Apoptosis Induced by Vitamin D Compounds in Breast Cancer Cells Is Inhibited by Bcl-2 but Does Not Involve Known Caspases or p53

Ida Stenfeldt Mathiasen, Ulrik Lademann, and Marja Jäättelä

Apoptosis Laboratory, Institute of Cancer Biology, Danish Cancer Society, DK-2100 Copenhagen, Denmark

ABSTRACT

The hormonally active form of vitamin D₃, 1,25-dihydroxyvitamin D₃, and its two analogues, EB 1089 and CB 1093, are novel putative anticancer agents with an interesting profile of induction of growth inhibition, differentiation, and apoptosis in tumor cells. To study the signaling pathways mediating these events, we used two human breast cancer cell lines: MCF-7 cells, expressing a wild-type p53 tumor suppressor protein, and T47D cells, lacking a functional p53. Vitamin D compounds induced a growth arrest followed by apoptosis in both cell lines at concentrations ranging from 1 to 100 nM, indicating that p53 is not necessary for growth-inhibitory effects induced by vitamin D compounds. Surprisingly, apoptosis induced by these compounds occurred also independently of known caspases. Inhibition of caspase activation by overexpression of a cowpox-derived caspase inhibitor CrmA or by addition of inhibitory peptides acetyl-Asp-Glu-Val-Asp-aldehyde (200 μM), acetyl-Ile-Glu-Thr-Asp-aldehyde (50 μM), and Z-Val-Ala-d,L-Asp-fluoromethylketone (1 μM) showed no effect on the induction of growth arrest or apoptosis by vitamin D compounds under assay conditions in which apoptosis induced by TNF or staurosporine was effectively inhibited. Moreover, overexpression of caspase-3 in MCF-7 cells had no sensitizing effect to vitamin D compounds, and neither caspase-3-like protease activity nor cleavage of a caspase substrate poly(ADP)ribose polymerase was detected in lysates from apoptotic cells following the treatment with these compounds. Contrary to CrmA, overexpression of an antiapoptotic protein Bcl-2 in MCF-7 cells conferred a nearly complete protection from apoptosis induced by vitamin D compounds. Taken together, these data indicate that vitamin D compounds induce apoptosis via a novel caspase- and p53-independent pathway that can be inhibited by Bcl-2. This may prove useful in the treatment of tumors that are resistant to therapeutic agents that are dependent on the activation of p53 and/or caspases.

INTRODUCTION

An important aim of cancer research is to find therapeutic compounds with fewer side effects than the presently used cytostatic/cytotoxic agents. This has led to the studies on the effectiveness of various naturally occurring differentiation-inducing agents as potential anticancer drugs. The hormonally active form of vitamin D₃, 1,25(OH)₂D₃, is well known for its regulatory role in calcium homeostasis, but it also plays an important role in the modulation of proliferation and differentiation of several malignant cell types (1, 2). In addition to its growth-inhibitory effect, 1,25(OH)₂D₃ has recently been shown to induce apoptosis in breast cancer, colon cancer, and glioma cell lines (3–9). The ability of 1,25(OH)₂D₃ to induce growth arrest, differentiation, and apoptosis provides it with promising therapeutic potential for the treatment of cancer. The use of the natural compound is, however, limited because its administration in therapeutic doses causes deleterious side effects due to disturbances in calcium homeostasis (10). Therefore, synthetic analogues have been developed with stronger growth-regulatory effects combined with reduced effects on calcium metabolism. EB 1089 and CB 1093 are two such synthetic vitamin D analogues (9, 11). EB 1089 and CB 1093 are ~10- and 100-fold more potent inducers of apoptosis in MCF-7 breast carcinoma cells than is 1,25(OH)₂D₃, respectively (9). Moreover, these compounds are able to cause regression of rat mammary tumors and to induce apoptosis in MCF-7 xenografts in mice without the induction of toxic side effects (7, 9, 12). Thus, these two analogues represent novel possible anticancer drugs with an interesting profile of growth-regulatory effects.

