup.com/endo/article-abstract/141/1/10/2987740/13)
observed no consistent decrease in Bcl-2 expression in LNCaP-Bcl-2 cells after treatment with calcitriol (Fig. 5B). We compared the response of LNCaP-Bcl-2 cells to calcitriol as well as their ability to recover from treatment after the removal of hormone to the parental cell line. Like the parental cells (Fig. 1), LNCaP-Bcl-2 cells (Fig. 5C) were strongly growth inhibited by calcitriol, but unlike the parental cells in which growth is completely halted after treatment with calcitriol, cells overexpressing Bcl-2 grow slowly in the presence of calcitriol. Additionally, the recovery of LNCaP-Bcl-2 after 6 days of treatment was rapid when contrasted with the recovery in the parental cell line (compare with Fig. 1).

Because Bcl-2 overexpression altered the response of the cells to calcitriol, we next assessed whether alterations in the cell cycle or apoptotic index were occurring. As shown in Table 2, LNCaP-Bcl-2 cells, treated with calcitriol and analyzed using terminal transferase labeling as described for the cells in Table 1, did not show enhanced DNA fragmentation compared with cells treated with vehicle. However, LNCaP-Bcl-2 cells retain some capacity to undergo DNA fragmentation as treatment with 10 nM TPA causes labeling, consistent with other reports that overexpression of Bcl-2 is sufficient to block apoptosis through some pathways but not through others (32).

Although Bcl-2 overexpression blocks calcitriol induced apoptosis in LNCaP cells, the cells remain strongly responsive to calcitriol. We next asked whether hormone treatment causes LNCaP-Bcl-2 cell accumulation in G0/G1 as we have found for the parental line (12) and Fig. 2. As shown in Table 3, we found a substantial increase in the number of LNCaP-Bcl-2 cells in G1 (+12%), but this was much less than that of the parental cell line (+38%). Moreover, the LNCaP-Bcl-2 cells continue to express Ki67 (Fig. 6C), a nuclear antigen that is only expressed in proliferating cell populations (40). In contrast, calcitriol treatment of LNCaP cells abrogates Ki67 expression (Fig. 6C).

**G1 accumulation occurs before DNA fragmentation**

Because our flow cytometry experiments on the LNCaP-Bcl-2 cells indicated that apoptosis was unnecessary to cause primary growth inhibition, we next determined whether cell cycle accumulation and apoptosis were happening concurrently in the parental cell line. We compared the DNA profile of LNCaP cells treated with 100 nM calcitriol over a 72 h period of time. Table 4 shows that calcitriol causes a substantial accumulation of cells in G1 after 48 h of treatment, whereas an increase in sub G1 is not observed until after 72 h of treatment.

**Discussion**

Although there are numerous studies demonstrating that calcitriol inhibits the growth of prostate cancer cells (7, 12, 41), the mechanisms by which calcitriol causes these effects...
TABLE 2. Calcitriol does not induce DNA fragmentation in LNCaP-Bcl-2 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% TdT-labeled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>100 nM Calcitriol</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>10 nM TPA</td>
<td>13.0 ± 0.1*</td>
</tr>
</tbody>
</table>

Cells were treated with the indicated compounds for either 6 days (ethanol and calcitriol) or 24 h (TPA) and analyzed with a Profile I Flow cytometer. Green fluorescence representing terminal transferase labeling was quantified. The experiment was performed at least three times, and a representative experiment is shown. Numbers represent the mean ± SD of three replicates. TPA with Phorbol 12-myristate 13-acetate; TdT, terminal transferase.

*P ≤ 0.05 compared with ethanol-treated cells.

