

# Apoptosis Is Induced by the Active Metabolite of Vitamin D<sub>3</sub> and Its Analogue EB1089 in Colorectal Adenoma and Carcinoma Cells: Possible Implications for Prevention and Therapy

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## ABSTRACT

Vitamin D<sub>3</sub> is believed to reduce the risk of colon cancer, and serum levels inversely correlate with colorectal cancer incidence. The active metabolite, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, has previously been shown to inhibit growth and promote differentiation of colon cancer cells. The vitamin D analogue, EB1089, is currently under clinical trial in a variety of cancers because of its growth-inhibitory effects *in vitro* and reduced hypercalcemic effects *in vivo*. The mechanism of growth inhibition by EB1089, however, remained to be determined. In this study we examined the effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 on five colorectal tumor cell lines (two adenoma and three carcinoma) to determine the mechanism of growth inhibition and to ascertain whether premalignant adenoma cells were responsive to these agents. 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> and EB1089 induced p53-independent apoptosis in adenoma and carcinoma cell lines in a dose-dependent manner between 10<sup>-10</sup> and 10<sup>-6</sup> M. EB1089, as well as inducing apoptosis, increased the proportion of cells in the G<sub>1</sub> phase, particularly in the adenoma cell lines. In two of the three carcinoma cell lines (SW620 and PC/JW), levels of apoptosis induced by EB1089 were similar or greater than those induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Although the carcinoma cell line HT29 was relatively resistant to apoptosis induced by EB1089 compared with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, EB1089 markedly inhibited cell yields. These observations offer promise for the clinical use of EB1089. To determine whether the induction of apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 was potentially via a differentiation pathway, alkaline phosphatase activity was measured as a marker of differentiation. Induction of alkaline phosphatase was observed in the floating apoptotic cells as well as in the adherent population. A link between the induction of differentiation and apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 is suggested by the occurrence of apoptosis subsequent to the induction of differentiation. To investigate the molecular pathway to apoptosis induction, members of the Bcl-2 family of proteins were examined (Bcl-2, Bcl-x, Bax, and Bak). Decreased Bcl-2 was observed in some cell lines, particularly in response to EB1089, but was not essential for apoptosis. Levels of the proapoptotic protein Bak, however, were consistently increased in all of the five cell lines in association with apoptosis induced by either agent. The results implicate Bak protein in the induction of apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or its analogue EB1089. The ability of EB1089 to induce apoptosis in colorectal carcinoma cells suggests that this or other vitamin D analogues may prove clinically effective for the treatment of colorectal cancer. Furthermore, the fact that it induces cell cycle arrest and apoptosis in the premalignant adenoma cells may suggest an application in colorectal cancer chemoprevention.

## INTRODUCTION

That colorectal cancer remains the second most common cause of cancer death in the western world emphasizes the requirement for more effective treatments of advanced disease and chemopreventive

strategies. More than 80% of advanced colorectal cancers have p53 mutations, and, therefore, identification of agents that induce apoptosis by a p53-independent mechanism may provide novel chemotherapeutics to augment the currently used regimen of 5-fluorouracil treatment. One approach to identifying candidate chemotherapeutic agents is to consider ways of enhancing the effectiveness of compounds that have been reported to be chemopreventive against colorectal cancer. Epidemiological studies have suggested that vitamin D<sub>3</sub> may reduce the risk for cancer of the colon (1, 2). In support of this, vitamin D<sub>3</sub> is chemopreventive and growth inhibitory to colonic tumors in animal model systems (3–5). Conversely, vitamin D-deficient mice develop hyperplasia and hyperproliferation within colonic crypts, along with a reduced rate of epithelial cell migration up the crypts (6). *In vitro*, the active metabolite of vitamin D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, is growth inhibitory to colonic adenocarcinoma cells, promoting cellular differentiation (7–9). In familial adenomatous polyposis patients, it has been demonstrated to inhibit proliferation of rectal adenomas (9). 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> has been reported to induce apoptosis in breast cancer cell lines (10). Furthermore, there has been a brief report that 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induces apoptosis in HT29 colon cancer cells (11), but the mechanisms of apoptosis have not been determined, and there have been no investigations into whether 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induces apoptosis in premalignant colon cells. These properties of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> could be responsible for the reported cancer preventive effects of the vitamin, particularly if 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induces apoptosis in premalignant adenoma cells.

The growth-inhibitory properties of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> could potentially be harnessed therapeutically. However, dose-limiting effects on calcium metabolism preclude its clinical use in the treatment of cancer. Emphasis has, therefore, been placed on the development of structurally related analogues that retain potent antiproliferative activity but have reduced hypercalcemic effects. One such analogue is EB1089, which, in breast cancer cells, has a 100-fold greater antiproliferative effect than 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (12) and has been shown to block cell cycle progression in the G<sub>1</sub> phase by means of p21 up-regulation (13). In addition, EB1089 has been reported to induce apoptosis in myeloma cells (14) and in breast cancer cells (15). The mechanisms of its growth-inhibitory properties on colorectal cancer cells remain undefined. In particular, there have been no reports of whether EB1089 induces apoptosis in colorectal tumor cells.

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> behaves as a steroid hormone and regulates gene transcription through a nuclear receptor, the VDR,<sup>2</sup> a member of the steroid/thyroid hormone receptor superfamily. The VDR is a ligand-activated nuclear receptor that binds to specific DNA binding sites known as VDREs to activate or repress the transcription of target genes that regulate a variety of biological processes (16).

<sup>2</sup> The abbreviations used are: VDR, vitamin D receptor; VDRE, vitamin D responsive element; ALP, alkaline phosphatase.

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These VDREs may be direct repeats of hexameric core-binding motifs separated by three nucleotides (DR3) or inverted palindromic repeats separated by 9 nucleotides (IP9; Ref. 17). EB1089 has been shown to preferentially target IP9-type VDREs (12). The VDR is thought to act preferentially in a heterodimeric complex with the retinoid X receptor (RXR) but may also act as a homodimer (17) or in association with the RAR all-*trans* retinoic acid receptor (16) or the thyroid hormone receptor (T3R; Ref. 18). The VDR is expressed in colonic epithelial cells (19, 20), which suggests that the colon is an important target organ for vitamin D<sub>3</sub> action. The VDR is also expressed in colorectal cancer tissues irrespective of Dukes' stage or degree of differentiation (21). Effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> mediated by the VDR are referred to as the genomic actions of vitamin D<sub>3</sub>. Additional effects, such as rapid increases in intracellular calcium and induction of phospholipase A2 and protein kinase C activities, known as the nongenomic effects of vitamin D<sub>3</sub>, are proposed to be mediated by the action of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> on a putative plasma membrane receptor (22).

The aim of this study was to determine the mechanisms of growth inhibition by the active metabolite of vitamin D (1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>) and its analogue EB1089 on colorectal adenoma as well as carcinoma cells, initially to determine whether this involves cell cycle arrest, differentiation, and/or apoptosis. Two premalignant adenoma cell lines and three adenocarcinoma cell lines were used to determine whether adenoma cells were responsive and to compare their response with that of carcinoma cells, because chemopreventive agents may act at different stages of the adenoma to carcinoma sequence. For example, salicylate (the active metabolite of aspirin) is protective against colorectal cancer and induces more extensive apoptosis in carcinoma cells than in adenoma cells (23). In this paper, we demonstrate that 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 induce p53-independent apoptosis subsequent to the induction of the differentiation marker, ALP, in all of the five colorectal tumor cell lines. These observations may, in part, explain the epidemiological link between vitamin D and a reduced risk for colorectal cancer and offer promise for the use of vitamin D analogues such as EB1089 for the treatment or prevention of colorectal cancer. Furthermore, we show that elevated Bak levels may provide a molecular basis for the induction of apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089.