Agents that induce apoptosis in cancer cells have recently attracted great attention. The process of apoptosis can be triggered by pleiotropic ways, including activation of cell surface death receptors of TNFR family, γ-irradiation, oxidative stress, various chemotherapeutic agents, and so on (13–17). The known signaling pathways induced by various apoptotic stimuli converge into a common death pathway either at a mitochondrial step or finally at a step at which Asp-Glu-Val-Asp (DEVD)-specific caspase-3-like cysteine proteases are activated (13–17). Active caspase-3-like proteases cleave a limited set of cellular proteins, and the resulting inactivation/activation of substrates supposedly leads to the typical apoptotic morphology of the dying cell (15). Although the activation of a caspase cascade has been considered a hallmark of apoptosis, apoptotic pathways not requiring known caspases have also been suggested. For example, a proapoptotic protein Bax, which targets mitochondrial membranes; a topoisomerase I poison, camptothecin; and nitric oxide have been reported to induce apoptosis-like cell death without caspase activation (18–20). Thus, other proteases than caspases may be able to induce apoptotic morphology. Indeed, serine proteases, cathepsins, and calpains have been implicated as mediators or comediators of apoptotic proteolysis induced by various stimuli, including TNFR-related death receptors, bile salt, irradiation, T-cell receptor activation, and camptothecin (19, 21–24).

Tumor cells express several proteins that suppress apoptosis and, thereby, render them resistant to various forms of therapy. The Bcl-2 family contains at least 15 proteins that can be divided in proapoptotic and antiapoptotic members (17). Several family members like antiapoptotic Bcl-2 and Bcl-xL contain a COOH-terminal hydrophobic membrane anchor domain that may target them to various intracellular membranes, including mitochondrial membranes, endoplasmic reticulum, and perinuclear membrane (17). Mitochondrial Bcl-2 may exert its antiapoptotic function by inhibiting the apoptosis-associated mitochondrial changes, whereas Bcl-2 localized to the endoplasmic reticulum may contribute to the regulation of apoptosis by inhibiting calcium fluxes and maintaining calcium homeostasis (16, 17).

The induction of apoptosis by 1,25(OH)₂D₃ and its synthetic analogues differs from most other apoptotic stimuli by having much slower kinetics; e.g., in MCF-7 breast cancer cells, the apoptotic morphology occurs first after 3 days of treatment with 1,25(OH)₂D₃ (7–9). This delay suggests that cellular changes must occur prior to the...
onset of apoptosis. Bcl-2 protein level has been observed to be down-regulated during treatment of MCF-7 breast cancer cells with vitamin D compounds, and this may be important for the susceptibility of the cells to apoptosis (7–9, 25). Vitamin D compounds have also been reported to up-regulate the levels of several cell cycle- and apoptosis-associated proteins in MCF-7 cells. Among these proteins are: p53 tumor suppressor protein, capable of inducing both growth arrest and apoptosis; two inhibitors of cyclin-dependent kinases, p21waf1 and p27kip1; and two proteins also induced during mammalian gland regression, namely, the active forms of cathepsin B and clustatin (7, 25–29). Moreover, cotreatment of MCF-7 cells with 1,25(OH)2D3 sensitizes them to TNF-induced apoptosis in a protein synthesis-dependent manner, suggesting a possible cross-talk between these two pathways (30).

The aim of this study was to gain more insight into the signaling pathways involved in apoptosis induced by 1,25(OH)2D3 and its synthetic analogues EB 1089 and CB 1093. We investigated the effect of well-characterized antiapoptotic proteins and peptides on apoptosis induced by vitamin D compounds in MCF-7 and T47D human breast cancer cells expressing wild-type and mutant p53, respectively.

**MATERIALS AND METHODS**

**Cell Lines, Transfection, and Culture Conditions.** The MCF-7S1 and MCF-7S8 cell lines are subclones of MCF-7 breast carcinoma cells derived by limiting dilution and selection for sensitivity to TNF-induced apoptosis (31). MCF-Fas-V2, MCF-Fas-Bcl2-2, and MCF-Fas-BclXl-2 cells are MCF-7 cells that were successfully transfected first with Fas expression plasmid and then with pEB5-7 vector or the same vector containing the cDNAs encoding human Bcl-2 or Bcl-Xl, respectively (31). MCF-CmA-K6, MCF-CmA-K7, and MCF-CmA-K6 cell clones of MCF-7S1 cells expressing wild-type CmA (K6 and K7) or its inactive mutant (M6; Ref. 32). The MCF-FADD-DN cells are MCF-7 cells overexpressing a dominant negative form of FADD protein (33). MCF-Casp3 and MCF-vector cells were obtained by transfecting MCF-7S1 cells with pcDNA-3-Casp3 or an empty cDNA-3 vector, respectively, as described previously (31). All MCF-7-derived cells were propagated in RPMI 1640 with glutamax (Life Technologies, Ltd., Paisley, United Kingdom) supplemented with 5% FCS (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin, and streptomycin and/or the required selection antibiotics, G418 and hygromycin. Selection antibiotics were omitted during experiments. The human breast cancer cell line T47D was propagated in RPMI 1640 with glutamax supplemented with 10% FCS, 250 ng/ml bovine pancreatic insulin (Boehringer Mannheim, Mannheim, Germany), penicillin, and streptomycin.