TABLE 3. Effect of calcitriol on cell cycle distribution in LNCaP and LNCaP-Bcl-2 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%G0/G1</th>
<th>%S/G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtOH</td>
<td>43.6 ± 7.6</td>
<td>53.9 ± 8.4</td>
</tr>
<tr>
<td>100 nM Calcitriol</td>
<td>81.8 ± 6.1*</td>
<td>12.6 ± 3.9</td>
</tr>
<tr>
<td>LNCaP-Bcl-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtOH</td>
<td>47.0 ± 4.5</td>
<td>53.0 ± 4.5</td>
</tr>
<tr>
<td>100 nM Calcitriol</td>
<td>59.2 ± 4.1*</td>
<td>40.0 ± 2.9</td>
</tr>
</tbody>
</table>

LNCaP-Bcl-2 cells treated with calcitriol accumulate to a lesser extent in G0/G1. The cells were treated with either ethanol or calcitriol for 6 days, pulse labeled with BrdU for 15 h, fixed, incubated with anti-BrdU and propidium iodide, and analyzed by flow cytometry. S/G2/M represent cells that fluoresce green as a result of incorporation of BrdU. Numbers represent the mean ± SD of three replicates in a typical experiment; the experiment was performed a minimum of three times. EtOH, Ethanol.

*P ≤ 0.05 compared with corresponding ethanol-treated cells.

Ki67 continues to be expressed in LNCaP-Bcl-2 cells treated with either ethanol (-) or 100 nM calcitriol (+). Both LNCaP and LNCaP-Bcl-2 cells were plated on coverslips and treated with 100 nM calcitriol for 6 days. Coverslips were fixed and Ki67 expression detected using a fluorescently tagged antibody. Cells were counterstained with propidium iodide to detect total cell population, and imaged using a Carl Zeiss Axiophot microscope at 10× magnification. A, Ki67 levels in ethanol-treated LNCaP cells; B, total cell population for A; C, Ki67 levels in calcitriol-treated LNCaP cells; D, total cell population for A; E, Ki67 levels in ethanol-treated LNCaP-Bcl-2 cells; F, total cell population for E; G, Ki67 levels in calcitriol-treated LNCaP-Bcl-2 cells; H, total cell population for G.

has not been determined. In initial experiments to determine how calcitriol is inhibiting the growth of LNCaP cells, we and others (12, 19) have shown that treatment of LNCaP prostate cancer cells with 10–100 nM concentrations of calcitriol causes accumulation of cells in the G0/G1 phase of the cell cycle. There has been some demonstration of changes in expression of cell cycle proteins with 100 nM calcitriol treatment that are consistent with a G1 accumulation (19). Although it is fairly universally accepted that calcitriol causes a G1 accumulation in LNCaP cells, there have been conflicting reports as to whether calcitriol induces apoptosis in the cells (19–21). Calcitriol induced apoptosis has been well characterized in MCF-7 breast cancer cells (17, 42), although calcitriol inhibits the growth of HL60 myeloid leukemic cells at 100 nM concentrations, it does not cause apoptosis (43). Hence, induction of apoptosis is clearly not a universal response of cancer cells to calcitriol.

We report here that LNCaP cells undergo apoptosis as observed by flow cytometry. We quantified the amount of apoptosis observed in the total cell population (floating and adherent) using terminal transferase to label the DNA fragments that are generated in apoptotic cells and found an approximately 5-fold increase in apoptotic cells after calcitriol treatment. Previous studies to determine whether calcitriol induces apoptosis in LNCaP cells both support and contradict our results. Zhuang et al. (19) did not detect apoptosis in an adherent cell population of LNCaP cells treated with 10 nM calcitriol when the cells were fixed with 4% paraformaldehyde and analyzed using terminal transferase labeling; however, the poorly adherent or floating cells (more likely to be apoptotic) would not have been retained using this technique. In a study devoted predominantly to the effects of calcitriol on androgen receptor and prostate specific antigen (PSA), Hsieh et al. (21) noted that when they examined the nonadherent portion of an LNCaP cell population treated with 10 nM calcitriol, a small increase (35%) in hypodiploid cells (characteristic of apoptosis) was found compared with the nonadherent portion of cell populations from control cells. However, no measurement of the percentage of the total treated cell population was performed (21). In contrast, Fife et al. (20) found that calcitriol treatment of LNCaP cells caused 100% apoptosis. Fife et al. also reported a sub-
Apoptosis is an active cell-mediated process in which Bcl-2 family members play an important role. To initially address the mechanism through which calcitriol induces apoptosis in LNCaP cells, we measured the effect of treatment on the expression levels of Bcl-2 family members. Although there are numerous signals involved in the induction of apoptosis, alterations in the levels of the Bcl-2 family members is a frequent feature of programmed cell death (44). Apoptotic response is often dependent on the ratio of apoptosis-inducing (Bax, Bcl-X,s) to apoptosis-protective members (Bcl-2, Bcl-XL, Mcl-1) (22). We report here the first evidence that Bcl-2 and Bcl-Xs protein levels but not Mcl-1, Bax, or Bcl-Xs are regulated by calcitriol in prostate cancer cells. There have been reports of down-regulation of Bcl-2 by calcitriol in breast (17, 42) and leukemic (43) cells, although down-regulation in HL60 leukemic cells was not associated with induction of apoptosis (43). However, the failure of calcitriol to decrease Bcl-2 protein expression in HeLa and DU145 cells despite the expression of functional VDR, suggests that regulation of Bcl-2 by calcitriol is a complex event requiring more than a functional VDR. Interestingly, neither the DU145 cell line nor the HeLa cell line is growth inhibited by calcitriol; however, some analogs of calcitriol slightly inhibit DU145 cell growth (45–47). This has been suggested to be due to decreased susceptibility to metabolism by the 25-hydroxyvitamin D 24-hydroxylase (48).