## MATERIALS AND METHODS

### Cell Lines and Culture

The adenoma cell lines used were AA/C1 and RG/C2. These cells were capable of growth after trypsinization to single cells and are derivatives of the PC/AA (24) and S/RG cell lines (25). The carcinoma cell lines used were HT29 (26); SW620, derived from a lymph node metastasis (27); and PC/JW, derived from a patient with familial adenomatous polyposis (25). The p53 status of each of these cell lines is known and is as follows: (a) AA/C1: wild-type p53 (28); (b) RG/C2: 282 (Arg-Trp) (29); (c) PC/JW: p53 null (29); and (d) HT29 and SW620: 273 (Arg-His) (30). All of the cell lines were grown in DMEM (Life Technologies, Inc.) containing 20% fetal bovine serum (Life Technologies, Inc., batch-selected), glutamine (2 mM), insulin (0.2 units/ml), hydrocortisone sodium succinate (1  $\mu$ g/ml), penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml). Cells were grown in T25 flasks and were routinely passaged using 0.1% trypsin (Difco)/0.1% EDTA (AnalaR, BDH).

### Treatment with 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> or EB1089

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> and EB1089 were synthesized in the Department of Chemical Research (Leo Pharmaceuticals, Ballerup, Denmark). The compounds were dissolved in propan-2-ol at 4 mM. Dilution was performed in ethanol to give stock solutions of 2  $\times$  10<sup>-4</sup> M and aliquots of the stock solution were stored at -20°C. All of the solutions containing 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 were protected from the light.

Cells were seeded at 1  $\times$  10<sup>6</sup> cells per T25 flask for HT29, SW620, PC/JW and RG/C2, and at 2  $\times$  10<sup>6</sup> cells per T25 flask for the AA/C1 cell line. These seeding densities yielded a similar number of cells at the day of treatment 3 days later. At the time of treatment, the cells were in the exponential growth phase. Cultures were treated with either 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089 at concentrations ranging from 10<sup>-10</sup> M to 10<sup>-6</sup> M in triplicate for harvests at each of three time points. Solvent-containing culture medium was used to treat the control flasks and to perform the serial dilutions, such that the same volume of solvent was present in all of the treatment conditions (0.5%). Preliminary experiments demonstrated that medium changing every day with fresh 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> resulted in a greater reduction in the adherent-cell yield. Therefore, for all of the experiments described in this paper, the medium was changed every day once treatment commenced. At each medium change, the cells floating in the medium were collected and counted using a counting chamber. Adherent-cell counts were obtained after 2, 4, and 7 days of treatment.

### Identification of Apoptosis

Acridine orange/ethidium bromide dual staining was used to identify apoptotic cells by staining the condensed chromatin. Unfixed cells were stained with 5  $\mu$ g/ml acridine orange together with 5  $\mu$ g/ml ethidium bromide in PBS for 10 min and were then viewed by fluorescence microscopy. Acridine orange stains early-stage apoptotic cells. Only late-stage apoptotic cells that have lost membrane permeability stain with ethidium bromide. Ethidium bromide is also useful in that it identifies cells that are necrotic, because they have lost membrane permeability but do not contain condensed chromatin. At least 300 cells were scored for each sample for three independent experiments.

To further demonstrate that the floating cells were apoptotic, 10<sup>6</sup> cells were used to prepare samples for DNA laddering by the method of Smith *et al.* (31). As a positive control, mouse thymocytes, treated with 10<sup>-7</sup> M dexamethasone for 16 h, were used. Cells were lysed and digested for 1 h at 50°C in 20  $\mu$ l of a solution containing 5 mM EDTA, 25 mM Tris-HCl (pH 8.0), 5 mg/ml lauryl sarcosine, and 0.5 mg/ml proteinase K. RNase A (10  $\mu$ l) from a stock solution of 0.5 mg/ml was added to the samples, which were incubated for an additional hour at 50°C. The samples were heated to 70°C, and 10  $\mu$ l of melted loading buffer, containing 10 M EDTA, 1% (w/v) low-melting-point agarose, 40% (w/v) sucrose, and 0.25% bromophenol blue, were added to each sample. The samples were immediately dry-loaded into a gel of 2% agarose type II (Sigma) and 0.7% Infinity Agarose Enhancer (Oncor). To make up the gel, the agarose and enhancer were slurried in 7 ml of ethanol and then made up to 70 ml with Tris acetate buffer [40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA (pH 8.1)] and supplemented with 0.5  $\mu$ g/ml ethidium bromide. The gel was run at 40 V for 5 h in Tris-acetate buffer.

### Cell Cycle Analysis

One million trypsinized adherent cells were washed in PBS, fixed in 70% ethanol, and stored at -20°C for 1 week. In preparation for cell cycle analysis, the cells were pelleted, resuspended in 1 ml PBS containing 0.2 mg/ml propidium iodide (Sigma) and 0.15  $\mu$ g/ml RNase (Sigma), and incubated at 37°C for 30 min in the dark. Cells were then left overnight at 4°C in the dark before analysis on a fluorescent-activated cell sorter (Becton Dickinson). Cells were excited with a single 488-nm argon laser, and the fluorescence was detected through a 585  $\pm$  22-nm filter. Linear red-orange fluorescence (FL3) data were collected in list format to 10,000 total events, and the distribution of cells within the cell cycle was estimated using a CellFIT software program, based on a rectangular S-phase model.

### SDS-PAGE Western Blotting

**Sample Preparation for SDS-PAGE Western Blotting Detection of Bcl-2 Family of Proteins.** Trypsinized adherent cells (10<sup>6</sup>) were resuspended in 50  $\mu$ l of gel sample buffer [0.125 M Tris-HCl (AnalaR, BDH; pH6.8), 20% (v/v) glycerol, 2% (w/v) SDS, 10% (v/v)  $\beta$ -mercaptoethanol, and 0.25% (w/v) bromophenol blue], and the tubes were placed in a boiling-water bath for 5 min. Samples were stored at -20°C before electrophoresis.

**SDS-PAGE.** Resolving gel was made up at 12.5% Protogel [30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide; Bio-Rad] and stacking gel at 4.5%.

Gels were run in a buffer solution of 192 mM glycine (AnalaR, BDH), 25 mM Tris (AnalaR, BDH), and 1% SDS and were then electroblotted onto Immobilon polyvinylidene difluoride (PVDF) membrane in a transfer buffer of 192 mM glycine (AnalaR, BDH), 10 mM Tris (AnalaR, BDH), and 20% (v/v) methanol (AnalaR, BDH).

