Vitamin D<sub>3</sub>-induced Apoptosis of Murine Squamous Cell Carcinoma Cells

SELECTIVE INDUCTION OF CASPASE-DEPENDENT MEK CLEAVAGE AND UP-REGULATION OF MEKK-1*

Received for publication, November 6, 2000, and in revised form, April 6, 2001 Published, JBC Papers in Press, April 30, 2001, DOI 10.1074/jbc.M010101200

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Vitamin D<sub>3</sub> inhibits cell growth and induces apoptosis in several human cancer lines in vitro and in vivo. However, little is known about the molecular events involved in vitamin D<sub>3</sub>-induced apoptosis. Here, we demonstrate that the growth-promoting/pro-survival signaling molecule mitogen-activated protein kinase (MEK) is cleaved in a caspase-dependent manner in murine squamous cell carcinoma (SCC) cells induced to undergo apoptosis by treatment with vitamin D<sub>3</sub>. Cleavage resulted in nearly complete loss of full-length MEK and ERK1/2 phosphorylation. ERK1/2 expression was affected only slightly. The phosphorylation and expression of Akt, a kinase regulating a second cell survival pathway, was also inhibited after treatment with vitamin D<sub>3</sub>. However, the pro-apoptotic signaling molecule MEKK-1 was up-regulated in both apoptotic and non-apoptotic cells with greater induction and partial N-terminal proteolysis of MEKK-1 observed in apoptotic cells. In contrast to vitamin D<sub>3</sub>, cisplatin and etoposide down-regulated Akt levels only modestly, did not promote significant loss of MEK expression, and did not up-regulate MEKK-1. We propose that vitamin D<sub>3</sub> induces apoptosis in SCC cells by a unique mechanism involving selective caspase-dependent MEK cleavage and up-regulation of MEKK-1. Additional evidence is provided that vitamin D<sub>3</sub>-induced apoptosis may be mediated via p38 MAPK.

1,25-Dihydroxycholecalciferol (vitamin D<sub>3</sub>)<sup>1</sup> is the active metabolite of vitamin D and exhibits anti-proliferative and differentiation-promoting activities in vitro toward a number of malignant cell types, including breast cancer cells (1, 2), prostate cancer cells (3, 4), colorectal adenoma and carcinoma cells (5), melanoma cells (6), and mouse myeloid leukemia (7–9). In addition, vitamin D<sub>3</sub> inhibits prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system (10) and displays anti-neoplastic activity in a variety of human xenograft tumor model systems (11, 12). Several vitamin D<sub>3</sub> analogues have also been developed and display similar activities in vitro (5, 13–15) and in vivo (16–18). Furthermore, we have demonstrated that vitamin D<sub>3</sub> inhibits the proliferation of squamous cell carcinoma (SCC) cells in vitro as well as the growth of newly transplanted or established SCC tumors in vivo (19, 20). Hence, the accumulated evidence clearly establishes the anti-proliferative activity of vitamin D<sub>3</sub> (and its analogues) against several cancer cell types and supports the examination of their potential usefulness as anti-cancer agents (reviewed in Refs. 12 and 21).