**Compounds.** The vitamin D compounds 1,25(OH)2D3, EB 1089, and CB 1093 were generous gifts from Lise Binderup and Christina Mørk Hansen (Leo Pharmaceutical Products, Ballerup, Denmark). The vitamin D compounds were stored as 4 mM stock solutions in isopropanol and further diluted in PBS (pH 7.4), 1 mM EDTA, 1 mM EGTA, 40 mM phenylmethylsulfonyl fluoride, and 4 mM DTT. The vitamin D compounds did not reduce the value per cell, indicating that the reduction in cell density earlier than 1,25(OH)2D3. The MTT tetrazolium assay was used to measure the activity of these two pathways (30).

**In Vitro Measurement of Caspase-3-like Protease Activity.** The activity of caspase-3 like enzyme activity was measured in vitro using a colorimetric probe DEVD-pNA (Biomol). Cells were seeded in Petri dishes and treated as indicated in "Results." After the end of the treatment period, the cell lysates were analyzed for activity in vitro DEVDase activity as described previously (35). Briefly, a cell lysate of 10–10000 cells/ml was incubated with 100 μM DEVD-pNA in buffer A [80 mM KCl, 10 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 40 μg/ml leupeptin, 25 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 4 mM DTT] was analyzed for protease content (30 min) using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Lysates containing equal amounts of proteases (corresponding to ~105 cells) were mixed in a microtiter plate with 150 μl of 100 μM DEVD-pNA in buffer B [100 mM HEPES (pH 7.5), 20% glycerol, 5 mM DTT, and 0.5 mM EDTA]. After 4–20 h incubation at 37°C, absorbance at 405 nm was measured in a microtiter plate reader (Molecular Devices).

**Immunoblot Analysis.** Immunoblot analyses were performed as described previously (32). The primary antibodies used were mouse monoclonal antibody C2-10, recognizing PARP, at a dilution of 1:15000 (36) and mouse monoclonal anti-caspase-3 antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000. The peroxidase-conjugated antiserum was diluted 1:10000. Chemiluminescence reactions were performed according to manufacturer’s instructions using ECL Western blot reagents (Amersham).

**RESULTS**

**Treatment of MCF-7S8 with Vitamin D Compounds Induces Growth Arrest followed by Induction of Apoptosis.** The human breast cancer cell line MCF-7S8 was treated with 1,25(OH)2D3, EB 1089, or CB 1093 at concentrations ranging from 1 to 100 nM for 1–7 days, and the kinetics of the induction of growth arrest and apoptosis were studied by thymidine incorporation assay and TUNEL staining, respectively. The combined effect of growth arrest and apoptosis on the cell density was measured by the MTT assay. The density of cells treated with vitamin D compounds started to decline at day 2 as compared with diluent-treated control cells (Fig. 1A). Treatment of MCF-7S8 cells with vitamin D compounds at the concentration of 100 nM for 6 days resulted in ~80% reduction in cell density (Fig. 1A). At 100 nM concentrations, no clear differences between the compounds could be seen in the efficacy, although EB 1089 and CB 1093 showed a tendency of reducing the cell density earlier than 1,25(OH)2D3. Moreover, at lower doses (1 nM and 10 nM) EB 1089 and CB 1093 were clearly more effective than 1,25(OH)2D3 (data not shown).
The antiproliferative effect measured by incorporation of thymidine could be detected in MCF-7S8 cells treated with all three compounds as soon as after 1 day of treatment, i.e., before any decrease in cell density (Fig. 1B). The antiproliferative effect was further enhanced by all three compounds until day 4, after which the thymidine incorporation levels stabilized just below 20% of that in the control culture.

Induction of apoptosis by vitamin D compounds in MCF-7S8 cells was delayed for several days, occurring clearly after the cells had been growth-arrested (Fig. 1C). CB 1093, EB 1089, and 1,25(OH)2D3 seemed to display a slight difference in the timing for the onset of apoptosis, the synthetic analogues being faster than 1,25(OH)2D3 (Fig. 1C). All compounds resulted, however, in ~14% apoptotic cells on days 6 and 7 of the treatment.

In all three assays, the compounds were tested at 1, 10, and 100 nM, and in all cases, dose-dependent responses were seen (data not shown). These data also confirmed earlier results showing that CB 1093 is more potent than EB 1089, which is more potent than 1,25(OH)2D3 in inducing both growth arrest and apoptosis (9).