**Immunoblotting for the Bcl-2 Family of Proteins.** Antibodies used were as follows: (a) Bcl-2 mouse monoclonal clone 124 (kindly provided as culture supernatant by David Mason, University of Oxford, United Kingdom), used at a dilution of 1:100; (b) Bax N20 rabbit polyclonal (Santa Cruz), used at a dilution of 1:500; (c) Bak G23 rabbit polyclonal (Santa Cruz) used at a dilution of 1:500; (d) Bak monoclonal clone G317-2 (PharMingen) used at a dilution of 1:1000; and (e) Bcl-x L19 rabbit polyclonal (Santa Cruz) used at a dilution of 1:500. A mouse monoclonal  $\alpha$ -tubulin antibody (Sigma) was used to assess equal loading of the gels at 1:1000. Membranes were blocked in milk block buffer [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 4% nonfat dried milk] for 1 h or overnight at 4°C. Incubations in primary antibody were conducted for 4 h at room temperature or overnight at 4°C. Membranes were rinsed in distilled water, washed twice for 10 min in milk-block buffer and then twice for 10 min in Tween buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% Tween 20 (w/v); Sigma], followed by an additional 10 min in milk-block buffer. Secondary antibodies were antimouse or antirabbit horseradish peroxidase-conjugated antibodies (Sigma). Membranes were incubated in the appropriate secondary antibody at 1:1000 for 45 min at room temperature. The membranes were then washed for 20 min in milk-block buffer and for 10 min in Tween buffer. After rinsing in distilled water, peroxidase activity was detected

using an Enhanced ChemiLuminescence (ECL) detection kit (Amersham) following the manufacturers' protocol.

#### Measurement of Differentiation: The ALP Assay

This assay was used to determine the effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089 on colonic cell differentiation. ALP activity has previously been used as a measure of colonic cell differentiation in response to butyrate and was used to demonstrate that butyrate induces apoptosis by a differentiation-dependent pathway in colonic tumor cells (32, 33). Triplicate flasks were set up to harvest for the assay in parallel with the flasks for the cell counts, and the cultures were treated as described above. Medium was removed from the flasks, which were each washed with 2.5 ml of 0.15 M NaCl and then incubated for 5 min at 37°C in 0.25% sodium deoxycholate (Sigma). Cells were removed from the flasks using a cell scraper, and the lysate was centrifuged at 4000 rpm for 5 min. For measurement of ALP activity, an ALP reagent kit (Sigma) was used following the method of Bowers and McComb (34). Reaction mixture (1 ml) consisting of 7 mM p-nitrophenyl phosphate, 0.1 M sodium bicarbonate, and 5 mM MgCl<sub>2</sub> was added to spectrophotometer cuvettes containing 20  $\mu$ l of sample lysate. The absorbance was measured at 410 nm using a UV-Unicam spectrophotometer for 1 min at 30°C. Protein content for each sample was measured by the Bradford colorimetric method (35) with reference to BSA standards (Bio-Rad). The absorbance was measured at 595 nm using an Ultrospec III spectrophotometer (Pharmacia). The calculation of protein content per sample allowed the ALP activity to be expressed as units per  $\mu$ g of protein.

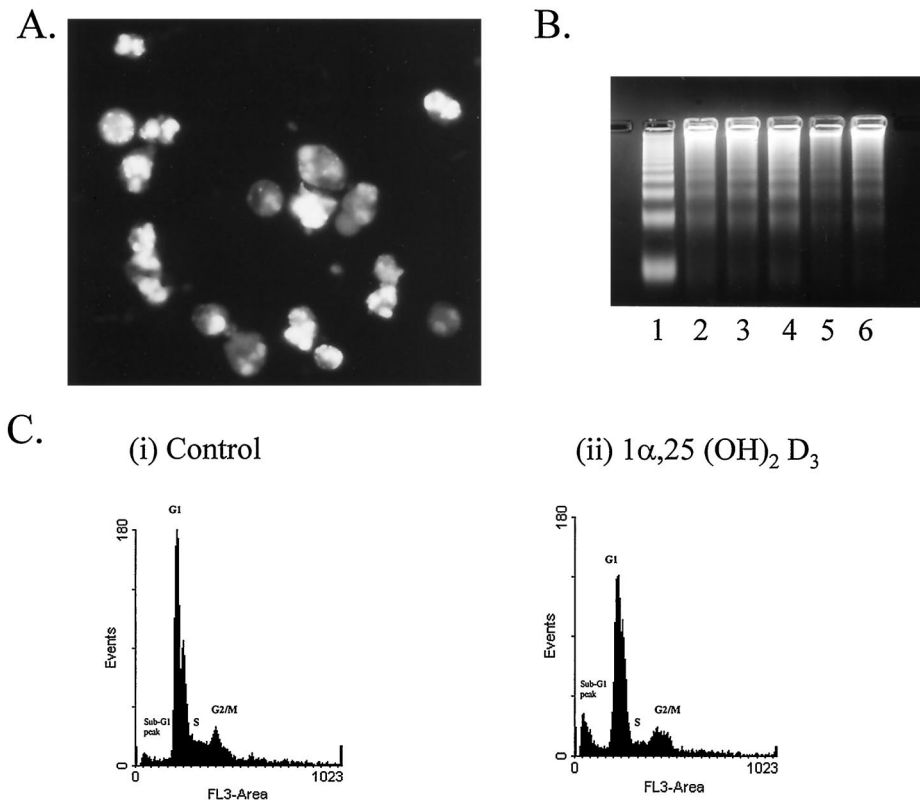


Fig. 1. Induction of apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Apoptotic cells were rapidly shed into the culture medium. For all of the experiments in this study, attached and floating cells were examined separately. *A*, fluorescence microscopy of acridine orange/ethidium bromide dual-stained floating cells demonstrating apoptotic morphology with condensed chromatin. As an example, floating RG/C2 adenoma cells from cultures treated with 10<sup>-6</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> are shown. *B*, floating cells of both control and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-treated cultures produced DNA ladders on electrophoresis. To obtain sufficient floating cells for DNA analysis, 0.33  $\times$  10<sup>6</sup> floating cells from harvests on days 5, 6, and 7 were pooled. *Lane 1*, mouse thymocytes treated for 16 h with 10<sup>-7</sup> M dexamethasone to induce synchronous apoptosis; *Lane 2*, PC/JW floating cells from control cultures; *Lane 3*, PC/JW cells from cultures treated with 10<sup>-10</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; *Lane 4*, PC/JW floating cells from cultures treated with 10<sup>-8</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; *Lane 5*, PC/JW floating cells from cultures treated with 10<sup>-7</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; *Lane 6*, PC/JW floating cells from cultures treated with 10<sup>-6</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Adherent cells from control flasks reproducibly do not produce a DNA ladder, the DNA remaining as a single band at the top of the gel (data not shown). *C*, demonstration of a sub-G<sub>1</sub> peak of apoptotic cells by flow cytometric analysis of the adherent-cell population in the carcinoma cell line, PC/JW. In PC/JW, the adherent-cell population included cells with sub-G<sub>1</sub> DNA content, and the proportion of cells in this sub-G<sub>1</sub> peak increased with the increasing concentration of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. The results from day 7 of treatment (when the percentage of adherent cells that were apoptotic was maximal) with 10<sup>-6</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> are shown. The sub-G<sub>1</sub> peak was confirmed to represent apoptotic cells by the examination of the ethanol-permeabilized, propidium iodide-stained cells for apoptotic morphology. The proportion of morphologically apoptotic cells reflected the proportion in the sub-G<sub>1</sub> peak (1.5% in the controls and 8.5% in the cultures treated with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>).