On the cellular level, vitamin D<sub>3</sub> has been shown to exert its anti-proliferative effects by inhibiting cell cycle progression and/or by promotion of programmed cell death (apoptosis). G<sub>1</sub> arrest induced by vitamin D<sub>3</sub> or vitamin D<sub>3</sub> analogues has been attributed to a number of molecular changes, including induction of the cyclin-dependent kinase (cdk) inhibitors p21<sup>Waf1</sup> and p27<sup>Kip1</sup>, inhibition of cdk2 activity, hypophosphorylation of the retinoblastoma protein (Rb), and suppression of E2F activation (4, 18, 20, 22–24). Vitamin D<sub>3</sub>-induced apoptosis, while being demonstrated for several cancer cell lines derived from various tissues, including breast (2, 3, 17, 25, 26), prostate (3, 27), colon (5, 13), and skin (6) in vitro, as well as breast cancer cells in vivo (28), is less well defined. Levels of the anti-apoptotic molecule Bcl-2 have been shown to be reduced after vitamin D<sub>3</sub> treatment in some cell lines, including MCF-7 human breast cancer cells (17, 26, 28) and LNCaP human prostate cancer cells (27). Furthermore, vitamin D<sub>3</sub>-induced apoptosis in these cell lines is blocked by overexpression of Bcl-2 (27, 29). Although these results suggest a possible role for Bcl-2 down-regulation in vitamin D<sub>3</sub>-induced apoptosis, it is not clear whether reduction of Bcl-2 expression is sufficient of itself to induce apoptosis. In addition, vitamin D<sub>3</sub>-induced apoptosis of colon cancer cells is not dependent on Bcl-2 down-regulation but is more tightly associated with up-regulation of the pro-apoptotic molecule Bak (5). Another body of work suggests that vitamin D<sub>3</sub> induces insulin-like growth factor binding proteins, which bind to insulin-like growth factor-1 receptor, and thereby promote apoptosis (30–32). Finally, Mathiasen et al. (29) recently reported that sensitivity of MCF-7 cells to vitamin D<sub>3</sub>-induced apoptosis does not depend on expression of a functional p53 tumor suppressor protein and does not involve the activation of known caspases. Thus far, the involvement of stress and survival signal transduction pathways in the mechanism of vitamin D<sub>3</sub>-induced apoptosis has not been carefully examined.

This paper is available on line at http://www.jbc.org

* This work was supported by National Institutes of Health Grants CA-67267 and CA-85142 and by Department of Defense Grant DAMD 17-98-1-8549. The costs of publication of this article were defrayed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: vitamin D<sub>3</sub>, 1,25-dihydroxycholecalciferol; SCC, squamous cell carcinoma; ERK, extracellular signal-regulated protein kinase; MEK, ERK kinase; MAPK, mitogen-activated protein kinase; MEKK-1, MAPK kinase kinase-1; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; JNK, c-Jun NH<sub>2</sub>-terminal kinase; FBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; cDDP, cisplatin.
The integration of multiple signals from numerous transduction pathways plays a critical role in regulating cell survival and execution of programmed cell death (reviewed in Refs. 33 and 34). Although activation of stress signals from the SEK1-JNK and the MKK3/6-p38 MAPK pathways can push the cell toward apoptosis (35–38), opposing signals generated from the MEK-ERK and phosphatidylinositol 3-kinase-Akt pathways antagonize the death signals and mediate survival (39–45). When the integrated signals from these pathways begin to favor apoptosis, caspases can become activated, resulting in the selective cleavage of a distinct set of proteins and greatly enhancing the potential toward commitment to apoptosis. Although cleavage of target proteins by caspases typically results in their inactivation, exceptions to this include the caspases themselves as well as two stress-signaling proteins, MEKK-1 and PAK2 (46, 47). MEKK-1 is a 196-kDa Ser/Thr kinase, which, upon phosphorylation, becomes activated and transduces stress signals that lead to the activation of JNK and p38 MAPK (48), thereby promoting apoptosis. Upon treatment of cells with genotoxic agents (e.g. etoposide or UV-C irradiation), MEKK-1 initially undergoes phosphorylation-dependent activation and is subsequently cleaved by caspase-3, releasing the N-terminal regulatory domain and producing a constitutively active C-terminal kinase domain (49). Similarly, MEKK-1 cleavage is also observed in cells induced to undergo apoptosis by Fas ligation (50) and by detachment from the extracellular matrix (anoikis) (46). Interestingly, cells expressing a non-cleavable MEKK-1 mutant are impaired in their ability to undergo apoptosis after treatment with DNA-damaging agents (although mutant MEKK-1 is still phosphorylated) (49). Thus, MEKK-1 cleavage is not only a common biological event observed during apoptosis induced by a variety of agents but appears to play a necessary role in the execution of the apoptotic program. Caspase-mediated cleavage of PAK2, a Ser/Thr kinase that regulates cytoskeletal changes in many cell types, also appears to play a role in the apoptotic process (47).