**Bcl-2 Inhibits Induction of Apoptosis by Vitamin D Compounds.** The delay in the induction of apoptosis by vitamin D compounds suggests that some cellular changes need to take place before the onset of apoptosis. Because the Bcl-2 protein has been described to be down-regulated in response to 1,25(OH)2D3, we tested whether its overexpression could influence the survival of MCF-7 cells following treatment with vitamin D compounds. For this purpose, we used MCF-Fas cells overexpressing Bcl-2 (MCF-Fas-Bcl2-2) together with the respective vector-transfected control cells (MCF-Fas-V2; Ref. 31). Overexpression of Bcl-2 in MCF-Fas cells had no significant effect on early growth-regulatory effects induced by up to 3 days of treatment with vitamin D compounds, whereas it inhibited almost completely the growth-regulatory effects observed after 3 days (Fig. 2A). These results suggested that Bcl-2 inhibits apoptosis induced by vitamin D compounds without affecting the growth-inhibitory effects. To ensure that the difference in cellular response between the cell lines was, indeed, the result of the antiapoptotic function of Bcl-2, we tested the ability of vitamin D compounds to induce apoptosis in these cells by TUNEL method. Treatment of MCF-Fas-V2 cells with 1,25(OH)2D3, EB 1089, or CB 1093 at the concentration of 100 nM for 6 days resulted in a significant (P < 0.05, Student’s t test) increase in the number of apoptotic cells (Fig. 2B and data not shown). In Bcl-2-overexpressing MCF-Fas-Bcl2-2 cells, all vitamin D compounds failed to induce significant apoptosis (Fig. 2B). Similar results were obtained with MCF-Fas cells overexpressing Bcl-xL (data not shown).

The MCF-Fas-V2 cells overexpressing the death receptor Fas were more susceptible to induction of apoptosis by vitamin D compounds as compared with MCF-7S8 cells with low Fas expression (Figs. 1C and 2B). This could be due to a clonal variation or an involvement of Fas in the apoptosis signaling pathway induced by vitamin D compounds. To test the latter possibility, we tested the effect of the inhibition of Fas pathway on apoptosis induced by vitamin D compounds using MCF-7 cells expressing a dominant negative mutant of Fas-associated death domain protein (MCF-FADD-DN; Ref. 33). Although the MCF-FADD-DN cell line was protected against apoptosis induced by activation of Fas or TNFR (33), apoptosis induced by vitamin D compounds was not affected (data not shown). Thus, the slightly higher sensitivity to apoptosis induced by vitamin D compounds seen in the MCF-Fas cell clone is not likely to be due to a Fas-induced activation of the FADD-dependent apoptotic pathway.

**The Antiapoptotic Protein CrmA Does Not Inhibit Apoptosis Induced by Vitamin D Compounds.** The cowpox-derived inhibitor of caspases CrmA has proven inhibitory against various apoptotic stimuli (37–40). To test the ability of CrmA to inhibit apoptosis
induced by vitamin D compounds, we used MCF-7-derived cell lines expressing CrmA (MCF-CrmA-K6 and MCF-CrmA-K7) together with a control cell line (MCF-CrmA-M6), which expresses an inactive mutant form of CrmA (32, 37). These three cell lines were treated with graded doses of 1,25(OH)2 D3 for 7 days. 1,25(OH)2 D3 induced a dose-dependent reduction in the cell density in all three cell clones (Fig. 3A). The two cell lines MCF-CrmA-K6 and MCF-CrmA-K7, each expressing the functional form of CrmA did not show any degree of protection against 1,25(OH)2 D3-induced antiproliferative effects as compared with the reference cell line, MCF-CrmA-M6 (Fig. 3A). Similarly, the treatment with EB 1089 or CB 1093 resulted in a dose-dependent response without any protection against the inhibition of cell growth as a result of CrmA overexpression, as measured by MTT cell density assay (data not shown). Therefore, we tested whether known caspase substrates are cleaved in MCF-7S1 cells after 5-day treatment with 10 ng/ml TNF (Fig. 4C). These data show that the activation of known caspases is not needed for induction of growth inhibition or apoptosis in cells treated with vitamin D compounds.