## RESULTS

**Establishment of Methods to Determine Whether Apoptosis Is Induced by 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> and Its Analogue EB1089 in Colorectal Adenoma and Carcinoma Cell Lines.** In the five cell lines, both 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 induced growth inhibition and cell shedding into the medium. We have previously demonstrated that the majority of colon cells that are shed into the medium, either spontaneously or by treatment with agents such as butyrate or radiation (36, 37), are apoptotic. Because of rapid detachment of cells entering apoptosis, very few apoptotic cells were seen in the adherent population. The proportion of cells floating in the medium can, therefore, be used as a measure of the extent of apoptosis in the cultures if the floating cells are confirmed to be morphologically apoptotic. Furthermore, the floating cells produce a DNA ladder indicative of apoptotic oligonucleosomal DNA cleavage. Adherent cells, by contrast, rarely demonstrate this DNA cleavage pattern, because oligonucleosomal fragmentation of DNA is a late event in apoptosis. To determine whether the cells that were shed in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089 represented apoptosis induced by the treatment, the adherent and floating cells were separately examined for apoptotic morphology using acridine orange/ethidium bromide dual staining. At each time point (2, 4, and 7 days of treatment) and for each agent, the floating cells collected from the treated cultures had a greater proportion of cells with the condensed chromatin pattern definitive of apoptosis (38) than the control cultures (Fig. 1A). In each case, there was a dose-dependent increase in the apoptotic index of the floating cells. This was true even for the top concentration of 10<sup>-6</sup> M, which indicated that the pronounced increases in the proportion of cells that were floating in the medium of cultures receiving the top concentration were attributable to the induction of apoptosis and not attributable to necrosis. To further confirm this observation, DNA gel electrophoresis was used to demonstrate DNA laddering indicative of internucleosomal fragmentation in the floating cells (Fig. 1B). In the PC/JW carcinoma cell line, this was accompanied by an increase in the apoptotic index of the adherent population that was observed (a) by fluorescent microscopy (data not shown); and (b) as a similar proportion of adherent cells having a sub-G<sub>1</sub> content on flow cytometric analysis (Fig. 1C). For example at the 7-day time point, the percentage of adherent cells that were apoptotic was 8.5% in the cultures treated with 10<sup>-6</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> compared with 1.5% in the control cultures. For the other four cell lines, apoptosis in the form of chromatin condensation, DNA laddering, or reduced DNA content was only seen in the floating cell populations. For each cell line treated with each agent, the floating cells were confirmed to be apoptotic by acridine orange/ethidium bromide dual staining and by DNA laddering. Once we were satisfied that the cells that were shed into the medium of the treated cultures represented apoptosis, we used the proportion of cells floating in the medium as a measure of the extent of apoptosis taking place in the cultures, as we had done for previous studies of the differentiation agent, sodium butyrate (36).

**1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> Treatment Reduces the Attached Cell Yield and Induces Apoptosis.** Cells shed into the medium were counted every day to give an indication of the timing, and the total extent of apoptosis and adherent-cell yields were obtained at days 2, 4, and 7 of treatment. Daily treatment with fresh 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> was required for the optimal induction of apoptosis. The data shown in Fig. 2 are the results obtained after 7 days of treatment, and give an overall effect of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> treatment on adherent-cell yield and the extent of apoptotic cell shedding. The proportion of cells shed into the medium was the cumulative number of floating cells collected each day as a proportion of the total

numbers of cells at the time of adherent-cell harvest (attached cell yield plus the total number of floating cells generated during the treatment time). To enable the effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> on the five different cell lines to be easily compared, the adherent-cell yield is presented as a percentage of the control yields, and the proportion of cells shed into the medium is expressed as a fold control value. Individual (daily) floating cell numbers and the adherent-cell yields for days 2 and 4 are not shown. 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> was growth inhibitory and induced apoptosis in all of the five cell lines (Fig. 2). The carcinoma cell lines HT29 and SW620 were highly sensitive to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-induced apoptosis at 10<sup>-6</sup> M. Although apoptosis was obtained after only 2 days of treatment in all of the five cell lines, there was initially differential sensitivity to apoptosis with a greater extent of apoptosis in the adenoma cell lines, and, therefore, a longer treatment time (7 days) was required to demonstrate the extent to which 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> was able to induce apoptosis in the carcinoma cell lines. By day 7 of treatment with fresh vitamin applied each day, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induced apoptosis in each of the five cell lines.

**EB1089 Treatment Reduces the Attached Cell Yield and Induces Apoptosis.** EB1089 treatment was carried out in the same way as in the experiments for 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. EB1089 reduced the adherent-cell yield (Fig. 3A) and induced apoptosis in each cell line in a dose-dependent manner, although HT29 was relatively resistant to EB1089-induced apoptosis (Fig. 3B). Interestingly, as was observed with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> at day 4, the adenoma cell lines were more sensitive than the carcinoma cell lines to apoptosis induced by EB1089 early in the time course (data not shown). The carcinoma lines varied in their apoptotic responses to EB1089. By day

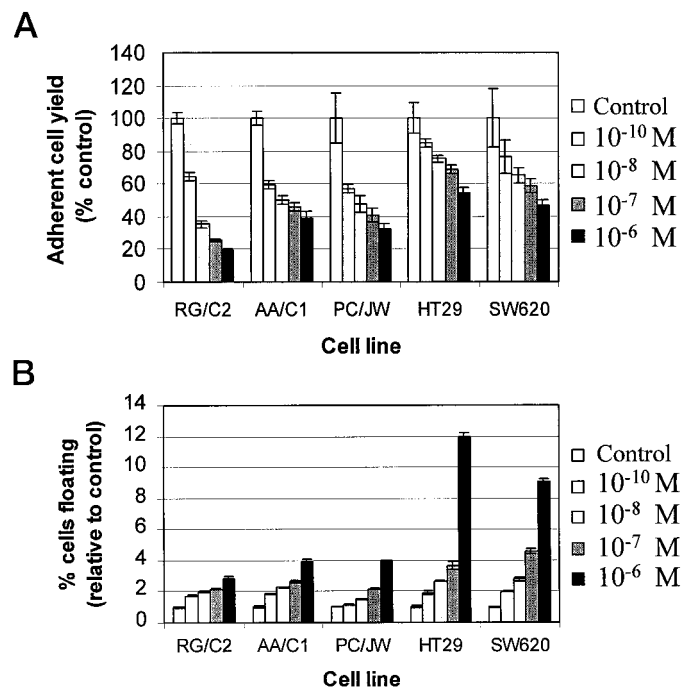


Fig. 2. The effect of 7 days' treatment with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> on adherent-cell yield and cell shedding. A, adherent-cell yields of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-treated cultures relative to the control cultures after 7 days of treatment. To compare the extent of growth inhibition in the five cell lines, the adherent-cell yields are expressed as a percentage of the control. The data shown are means  $\pm$  SD of at least three experiments. B, proportion of cells floating in the medium used as a measure of the extent of apoptosis induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> in the cultures over a 7-day treatment period. To compare the extent of apoptosis in the five cell lines, the proportion of cells floating in the control cultures was normalized to 1. The graphs, therefore, depict fold control values. For each cell line and concentration, the floating cells were confirmed to be apoptotic by fluorescence microscopy and DNA laddering as described in "Materials and Methods". The data shown are means  $\pm$  SD of at least three experiments.