We have investigated the mechanism by which vitamin D₃ induces apoptosis in SCC cells, particularly with respect to its effects on survival and stress signaling pathways. We report here that vitamin D₃ induces the caspase-dependent cleavage of MEK, not previously described, resulting in nearly complete loss of MEK expression and ERK1/2 signaling. Moreover, Akt signaling is potently inhibited in cells induced to undergo apoptosis by vitamin D₃. Furthermore, vitamin D₃ strongly induces MEKK-1 expression in cells prior to onset of apoptosis and subsequently promotes N-terminal proteolysis such that numerous MEKK-1 fragments are observed in the apoptotic cells. Finally, direct comparison of vitamin D₃ with genotoxic agents revealed that the molecular events described above, with the exception of Akt inhibition, were selective for vitamin D₃.

**EXPERIMENTAL PROCEDURES**

**Culture and Treatment of SCC Cells**—Murine squamous cell carcinoma (SCC) cells (either 3 × 10⁵ or 6 × 10⁵ cells/T-75 flask) were plated in RPMI 1640 medium supplemented with 12% fetal bovine serum (FBS), unless otherwise noted (see Fig. 2 below). After 1 or 2 days, the cells were treated at sub-confluence with either vehicle (0.001% ethanol, EtOH), 10 nm 1,25-dihydroxycholecalciferol (1,25-D₃), 1 μg/ml cisplatin, or 10 μM etoposide. After 2 days of treatment, medium in the flasks was gently swirled to suspend floating (apoptotic) cells, which were then harvested by centrifugation, and washed once with ice-cold PBS. Attached (non-apoptotic) cells were rinsed once with PBS and either scraped off the flask surface into PBS and placed in a separate tube (for Western blotting) or trypsinized, suspended in PBS, and combined with the floating cells (for YO-PRO-1 staining). For experiments assessing the role of caspases in vitamin D₃-induced MEK cleavage, cells were treated with either vehicle or 10 nm vitamin D₃ in the presence or the absence of either 20 μM Z-DEVD-FMK or 20 μM Z-VADOMe-FMK and the cells were harvested, as described above.

**Western Blotting**—Both attached and floating cell populations were then lysed in buffer containing the following: 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotonin, 10 μg/ml trypsin inhibitor, 1 mM benzamidine, 5 mM N-ethylmaleimide, 2 mM vanadate, 2 mM EGTA, 12 mM β-glycerophosphate, 10 mM NaF, and 10 mM orthovanadate. Cell lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C, and aliquots were incubated with an equivalent volume of 2× SDS-PAGE sample buffer for at least 4 min at 100 °C. Equivalent amounts of protein from each sample were electrophoresed on either 7.5% SDS-PAGE (for PARP and MEKK-1) or 12.5% SDS-PAGE (for all other antibodies) and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (T-TBS) and then probed with either anti-PARP (PharMingen, 65196E), anti-phospho-MEK1/2 (New England BioLabs, 9121S), anti-MEK1/2 (New England BioLabs, 9122), anti-ERK1/2 (Santa Cruz, sc-7383), or anti-ERK1/2 (Upstate Biotechnology, 06-182), anti-phospho-Akt (New England BioLabs, 9271), anti-Akt (New England BioLabs, 9272) anti-MEK1 (Santa Cruz, sc-252), anti-SEKI (Santa Cruz, sc-964), anti-phospho-p38 MAPK (New England BioLabs, 9211), anti-phospho-PARP (Santa Cruz, sc-353), anti-phospho-e-Jun (Santa Cruz, sc-822), or anti-actin (Oncogene, CP01) in 3% nonfat dry milk in T-TBS. Anti-phospho-MEK, anti-phospho-ERK1/2, anti-phospho-Akt, and anti-phospho-p38 MAPK antibodies were used to detect MEK phosphorylated at serine 218, 219, 221, ERK1/2 phosphorylated at tyrosine 204, Akt phosphorylated at serine 473, and p38 MAPK phosphorylated at threonine 180 and tyrosine 182, respectively, sites of phosphorylation necessary for full activation of these kinases. The immunogen used to generate the anti-MEK-1 antibody (sc-252) was a 22-aa amino acid peptide, the sequence of which corresponded to a region lying within the last 50 amino acids of the rat MEK-1 C terminus (identical in sequence to that region found in the mouse MEKK-1 C terminus). Positive antibody reactions were visualized using an appropriate horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) (PerkinElmer Life Sciences) for PARP immunoblotting, ECL SuperSignal (Pierce) was used.