The above data do not show whether apoptotic caspases are activated, although they are not needed, for the induction of apoptosis by vitamin D compounds. To test the ability of vitamin D compounds to activate caspase-3-like proteases, we prepared cell lysates from MCF-7S8 cells after 5-day treatment with 100 nm 1,25(OH)2 D3, EB 1089, or CB 1093. As a positive control, we used a cell lysate from MCF-7S8 cells treated with 10 ng/ml TNF for 24 h. Although the lysate from TNF-treated cells showed a 14-fold increase in the DEVADase activity, the lysates treated with the vitamin D compounds for 5 days had DEVADase activity levels that were indistinguishable from cell lysates from the diluent-treated control cells (Fig. 4D). In the case of a rapid, transient activation of caspases, we could have missed an activation peak by choosing a wrong time point for the assay. Therefore, we tested whether known caspase substrates are cleaved in MCF-7S8 cells treated with inhibitory peptides did not influence the growth-inhibitory effects of vitamin D compounds. Moreover, ZVAD-fmk, a potent general inhibitor of caspases added to cells at the final concentration of 1 μM at the beginning and at day 3 of treatment with 100 nm 1,25(OH)2 D3 or 10 ng/ml TNF had no effect on 1,25(OH)2 D3-induced reduction in cell density despite the almost complete inhibition of TNF-induced cell death (Fig. 4B). Similarly, 1 μM ZVAD-fmk failed to inhibit the antiproliferative effects induced by a 6-day treatment with 100 nm EB 1089 or CB 1093 (data not shown). The inability of ZVAD-fmk to inhibit apoptosis induced by vitamin D compounds was also confirmed by a TUNEL assay, in which cells treated with vitamin D compounds alone or in combination with ZVAD-fmk had similar percentage of apoptotic cells, whereas ZVAD-fmk effectively inhibited apoptosis induced by 10 ng/ml TNF (Fig. 4C). These data show that the activation of known caspases is not needed for induction of growth inhibition or apoptosis in cells treated with vitamin D compounds.

**Known Caspases Are Not Involved in Apoptosis Induced by Vitamin D Compounds.** CrmA is a more potent inhibitor of the inflammatory caspase-1-like proteases than actual apoptotic proteases resembling caspase-8 or caspase-3 (15, 41, 42). To investigate more directly whether these apoptosis-specific enzymes mediate cell death induced by vitamin D compounds, we added the inhibitory peptides IETD-CHO and DEVD-CHO into cell cultures induced to undergo apoptosis by vitamin D compounds. MCF-7S8 cells were treated with 100 nm 1,25(OH)2 D3 or CB 1093 for 5 days, and the inhibitory peptides were added at day 3 of the treatment, i.e., before any apoptotic changes were apparent. This timing was chosen to ensure that putative caspases were inhibited in the onset of apoptosis without the cells having time to degrade the peptides. As shown in Fig. 4A, treatment of MCF-7S8 cells with inhibitory peptides did not influence the growth-inhibitory effects of vitamin D compounds. Moreover, ZVAD-fmk, a potent general inhibitor of caspases added to cells at the final concentration of 1 μM at the beginning and at day 3 of treatment with 100 nm 1,25(OH)2 D3 or 10 ng/ml TNF had no effect on 1,25(OH)2 D3-induced reduction in cell density despite the almost complete inhibition of TNF-induced cell death (Fig. 4B). Similarly, 1 μM ZVAD-fmk failed to inhibit the antiproliferative effects induced by a 6-day treatment with 100 nm EB 1089 or CB 1093 (data not shown). The inability of ZVAD-fmk to inhibit apoptosis induced by vitamin D compounds was also confirmed by a TUNEL assay, in which cells treated with vitamin D compounds alone or in combination with ZVAD-fmk had similar percentage of apoptotic cells, whereas ZVAD-fmk effectively inhibited apoptosis induced by 10 ng/ml TNF (Fig. 4C). These data show that the activation of known caspases is not needed for induction of growth inhibition or apoptosis in cells treated with vitamin D compounds.
specific caspases. Furthermore, the inability of vitamin D compounds to induce DEVDase activity in MCF-7 cells is not due to the lack of caspase-3 in this cell line.

**Vitamin D Compounds Induce Growth Arrest and Caspase-independent Apoptosis in T47D Breast Cancer Cells Lacking the Functional p53.** On the basis of the inability of vitamin D compounds to induce apoptosis in several breast cancer cells lacking the functional p53 proteins, it has been suggested that p53 may be involved in the signaling pathways induced by these compounds (44). Moreover, vitamin D compounds have been reported to up-regulate the level of p53 protein and to induce its translocation into the nuclei of sensitive MCF-7 cells expressing the wild-type p53 (7, 44). To test whether p53 is required for the growth regulatory effects of vitamin D compounds, we tested their ability to inhibit DNA synthesis and induce apoptosis in a human breast cancer cell line T47D that lacks the functional p53 protein (45). T47D cells were treated with 1,25(OH)2 D3, EB 1089, or CB 1093 (100 nM) over a time course of 7 days to study the kinetics of the reduction in cell density, inhibition of DNA synthesis, and induction of apoptosis. The cell density of T47D cells treated with vitamin D compounds started to decline on the third day of the treatment, and at day 6, the cell density of the treated cells was 40–50% of that of the control cells (Fig. 6A). No obvious differences in the efficacy could be seen between the vitamin D compounds at the concentration of 100 nM. As was the case in MCF-7S8 cells, CB 1093 was, however, more potent than EB 1089, and both synthetic analogues were considerably more potent than 1,25(OH)2 D3 when added at lower concentrations, i.e., 1 and 10 nM (data not shown).