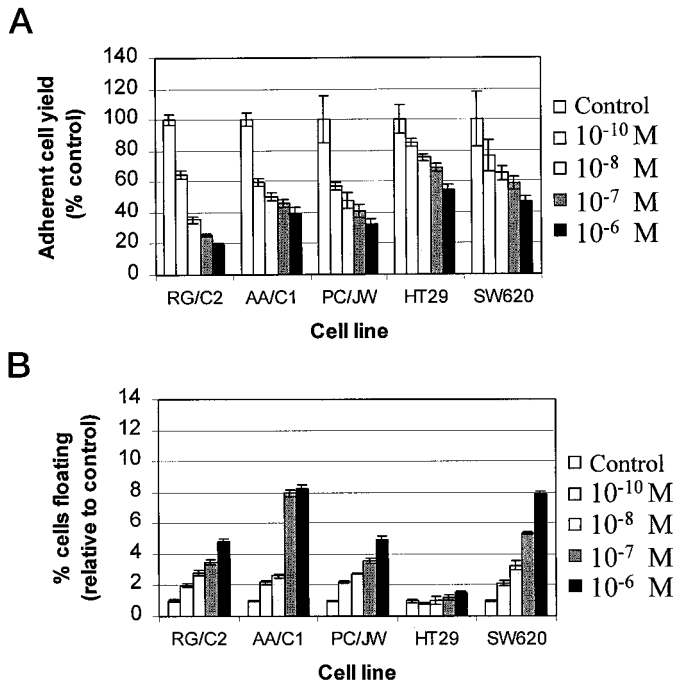


Fig. 3. The effect of 7 days' treatment with EB1089 on adherent-cell yield and cell shedding. *A*, adherent-cell yields of EB1089-treated cultures relative to the control cultures after 7 days of treatment. To compare the extent of growth inhibition in the five cell lines, the adherent-cell yields are expressed as a percentage of the control. The data shown are means  $\pm$  SD of at least three experiments. *B*, proportion of cells floating in the medium used as a measure of the extent of apoptosis induced by EB1089 in the cultures over a 7 day treatment period. To compare the extent of apoptosis induced by EB1089 in the five cell lines, the proportion of cells floating in the control cultures was normalized to 1. The graphs, therefore, depict fold control values. For each cell line and concentration of EB1089, the floating cells were confirmed to be apoptotic by fluorescence microscopy and DNA laddering as described in "Materials and Methods". The data shown are means  $\pm$  SD of at least three experiments.

7 of treatment, HT29 and SW620 had comparable levels of growth inhibition (Fig. 3A); however, SW620 demonstrated a marked increase in apoptosis (>8-fold higher for the highest concentration than for the control), whereas HT29 demonstrated relatively low levels of apoptosis (<2-fold the control for the highest concentration; Fig. 3B). Thus, growth inhibition did not always correlate with apoptosis.

**EB1089 Induces a Greater G<sub>1</sub> Arrest Than 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub>.** To determine whether growth inhibition was attributable to an arrest in a particular phase of the cell cycle as well as to the

induction of apoptosis, cell cycle profiles were obtained by flow cytometric analysis. After 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> treatment, a G<sub>1</sub> arrest was evident only with the highest concentration (10<sup>-6</sup> M) in RG/C2, AA/C1, and PC/JW (Fig. 4A shows data from day 7). The most sensitive cell line was the adenoma cell line RG/C2, for which there was a progressive increase in the proportion of cells in the G<sub>1</sub> phase and a reduction in the proportion of cells in the S phase and G<sub>2</sub>-M from day 2 onwards (data not shown). Inasmuch as a cell cycle arrest was only observed when using the highest concentration of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, this does not account for the growth inhibition by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>.

The analogue EB1089 induced a G<sub>1</sub>-S arrest in the two adenoma cell lines RG/C2 and AA/C1. This was evident at 10<sup>-10</sup> M and 10<sup>-8</sup> M and was pronounced at 10<sup>-7</sup> M and 10<sup>-6</sup> M (Fig. 4B). The carcinoma cell lines (including HT29, which was insensitive to the induction of a cell cycle arrest with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>) showed an increase in G<sub>1</sub>:S phase ratio but only with the top concentration of EB1089. The G<sub>1</sub>:S phase ratio of the carcinoma cell lines at the highest concentration of EB1089 (10<sup>-6</sup> M) was lower than that of the adenoma cell lines at 10<sup>-7</sup> M.

In PC/JW, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 induced a sub-G<sub>1</sub> peak (example shown in Fig. 1C). This increased with both time and concentration in this particular cell line and is indicative of apoptosis in the adherent-cell population. The percentage of cells in the sub-G<sub>1</sub> peak was consistent with the percentage of cells with apoptotic morphology (visualized in the propidium iodide-stained cells under the fluorescence microscope). Because no sub-G<sub>1</sub> peak was observed in the adherent populations of the other four cell lines after treatment with either agent, this was not used to assess the extent of apoptosis in all of the cultures.

**1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> and EB1089 Induce Expression of the Proapoptotic Protein, Bak, in Association with the Induction of Apoptosis.** Three experiments were performed for each of the five cell lines, and, for these experiments, samples of the adherent cells were run for Western blot analysis of the Bcl-2 family of proteins. The proteins that we chose to investigate in this study were the antiapoptotic proteins Bcl-2 and Bcl-x<sub>L</sub> and the proapoptotic proteins Bax and Bak. The five cell lines had different basal levels of Bcl-2 protein. RG/C2 and SW620 had high levels, PC/JW and HT29 had moderate levels, and AA/C1 did not express detectable levels of Bcl-2. All of the cell lines expressed similar levels of Bcl-x<sub>L</sub>, Bax, and Bak. For all of the blots in this study, protein

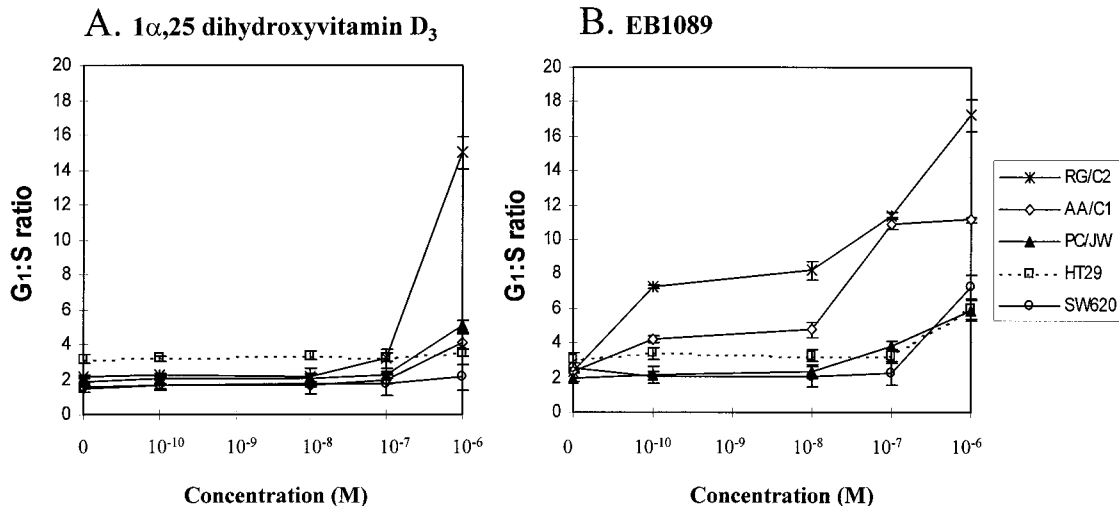


Fig. 4. G<sub>1</sub>:S phase ratios of cultures treated for 7 days with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (A) or the analogue, EB1089 (B). Higher G<sub>1</sub>:S phase ratios are indicative of cell cycle arrest in the G<sub>1</sub> phase of the cell cycle. The results are means  $\pm$  SD of three experiments.