It is important to note that, although both the anti-phospho-MEK and anti-MEK antibodies recognized both MEK isoenzymes (MEK1 and MEK2), these two proteins were typically not completely resolved under the electrophoresis conditions used for this work. Both proteins, however, appeared to behave in a similar manner in response to the various treatment conditions described above. Therefore, in this report, we have represented both of the isoenzymes together by denoting them as “MEK” in the text and in the figures.

**YO-PRO-1 Staining**—As described above, at the time of harvest, floating cells were first removed with the medium and placed in a conical tube; attached cells were rinsed once, trypsinized, suspended in RPMI 1640 medium containing 12% FBS, and combined with the floating cells. The cells were then pelleted and resuspended in PBS to a concentration of 1.0–1.5 × 10⁷ cells/ml, and the percentage of apoptotic cells was measured using the Vybrant Apoptosis Assay Kit #4 (Molecular Probes). According to the manufacturer’s instructions, 1.0 μl each of YO-PRO-1 dye and propidium iodide solutions was added to 1.0-ml aliquots of suspended cells, allowed to incubate for at least 20 min, and subsequently analyzed by flow cytometry.

**RESULTS**

**Vitamin D₃ Induces Apoptosis in Murine Squamous Cell Carcinoma (SCC) Cells**—We have reported previously that vitamin D₃ predominantly induces murine SCC cells to undergo cytoplasmic spreading (flattening) and become growth-arrested in G₁ phase of the cell cycle (19, 51). However, a significant population of cells is also observed detached and floating in the culture medium after vitamin D₃ treatment and possibly represents apoptotic cells. To determine whether this population of cells exhibits characteristics of apoptosis, SCC cells were treated with vitamin D₃ (10 nm) for 2 days, and stained for apoptosis with various assays as shown in Fig. 3A. Vitamin D₃ induced a fraction of the cells to remain attached and to spread while another fraction became detached and exhibited cellular condensation and membrane blebbing, characteristics of cells undergoing apoptosis. The cells were then harvested, stained with propidium iodide and YO-PRO-1, and subjected to flow cytometric analysis. YO-PRO-1 is a fluorescent dye taken.
Vitamin D₃-induced apoptosis in murine squamous cell carcinoma (SCC) cells 
in vitro. SCC cells were treated with either vehicle (<0.001% ethanol, EtOH) or 10 nM vitamin D₃ (1,25-D₃) in RPMI medium containing 10% FBS, as described under “Experimental Procedures.” After 2 days, cells were photographed (A) and/or processed for either YO-PRO-1/propidium iodide staining followed by flow cytometric analysis (B) or Western blotting (C) as described under “Experimental Procedures.” Photographs show that vitamin D₃ causes SCC cells both to flatten (G₁ arrest) and to enter apoptosis as visualized by bright cells that exhibit cellular condensation and membrane blebbing. Results from flow cytometric analysis were plotted such that FL1 represents fluorescence of propidium iodide and FL2 represents the fluorescence of YO-PRO-1. This analysis demonstrates that vitamin D₃ significantly increases the percentage of cells in early apoptosis (from 0.2% to 11.0%), cells in late apoptosis/necrosis (from 13.7% to 28.7%), and total cells in apoptosis (from 13.9% to 39.7%). Immunoblot analysis with anti-PARP antibody indicates that cells that are detached (but not attached cells) are apoptotic. *, in vehicle-treated cultures, nearly all of the cells were attached to the flask and it was not possible to collect the very small number of floating cells for Western blot analysis. These results are representative of at least four independent experiments.