Zhao et al. (49) have proposed that the actions of calcitriol in LNCaP cells are through vitamin D receptor-mediated enhancement of the expression and consequent increased activity of the androgen receptor (49). In their study, calcitriol in combination with DHT (androgen receptor agonist) was more efficient at inhibiting the growth of LNCaP cells compared with either compound alone and the up-regulation of androgen receptor by calcitriol treatment was substantial in charcoal stripped serum (49). However, under conditions comparable to ours (FBS), androgen receptor only increased minimally from 378 to 436 fmol/mg protein (49). This change in expression is unlikely to be sufficient to cause down-regulation of Bcl-2. Moreover, calcitriol causes down-regulation of Bcl-2 in PC3 cells which lack androgen receptor. Thus, the factor(s) through which calcitriol affects Bcl-2 expression is probably not androgen receptor.

To our knowledge, this is the first report that Bcl-Xs is also regulated by calcitriol in any cancer cell line. Ratios of suppressors, like Bcl-2 and Bcl-Xs, to promoters often determines cell fate. Our data show that the ratio of expression of apoptosis promoting Bcl-2 family members to apoptosis protective family members is dramatically shifted in favor of the apoptosis promoters when cells are treated with calcitriol.

Our finding that artificial overexpression of Bcl-2 in LNCaP cells blocks calcitriol-induced apoptosis allowed us to assess the contribution of the apoptotic pathway to the overall growth inhibitory response to calcitriol. Although the Bcl-2 overexpressing cells are still extensively growth inhibited by calcitriol, the accumulation of cells in G0/G1 is less (~60% in G0 compared with ~80% in G1 in the parental cell line). This difference may be due to the parental line entering a G0 state with no Ki67 expression, whereas the Bcl-2 overexpressing cells continue to grow slowly, express Ki67, and recover rapidly once calcitriol is removed.

Several studies have shown that overexpression of Bcl-2 is characteristic of many hormone-independent prostate cancers (24, 50, 51), and much effort has been expended in developing techniques to reduce the level of Bcl-2 expression including the use of ribozymes (52). We show, here, that treatment with calcitriol is sufficient to down-regulate endogenous Bcl-2. However, concentrations of calcitriol (10–100 nm) required to induce maximal response in LNCaP cells cannot be used clinically, as these levels will induce side effects such as hypercalcemia. We and others have shown that the less calcemic analog, EB1089, can produce comparable growth inhibitory responses (12, 41) at concentrations that are much lower (0.1–1 nm) and do not induce hypercalcemia in vivo. Our preliminary studies indicate that lower concentrations of EB1089 elicit the same G1 accumulation in the cell cycle as does calcitriol (12) and our results (data not shown) also show that EB1089 induces apoptosis and down regulates Bcl-2. The finding that calcitriol can down regulate Bcl-2 and Bcl-Xs expression suggests that administration of a less calcemic analog such as EB1089 would be useful in inhibiting the growth of prostate tumors as well as in sensitizing tumor cells to treatments with other inducers of apoptosis.

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