loading was confirmed by reprobing for  $\alpha$ -tubulin. The effects of the top concentration of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 are summarized in Tables 1 and 2, respectively. The most consistent finding was that each of the five cell lines showed increased levels of Bak in response to either 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089. In response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, the increase in Bak protein was dose dependent in some cell lines (AA/C1 and HT29), and, in others, all of the concentrations up-regulated Bak protein to an apparently equal extent (RG/C2 and SW620; Fig. 5A). For PC/JW, a pronounced increase in Bak was detected with the top concentration only. The HT29 cell line showed an increase in Bak protein at days 4 and 7 only (*i.e.*, relatively late), paralleling the late induction of apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> in this cell line. In response to EB1089, Bak levels were increased with all of the concentrations of EB1089 at all of the time points in the adenoma and carcinoma cell lines (see Fig. 5A for examples). For PC/JW, the increase in Bak was detected at the day-2 time point, *i.e.*, the increase occurred earlier and at lower concentrations in response to EB1089 compared with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (Fig. 5A).

Changes in the levels of Bcl-2, Bcl-x<sub>L</sub>, and Bax were observed, but these changes were not consistent between cell lines (Tables 1 and 2). For example, Bcl-2 levels were reduced in response to EB1089 in the RG/C2 and PC/JW cell lines but were increased in HT29 and SW620 (Fig. 5B). Bax expression was increased at some of the time points after EB1089 treatment in AA/C1, RG/C2, and HT29 (Fig. 5B). Changes in Bcl-x<sub>L</sub> expression were small and the shorter proapoptotic form of Bcl-x, Bcl-x<sub>S</sub>, was not detected in any of the cell lines in control or treated samples (data not shown).

**1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> and EB1089 Induce ALP Activity in Colorectal Tumor Cells.** ALP activity has previously been used as a measure of colonic cell differentiation in response to butyrate and to demonstrate that butyrate induces apoptosis by a differentiation-dependent pathway in colonic tumor cells (32, 33). In these studies, the spontaneous floating cells had higher ALP activity than the adherent cells (indicating that at least some of the spontaneous floating cells are derived from a differentiated population), and treatment with the differentiation agent, butyrate, induced ALP activity in adherent and floating cell populations. To determine whether 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089 also induced apoptosis via a differentiation pathway, ALP activity was measured at days 4 and 7 of treatment as a reliable differentiation marker for colonic epithelial cells. ALP activity was measured in floating and adherent cells separately, so that we could detect not only whether differentiation was induced in the adherent-cell culture but also whether the floating apoptotic cells, induced by treatment with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or EB1089, had higher ALP activity than the corresponding adherent-cell populations. A higher ALP activity level would be consistent with the floating apoptotic cells representing terminally differentiated cells that would arise from the induction of differentiation in the adherent cells. In the control cultures for each of the five cell lines, the ALP activity was approximately 4-fold higher in the floating cells than in the adherent cells, as has been shown in previous studies (32, 33). In all of the five cell lines, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-treated cultures had higher ALP activity in both the adherent and floating cells, and the ALP activity increased with increasing 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> concentration (Fig. 6). It was noticeable that the cell line in which 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induced the least ALP activity in the adherent-cell population was the HT29 carcinoma cell line, although, as in the other cell lines, in the collected floating cells of the treated cultures, there were greater ALP activities than in those of the controls. Indeed, this finding is consistent with observations

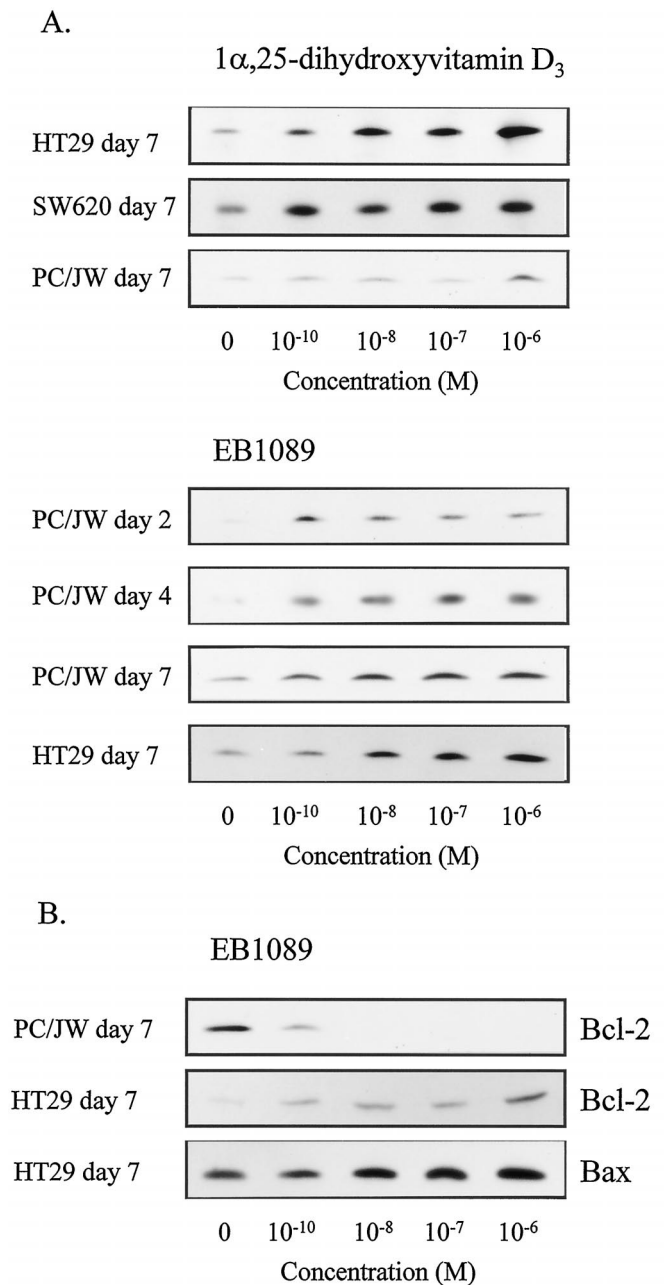


Fig. 5. The effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 on expression levels of Bcl-2 family members. Each of the blots shown were demonstrated to have equal protein loading by reprobing with the monoclonal antibody for  $\alpha$ -tubulin (Sigma). A, up-regulation of Bak protein by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089. Bak was increased in all of the five cell lines in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089. Bak blots for three cell lines (HT29, SW620, and PC/JW) with different extents of Bak up-regulation in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> are shown. Of the five cell lines, PC/JW showed the least up-regulation in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. For the PC/JW cell line, the up-regulation in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> occurred only in cells treated for 7 days with the top concentration. By contrast, EB1089 induced Bak up-regulation at all of the concentrations and at each of the three time points. In HT29, however, Bak was similarly up-regulated in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089. B, changes in Bcl-2 and Bax levels with EB1089 treatment. Bcl-2 was significantly reduced by all of the doses of EB1089 in PC/JW, whereas HT29 showed a consistent increase in Bcl-2 levels. The increase in Bcl-2 levels in HT29 cells with EB1089 treatment was accompanied by a consistent increase in the levels of Bax.

by Tanaka *et al.* (8) that very little ALP activity was induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> alone in HT29 cells. Similar patterns of ALP induction were obtained using EB1089 (data not shown), which indicated that, like 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, the analogue induces differentiation followed by apoptosis.