The inhibition of DNA synthesis in T47D cells was detected already after 1 day of treatment and was further enhanced by all three compounds until day 6, when thymidine incorporation was ~20% of that in control cultures (Fig. 6B). Apoptotic morphology appeared with a delay of several days when compared with the inhibition of DNA synthesis (Fig. 6C). CB 1093, EB 1089, and 1,25(OH)2 D3 displayed slight differences in the timing and extent of apoptosis induced, the synthetic analogues being more effective than 1,25(OH)2 D3 (Fig. 6C). At day 7, CB 1093 was most efficient in the induction of apoptosis, resulting in 16% apoptotic cells, whereas EB 1089 resulted in ~12% and 1,25(OH)2 D3 in just below 10% TUNEL-positive cells.

On the basis of the above data, it is clear that the induction of apoptosis by vitamin D compounds is not dependent on functional p53 and is not a specific feature of MCF-7 cells. T47D cells express caspase-3 in a similar level as transfected MCF-Casp3 cells (data not shown). Thus, we could use T47D cells as another model system to study the role of caspases in vitamin D-induced apoptosis. Treatment of T47D cells with up to 50 μM IETD-CHO, 200 μM DEVD-CHO, or 1 μM ZVAD-fmk (data not shown), concentrations capable of inhib-
CASPASE-INDEPENDENT APOPTOSIS BY VITAMIN D COMPOUNDS

The caspases, comprising a family of cysteine proteases, have been firmly established as major mediators of the execution phase of apoptosis. Cross-linking of death receptors (TNFR1, Fas, and death receptor 3) can directly activate caspases, whereas most other apoptotic stimuli lead to mitochondrial changes and a subsequent activation of the caspase cascade (13–16). We show that vitamin D compounds induce a growth arrest followed by typical morphological changes of apoptosis in MCF-7 and T47D cells. In our studies, apoptosis was defined by changes seen in light microscopy, i.e., shrinkage and rounding of the cells, as well as by TUNEL staining detecting DNA strand breaks. Moreover, others have described typical apoptotic changes, including DNA fragmentation, DNA strand breaks, pyknotic nuclei, chromatin condensation, and nuclear matrix reorganization as well as rounding, shrinkage, and detachment of MCF-7 cells treated with vitamin D compounds (8, 44, 46). Surprisingly, our data clearly show that the typical apoptotic morphology induced by vitamin D compounds occurs without activation of known caspases. This conclusion is based on following lines of evidence: (a) a cowpox virus-derived caspase inhibitor, CrmA, has no effect on apoptosis induced by vitamin D compounds in MCF-7S1 cells; (b) the caspase inhibitors, DEVD-CHO, IETD-CHO, and ZVAD-fmk, at concentrations that clearly inhibit TNF- and staurosporine-induced apoptosis, had no effect on apoptosis induced by vitamin D compounds in MCF-7S8 or T47D cells; (c) no caspase-3-like activity could be measured in apoptotic MCF-7S8 by an enzyme activity assay based

**DISCUSSION**

The caspases, comprising a family of cysteine proteases, have been firmly established as major mediators of the execution phase of apoptosis. Cross-linking of death receptors (TNFR1, Fas, and death receptor 3) can directly activate caspases, whereas most other apoptotic stimuli lead to mitochondrial changes and a subsequent activation of the caspase cascade (13–16). We show that vitamin D compounds induce a growth arrest followed by typical morphological changes of apoptosis in MCF-7 and T47D cells. In our studies, apoptosis was defined by changes seen in light microscopy, i.e., shrinkage and rounding of the cells, as well as by TUNEL staining detecting DNA strand breaks. Moreover, others have described typical apoptotic changes, including DNA fragmentation, DNA strand breaks, pyknotic nuclei, chromatin condensation, and nuclear matrix reorganization as well as rounding, shrinkage, and detachment of MCF-7 cells treated with vitamin D compounds (8, 44, 46). Surprisingly, our data clearly show that the typical apoptotic morphology induced by vitamin D compounds occurs without activation of known caspases. This conclusion is based on following lines of evidence: (a) a cowpox virus-derived caspase inhibitor, CrmA, has no effect on apoptosis induced by vitamin D compounds in MCF-7S1 cells; (b) the caspase inhibitors, DEVD-CHO, IETD-CHO, and ZVAD-fmk, at concentrations that clearly inhibit TNF- and staurosporine-induced apoptosis, had no effect on apoptosis induced by vitamin D compounds in MCF-7S8 or T47D cells; (c) no caspase-3-like activity could be measured in apoptotic MCF-7S8 by an enzyme activity assay based
on the cleavage of a colorimetric substrate of caspase-3-like proteases, DEVD-pNA; (d) a caspase substrate PARP remained intact in apoptotic MCF-7S8 and T47D cells treated with vitamin D compounds; and (e) finally, overexpression of caspase-3 did not sensitize MCF-7S1 cells to apoptosis or PARP cleavage induced by vitamin D compounds. Thus, vitamin D compounds seem to activate a caspase-independent pathway, resulting in apoptotic morphology. Interestingly, nitric oxide has recently been shown to induce a similar caspase-independent but Bcl-2-sensitive cell death in PC12 and HeLa cells (20).