Based on this type of analysis, vitamin D₃ increased the proportion of cells in early apoptosis (0.2% – 11.0%) and the total number of cells in apoptosis/necrosis (14% – 40%) (the average induction of total apoptotic/necrotic cells from four independent experiments was from 13 to 36%, or 2.8-fold) (Fig. 1B). Although flow cytometry detected significant basal levels of apoptosis in vehicle-treated cultures, no apoptosis could be detected in these cultures using the other two methods employed (morphological changes and caspase activation (see below)). Thus, it appears that the basal level of apoptosis in vehicle-treated cultures is artifactual and probably due to the trypsinization procedure used to prepare the cells for fluorescence-activated cell sorting analysis (see “Experimental Procedures”). Finally, we assessed induction of apoptosis by vitamin D₃ on the molecular level by examining caspase activation (i.e. by measuring the extent of cleavage of the caspase substrate poly(ADP-ribose) polymerase, or PARP). After treating SCC cells as described above, the floating cells were removed from the attached cells, and lysates of both cell populations were prepared (described under “Experimental Procedures”). The number of floating cells in the medium after vehicle treatment was low, and it was not possible to collect sufficient amounts to process for immunoblot analysis. As shown in Fig. 1C (lanes 1–2), lysates of vehicle-treated (attached) cells, as well as the attached cell population of vitamin D₃-treated cultures, exhibited no PARP cleavage. However, lysates of floating cells from cultures treated with vitamin D₃ demonstrated complete cleavage of PARP (116 kDa) to an 85-kDa fragment (Fig. 1C, lane 3). Taken together, these results indicate that vitamin D₃ induces programmed cell death in SCC cells. The fact that PARP cleavage was exclusively observed in lysates from vitamin D₃-treated, non-attached cells and that this population is easily isolated from the attached cell population affords the use of this model for investigating at the molecular level the role of signaling pathways in vitamin D₃-induced apoptosis.

Vitamin D₃ induces caspase-dependent cleavage of MEK in apoptotic cells. As shown in Fig. 2A, vitamin D₃ nearly completely inhibited MEK activation in the floating population of cells in the presence or the absence of serum but had little effect on MEK phosphorylation in the attached populations from either vehicle- or vitamin D₃-treated cultures. Results from immunoblotting with an anti-MEK antibody demonstrated that the vitamin D₃-induced decrease in MEK phosphorylation was not simply due to inhibition of its activation but to down-regulation of MEK protein itself (Fig. 2A). This result was confirmed using two other anti-MEK antibodies, which recognized distinct regions of the MEK protein (data not shown). Interestingly, the apoptotic, floating cells exhibited a lower molecular weight band that was also immunoreactive with the anti-MEK antibody (33 kDa in the presence of serum; 28 kDa in the absence of serum; Fig. 2A), suggesting that down-regulation of MEK protein levels might be due to MEK proteolysis.

Levels of phosphorylated/activated ERK1/2 were then analyzed and found to be virtually undetectable in the lysates of the floating cells (Fig. 2B), indicating that the MEK-ERK signaling pathway is blocked in these cells. It is perhaps noteworthy that vitamin D₃ also modestly inhibited ERK1/2 in vitamin D₃-treated attached cells (Fig. 2B) despite the fact that levels of phosphorylated MEK and intact MEK protein were unaffected (Fig. 2C). The molecular basis of vitamin D₃-induced ERK1/2 repression was exclusively observed in lysates from vitamin D₃-treated, non-attached cells and that this population is easily isolated from the attached cell population affords the use of this model for investigating at the molecular level the role of signaling pathways in vitamin D₃-induced apoptosis.