Table 1 Summary of the effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> on the expression of Bcl-2 family members

The results for the highest concentration (10<sup>-6</sup> M) are given for days 2, 4, and 7. Results were consistent among the three independent experiments for which Western blotting was performed.  $\alpha$ -tubulin antibody (Sigma) was used to reprobe each blot as a loading control.

	RG/C2			AA/C1			PC/JW			HT29			SW620		
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
Bcl-2	- <sup>a</sup>	-	-	×	×	×	-	↓	↓	-	↑	↑	↑	↑	↑
Bcl-x <sub>L</sub>	-	-	-	-	↑	↑	-	-	-	-	-	-	-	-	-
Bax	-	-	-	-	-	↑	-	-	-	-	-	-	-	-	-
Bak	↑	↑	↑	↑	↑	↑	↑	↑	↑	-	↑	↑	↑	↑	↑

<sup>a</sup> -, no change in protein levels; ×, no detectable protein expression; ↑, increased protein levels; ↓, decreased protein levels.

## DISCUSSION

In colonic epithelial cells, the induction of differentiation and apoptosis may be tightly linked processes that can be independent of p53 function, which offers promise for differentiation agents in colorectal cancer therapy. We have shown previously that the differentiation agent, butyrate, induces apoptosis in colorectal tumor cells, and that this is manifested as an increase in the proportion of cells in the cultures that are present in the form of floating cells. These floating cells have the morphological characteristics of apoptotic cells and produce a DNA ladder on gel electrophoresis, indicative of internucleosomal fragmentation of the DNA (36). There is evidence that butyrate induces apoptosis via a differentiation pathway in that ALP activity, a marker of enterocytic differentiation, is elevated in both adherent and floating cells of butyrate-treated cultures, and levels are higher in the floating apoptotic cells than in the corresponding adherent cells (32, 33). Butyrate-induced apoptosis occurs independently of wild-type p53 (36) and is characterized by decreased levels of Bcl-2 or increased levels of the proapoptotic member of the Bcl-2 family, Bak (39).

One of the questions that we wished to address was whether other agents known to induce differentiation in colonic epithelial cells would also induce apoptosis in colonic tumor cells and whether this was via a pathway of differentiation. We were interested in 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> because this steroid hormone had previously been shown to be growth inhibitory to colonic tumor cells (9). A number of studies of the effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> on the colonic carcinoma cell line HT29 have demonstrated the induction of differentiation by the steroid (40, 41) or potentiation of butyrate-induced differentiation (8). In the hyperproliferative skin disease, psoriasis, keratinocytes are induced to differentiate by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and analogues (42), and vitamin D compounds form an effective treatment. In mammary tumor cell lines, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induces apoptosis (10), whereas in the myeloid leukemia cell line, HL60, it has been reported to confer resistance to apoptosis induced by calcium ionophore and the chemotherapeutic drugs, arabinocytosine and etoposide (43). These observations led us to question whether the growth inhibition obtained in colonic carcinoma cells was attributable to apoptosis and/or a cell cycle arrest. We also questioned whether there was a link between differentiation and apoptosis and whether premalignant adenoma cells would undergo apoptosis in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>.

In all of the five cell lines (two adenoma and three carcinoma), 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induced growth inhibition, the differentiation marker ALP, and apoptosis. The apoptotic cells were shed into the medium. These cells were morphologically apoptotic and demonstrated internucleosomal DNA cleavage. In the PC/JW cell line, we have frequently noticed that, in response to different agents, detachment during apoptosis is delayed compared with the other four cell lines<sup>3</sup> and, in this cell line, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induced apoptosis that was detectable in the adherent cells as well as by an increase in the proportion of

apoptotic cells floating in the medium. ALP activity has previously been used to demonstrate that the induction of apoptosis in colonic tumor cells by the differentiation agent, butyrate, is via differentiation (32, 33). We used this same marker of colonic differentiation to investigate whether 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> also induced apoptosis via differentiation. ALP activity was increased by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> treatment in both the adherent- and floating-cell populations. The floating apoptotic cells had higher levels of ALP activity than the adherent cells, which indicated that apoptosis occurred by a process of differentiation and apoptosis akin to terminal differentiation. The adenoma cell lines were more sensitive to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-induced apoptosis at the earlier time points, but the carcinoma cell lines underwent greater apoptosis in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> after prolonged treatment (5–7 days' daily treatment with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>). In fact, apoptosis continued in the carcinoma cell lines after 2 weeks of continuous daily treatment (data not shown). The fact that colorectal carcinoma cells retain their response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> shows promise for therapeutic strategies using noncalcemic analogues of the hormone. Furthermore, the sensitivity of the adenoma cell lines to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-induced apoptosis may be important in the prevention of colon cancer. All five of the cell lines underwent apoptosis in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, irrespective of p53 status (see "Materials and Methods"), demonstrating p53-independent apoptosis.

Having shown that the active metabolite of vitamin D<sub>3</sub> induced apoptosis, we asked the important question as to whether the analogue EB1089 inhibited growth by the same mechanisms as the naturally occurring steroid. This analogue is undergoing clinical trials for the treatment of a variety of cancers because of its reduced hypercalcemic effects. EB1089 was investigated under the same experimental conditions and using the same five cell lines to determine whether it also induced apoptosis. The experiments were designed to compare the response of the cells to EB1089 and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and to investigate whether EB1089 was growth inhibitory and/or induced apoptosis in premalignant adenoma cells as well as in carcinoma cells. Studies with EB1089 showed that this analogue is more effective than 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> in inducing a G<sub>1</sub> arrest in colonic tumor cell lines. The G<sub>1</sub> arrest was evident at lower concentrations of EB1089 compared with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and also earlier during the treatment period. The adenoma cell lines were markedly more sensitive to EB1089-induced cell cycle arrest than the carcinomas. Furthermore, in the adenoma cell lines, RG/C2 and AA/C1, and in the carcinoma cell line, PC/JW, EB1089 treatment produced a greater reduction in attached cell yield at day 7 than 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. The most striking difference in the effect of the two agents was seen in the PC/AA/C1 adenoma cell line, in which 10<sup>-10</sup> M 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induced only 9% reduction in cell yield, whereas an equimolar concentration of EB1089 produced a 40% reduction. In the AA/C1 and PC/JW cell lines, EB1089 was also more effective in its induction of apoptosis than 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Although the carcinoma cell line HT29 was relatively resistant to apoptosis induced by EB1089 compared with 1 $\alpha$ ,25-

<sup>3</sup> Unpublished observations.