The mechanism by which vitamin D compounds induce apoptosis remains to be studied. Vitamin D effects in MCF-7 cells include up-regulation of the expression of cathepsin B, a cysteine protease that

Fig. 5. DEVD-specific caspases are not activated in MCF-7S8 cells or in MCF-Casp3 cells following treatment with vitamin D compounds. A, MCF-7S1 cells were transfected with a pcDNA3-Casp3 expression construct (MCF-Casp3) or pcDNA3 vector (MCF-Vector), by electroporation. Cell lysates from ~2 × 10^5 untreated cells or cells treated with 10 ng/ml TNF for 18 h were analyzed for the expression and cleavage of caspase-3 by immunoblot. B, the cell density of MCF-vector and MCF-Casp3 cells was measured by the MTT assay following treatment with 50 ng/ml TNF for 48 h (TNF) or with the indicated concentrations of 1,25(OH)_2D_3 (VD), EB 1089, or CB 1093 for 6 days. Columns, means of one representative experiment with triplicate determinations; bars, SD. C, the cleavage of a caspase substrate PARP was analyzed by immunoblot of lysates from ~2 × 10^5 MCF-7S8 and MCF-Casp3 cells treated for 6 days with 1% isopropanol (IP), 100 nM 1,25(OH)_2D_3 (VD) or 100 nM CB 1093 (CB) or for 24 h with 10 ng/ml TNF.

Fig. 6. Kinetics of growth inhibition (A), inhibition of DNA synthesis (B), and induction of apoptosis (C) in T47D cells treated with 100 nM 1,25(OH)_2D_3 (VD), EB 1089, or CB 1093 for 7 days. A, the cell density was measured by the MTT assay at indicated time points. Data points, means of three experiments with triplicate determinations. B, DNA synthesis was measured by the thymidine incorporation assay. Data points, means of two experiments with triplicate determinations. A and B, results are expressed as percentages relative to control cells treated with the diluent, 1% isopropanol. C, apoptosis was detected by TUNEL staining. The apoptotic index is expressed as a percentage of TUNEL-positive cells. Columns, means of two experiments with triplicate determinations; bars, SD. Control cells were treated with 1% isopropanol.
CASPASE-INDEPENDENT APOPTOSIS BY VITAMIN D COMPOUNDS

Fig. 7. Known caspases are not involved in apoptosis induced by vitamin D compounds in T47D cells. A, cell density of T47D cells was measured by the MTT assay following treatment with 50 nM staurosporine for 4 days (Stauro) or with 100 nM 1,25(OH)2D3 (VD), EB 1089, or CB 1093 for 6 days. The caspase inhibitors, IETD-CHO (50 μM) and DEVD-CHO (200 μM), were administered to the cells 3 h prior to the addition of staurosporine and at the third day of the treatment for the cells treated with vitamin D compounds. The results are expressed as percentages relative to control cells treated with 1% isopropanol. Columns, means of triplicate values from one representative experiment; bars, SD. B, the cleavage of caspase substrate PARP was analyzed by immunoblot of lysates from ∼2 × 10^5 T47D cells treated for 7 days with the diluent (1% isopropanol, IP), 100 nM 1,25(OH)2D3 (VD), or 100 nM CB 1093 (CB) or for 24 h with 10 μg/ml puromycin (Puro).

is also highly expressed during regression of mammary gland (8, 26). Thus, it is interesting to note that bile salt-induced apoptosis of hepatocytes is at least partially mediated by cathepsin B and campthothecin-induced apoptosis of hepatocellular carcinoma cells is mediated by proteases sensitive to tosyl-lysyl chloromethylketone, which is capable of inhibiting cysteine proteases (19, 23, 24). Therefore, it will be interesting to directly assess the abilities of cathepsin B and other cysteine proteases to induce apoptotic morphology in the absence of active caspases as well as their role in apoptosis induced by vitamin D compounds in breast cancer cells.