Vitamin D₃ induces caspase-dependent cleavage of MEK in apoptotic cells—The Ras-Raf1-MEK-ERK signaling pathway transduces signals from growth factor receptors to the nucleus, which can lead not only to mitogenesis and differentiation but also to survival (34). Some investigators studying vitamin D₃-induced apoptosis have reported that culturing cells under serum-free conditions enhances the apoptosis-promoting activity of vitamin D₃ (31). Thus, growth factor signaling may attenuate the apoptotic signal induced by vitamin D₃, and, conversely, vitamin D₃ may attenuate growth factor signaling as part of its mechanism to promote programmed cell death. We investigated the effects of vitamin D₃ on levels of activated MEK, a key mediator of signaling through the Ras-Raf1-MEK-ERK pathway, in SCC cells cultured in the presence or the absence of 12% fetal bovine serum (FBS). Significant apoptosis (based on morphology) was observed under both culture conditions, but levels of apoptosis were greater under serum-starved conditions (data not shown). Immunoblot analysis using an anti-phosho-MEK antibody that selectively detects activated MEK (described under “Experimental Procedures”) was then performed on lysates prepared from attached and non-attached cells. As shown in Fig. 2, vitamin D₃ nearly completely inhibited MEK activation in the floating population of cells in the presence or the absence of serum but had little effect on MEK phosphorylation in the attached populations from either vehicle- or vitamin D₃-treated cultures. Results from immunoblotting with an anti-MEK antibody demonstrated that the vitamin D₃-induced decrease in MEK phosphorylation was not simply due to inhibition of its activation but to down-regulation of MEK protein itself (Fig. 2A). This result was confirmed using two other anti-MEK antibodies, which recognized distinct regions of the MEK protein (data not shown). Interestingly, the apoptotic, floating cells exhibited a lower molecular weight band that was also immunoreactive with the anti-MEK antibody (33 kDa in the presence of serum; 28 kDa in the absence of serum; Fig. 2A), suggesting that down-regulation of MEK protein levels might be due to MEK proteolysis.
inhibition in attached cells is currently under investigation. ERK1/2 expression was unaltered in lysates of vitamin D$_3$-treated attached cells, and, in contrast to MEK, was only slightly reduced in floating cells (Fig. 2). Reduction of ERK1/2 expression was more apparent under serum-starved conditions and was coincident with the appearance of a putative 23-kDa fragment (Fig. 2). Because culturing cells in the presence of serum is more physiologically relevant and because serum starvation appeared to promote activation of proteases that were not necessary for vitamin D$_3$-induced apoptosis or loss of MEK protein, all subsequent experiments were performed in the presence of 12% FBS.

Because it is well established that programmed cell death often involves the activation of caspases, we examined whether these proteases might be responsible for vitamin D$_3$-induced MEK proteolysis. SCC cells were treated 2 days either with vehicle, vitamin D$_3$ alone, or vitamin D$_3$ in the presence of either DEVD-FMK or zVAD-FMK. Lysates were prepared, and immunoblot analysis was carried out using anti-MEK antibody. Whereas the caspase-3 inhibitor DEVD-FMK had little effect on vitamin D$_3$-induced MEK proteolysis, the pan-caspase inhibitor zVAD-FMK nearly completely blocked the loss of MEK protein and the production of the 33-kDa MEK fragment (Fig. 3). Thus, the decrease in MEK expression induced by vitamin D$_3$ treatment does not appear to be due to inhibition of MEK protein synthesis but rather the result of caspase-dependent MEK cleavage (although caspases may not cleave MEK directly). Caspase-dependent cleavage of MEK has not been previously reported. It is perhaps noteworthy that the apparent sum of the levels of intact MEK and the 33-kDa fragment in lysates of non-attached cells from vitamin D$_3$ cultures appears to be less than the total level of MEK in cells co-treated with vitamin D$_3$ and zVAD-FMK (Fig. 3, lanes 4 and 6). This suggests that the 33-kDa fragment is either less antigenic than intact MEK with the anti-MEK antibody used or that the 33-kDa fragment undergoes further proteolysis to yield products not recognized by the anti-MEK antibody or both. Finally, inhibition of MEK cleavage by zVAD-FMK resulted in the complete restoration of MEK and ERK1/2 phosphorylation/activation (Fig. 3), suggesting that MEK cleavage plays a critical role in the vitamin D$_3$-induced block of the MEK-ERK signaling pathway in detached cells.