Table 2 Summary of the effects of EB1089 on the expression of Bcl-2 family members

The results for the highest concentration (10<sup>-6</sup> M) are given for days 2, 4, and 7. Results were consistent among the three independent experiments for which Western blotting was performed.

	RG/C2			AA/C1			PC/JW			HT29			SW620		
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
Bcl-2	↓ <sup>a</sup>	↓	↓	×	×	×	↓	↓	↓	-	-	↑	↑	↑	-
Bcl-x <sub>L</sub>	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bax	-	-	↑	-	-	-	-	-	-	-	-	↑	-	-	-
Bak	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑

<sup>a</sup> ↓, decreased protein levels; ↑, increased protein levels; -, no change in protein levels; ×, no detectable protein expression.

dihydroxyvitamin D<sub>3</sub>, EB1089 reduced the adherent-cell yield of HT29 to a similar extent at concentrations of 10<sup>-10</sup> to 10<sup>-7</sup> M over the 7 days of treatment. The observation that EB1089 can induce apoptosis in adenoma as well as carcinoma cells supports the idea of using EB1089 for colon cancer chemoprevention, particularly given that, compared with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, higher doses of EB1089 can be tolerated *in vivo*.

To begin to determine how apoptosis is induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 in colorectal adenoma and carcinoma cells, we selected four members of the Bcl-2 family of apoptosis-regulatory proteins for study. The proapoptotic protein Bak was consistently up-regulated in association with apoptosis in all of the five colonic tumor cell lines. Bak increases in response to EB1089 were often more pronounced and earlier than in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. That 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> induces differentiation and Bak-mediated apoptosis is consistent with the gradient of expression of Bak in the crypt, with maximal expression in the mature, differentiated cells at the top of the crypt (44). In addition, we have data from SW620 cells treated with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 for 7 days showing that the VDR protein levels were increased, consistent with the binding of the ligand to the nuclear receptor (data not shown). However, additional studies are required to determine whether the induction of apoptosis by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> is via the VDR nuclear receptor and whether the VDR directly or indirectly induces Bak transcription.

EB1089 has been shown to down-regulate Bcl-2 in the breast cancer cell line MCF7 (45). In the colonic tumor cell lines RG/C2 and PC/JW, Bcl-2 was markedly down-regulated by EB1089; however,

the adenoma cell line AA/C1, which does not express Bcl-2, still underwent apoptosis in response to both 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089. Bcl-2 down-regulation is, therefore, only partly responsible for the induction of apoptosis by EB1089. Moreover, HT29 and SW620 did not show Bcl-2 down-regulation in response to EB1089. On the contrary, there was a small increase in Bcl-2 levels, which occurred late in the time course for HT29 and early for SW620. Interestingly, this inversely correlated with the time points at which most apoptosis occurred. Bcl-2 up-regulation may, in part, confer some resistance to EB1089-induced apoptosis and counter the effects of Bak up-regulation. It may represent a stress response by the cells, as has been described previously (46).

In conclusion, both 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 induce apoptosis in colorectal tumor cells. Apoptosis seems to occur subsequent to the induction of enterocytic (absorptive cell) differentiation. Up-regulation of Bak in response to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and EB1089 in five different colorectal tumor cell lines occurs in association with apoptosis, which suggests that this protein mediates at least part of the apoptotic response to these agents and that apoptosis is induced by similar mechanisms for both 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and its analogue, EB1089. Interest has focused on EB1089 because of its reduced hypercalcemic effects, which mean that, compared with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, higher doses of EB1089 can be achieved *in vivo*. In addition to inducing apoptosis, the analogue EB1089 induces a more pronounced growth arrest in the G<sub>1</sub> phase of the cell cycle than 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, particularly in the premalignant adenoma cells. These studies suggest that EB1089 may be an effective chemopreventive agent for individuals at high risk of

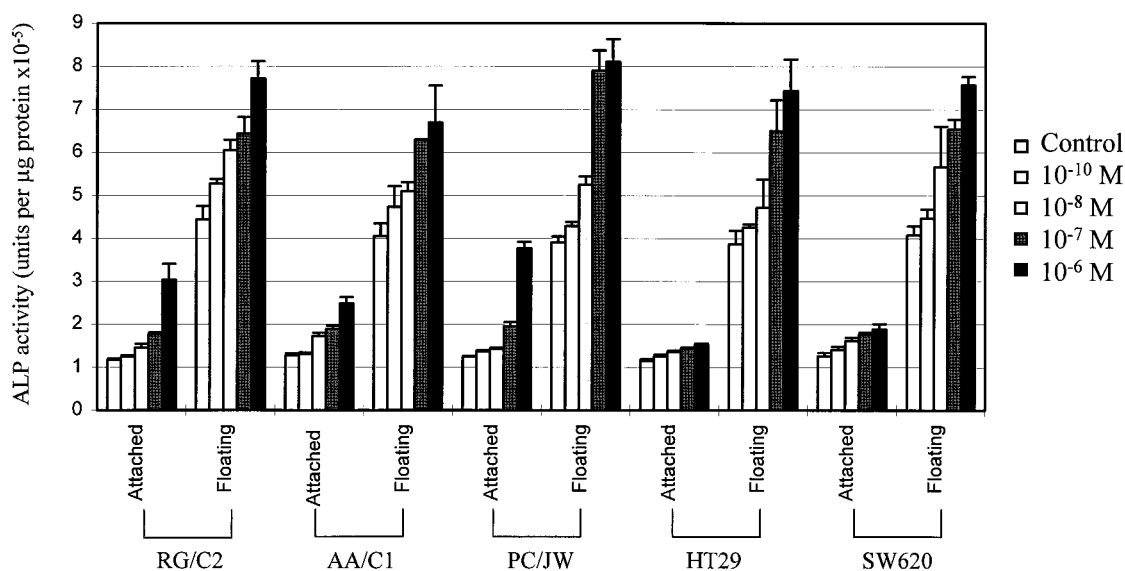


Fig. 6. ALP activity (units per  $\mu\text{g}$  of protein  $\times 10^{-5}$ ) in the adherent and floating cells of cultures after 7 days' treatment with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. ALP activity was used as a marker of absorptive cell differentiation. Results are means  $\pm$  SD of at least three experiments. For all of the five cell lines, higher ALP activity was detected in the floating cells than in the adherent cells (in control and treated cultures), and there was a dose-dependent increase in ALP activity in floating and adherent populations. Results obtained from cultures treated with EB1089 were similar.



developing colorectal cancer. Furthermore, EB1089 induces apoptosis (the extent of which increases with time of treatment) in colorectal cancer cells, including cells with mutant p53 and the SW620 cell line, which was derived from a lymph node metastasis (28). Therefore, EB1089 shows potential for the treatment of colorectal cancer in conjunction with current chemotherapy. Vitamin D analogues such as EB1089 have potential as therapeutic agents for colorectal cancer, and a greater understanding of their mechanisms of action may enable more effective clinical application.

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