Vitamin D compounds have been suggested to induce apoptosis via a Bcl-2-regulated pathway because they down-regulate the Bcl-2 protein level in sensitive target cells prior to the onset of apoptosis (7–9, 25). Our data showing that overexpression of Bcl-2 as well as that of a related antiapoptotic protein Bcl-xL clearly inhibited apoptosis induced by vitamin D compounds support the involvement of a Bcl-2-regulated pathway. Bcl-2 has been suggested to exert its antiapoptotic effect by inhibiting the fall of the mitochondrial transmembrane potential and the release of cytochrome c from the mitochondria and/or by regulating endoplasmic reticulum-associated calcium fluxes (14, 16, 17). It remains to be studied whether Bcl-2 exerts its protective effect against vitamin D compounds by protecting mitochondria, by regulating cytosolic calcium levels, or by an as yet unknown mechanism. Interestingly, it has been reported that apoptosis induced by vitamin D derivatives in MCF-7 cells is preceded by a depletion of intracellular calcium stores (6). Thus, it will be interesting to test whether Bcl-2 could function by inhibiting vitamin D effects on theemptying of intracellular calcium pools and thereby protect cells from apoptosis. The other step of the signaling cascade at which Bcl-2 has been reported to function is the release of cytochrome c from the mitochondria to the cytosol (14, 16, 17). The cytosolic cytochrome c has been shown to rapidly activate procaspase-9 and, thereby, the caspase cascade in several cell types (20, 47). Because vitamin D compounds failed to activate any known caspases in MCF-7 and T47D cells, it is unlikely that they induce the release of cytochrome c from the mitochondria, or if cytochrome c is released, its ability to activate downstream caspases must be inhibited. Taken together, our data suggest that, in addition, to the previously described protective mechanisms of Bcl-2, which result in the inhibition of the activation of caspases, it can also prevent apoptosis via a caspase-independent mechanism.

Several cytotoxic drugs induce apoptosis via a up-regulation of a cell surface death receptor Fas or a tumor suppressor protein p53 (13, 48, 49). FADD-dependent death pathways induced by Fas or related death receptors, TNFR1, and death receptor 3, are however, unlikely mediators of vitamin D effects as dominant negative mutant of FADD, an adapter molecule necessary for death receptor-induced apoptosis, had no effect on apoptosis induced by vitamin D compounds (13, 50). Furthermore, the fact that caspase inhibitors did not inhibit apoptosis induced by vitamin D compounds opposes the involvement of TNFR-related death receptors. We also show that p53 is not required for apoptosis induced by vitamin D compounds because T47D cells lacking a functional p53 undergo typical apoptotic changes following treatments with 1,25(OH)2D3, EB 1089, or CB 1093.

The ability of vitamin D compounds to induce apoptosis without the involvement of caspases, death receptors, and p53 may prove useful in therapy because several tumors have acquired resistance to other apoptosis-inducing agents by mutations either in p53 tumor suppressor protein or in proteins necessary for death receptor signaling (13, 48, 51, 52). Moreover, tumor cells overexpressing antiapoptotic proteins like IAPs inhibiting caspase activation as well as Hsp70 protecting cells from active caspases may remain sensitive to the caspase-independent cell death induced by vitamin D compounds (53–55).

ACKNOWLEDGMENTS

We thank Dorrit Lützhöft, Helle Mouritzen, and Birgit Poulsen for valuable technical assistance; Lise Binderup and Christina Mørk Hansen for the vitamin D compounds; Vishva Dixit for caspase-3, CrmA, and CrmA-mutant expression constructs; Marcus Peter for MCF-FADD-DN cells; and Guy Poirier for the anti-PARP antibody.

REFERENCES

CASPEP-INDEPENDEENT APOPTOSIS BY VITAMIN D COMPOUNDS


Apoptosis Induced by Vitamin D Compounds in Breast Cancer Cells Is Inhibited by Bcl-2 but Does Not Involve Known Caspases or p53

Ida Stenfeldt Mathiasen, Ulrikm Lademann and Marja Jäättelä


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/59/19/4848

Cited articles  This article cites 54 articles, 19 of which you can access for free at: http://cancerres.aacrjournals.org/content/59/19/4848.full#ref-list-1

Citing articles  This article has been cited by 27 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/59/19/4848.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.