**Vitamin D$_3$ Inhibits the Akt Survival-signaling Pathway**—In
addition to the MEK-ERK pathway, the phosphatidylinositol 3-kinase-Akt pathway also generates a significant survival signal. Akt was recently demonstrated to undergo caspase-dependent cleavage during apoptosis induced by treatment either with etoposide, ultraviolet-C exposure, or Fas ligation in human Jurkat leukemia cells (52). To assess whether this pathway is also affected in vitamin D3-induced apoptosis, immunoblot analysis was used to assess Akt phosphorylation and expression in lysates of cells treated with either vehicle or vitamin D3. Fig. 4 shows that vitamin D3 affected Akt in a manner similar to that of MEK, inducing a strong decrease in both its phosphorylation and expression. Inhibition of Akt by vitamin D3 was shown to occur via caspase-dependent cleavage of Akt in the attached cells.

Vitamin D3 Induces MEKK-1 Expression in Apoptotic and Non-apoptotic Cells and Promotes MEKK-1 Proteolysis in Apoptotic Cells—Although the above results clearly indicate that cells undergoing vitamin D3-induced apoptosis exhibit a block in the mitogenic/survival signaling pathways, this alone may not be sufficient to induce cells to enter programmed cell death. Therefore, in an effort to assess whether vitamin D3 treatment also activates stress signals that could directly promote apoptosis, SCC cells were treated with or without vitamin D3 and the expression of MEKK-1, an upstream activator of the JNK and the p38 MAPK stress pathways, was assessed by Western blot analysis. As shown in Fig. 5A, vitamin D3 significantly up-regulated MEKK-1 expression in both the attached and floating cell populations, with expression being greater in the floating, apoptotic cells. A second major immunoreactive band of slightly lower molecular mass (190 kDa; Fig. 5A, left panel, lane 3) compared with that of intact MEKK-1 (molecular mass = 196 kDa) was also detected in lysates of apoptotic, but not attached, cells. In addition, longer exposures of the x-ray film to the Western blot ECL signal revealed multiple minor immunoreactive bands in the lysates of floating cells (molecular mass range of 85–190 kDa) that were not observed in those of attached cells (Fig. 5A, right panel, compare lanes 2 and 3). Because the anti-MEKK-1 antibody recognizes an epitope lying very near the C terminus of the MEKK-1 protein (within the last 50 amino acids of the mouse MEKK-1 protein; see “Experimental Procedures”), these data indicate that the products formed represent MEKK-1 species that have undergone N-terminal proteolysis. It has been previously demonstrated that, upon treatment of cells with either genotoxic agents or Fas ligation, MEKK-1

![Figure 4](https://www.jbc.org/content/26369/42/26369/F4.large.jpg)

**Fig. 4.** Vitamin D3 inhibits Akt survival signaling via caspase-dependent Akt cleavage. Cells were treated 2 days with either vehicle or 10 nM vitamin D3 (A and B) in the presence or the absence of 20 μM Z-VAD(OMe)-FMK (zVAD) (B). Cells were then processed for immunoblotting with either anti-phospho-Akt or anti-Akt antibodies, as described under “Experimental Procedures.” It should be noted that, in some experiments, vitamin D3 partially inhibited phosphorylation of Akt in the attached cells.

![Figure 5](https://www.jbc.org/content/26369/42/26369/F5.large.jpg)

**Fig. 5.** Vitamin D3 induces MEKK-1 expression and N-terminal proteolysis in apoptotic cells. A, cells were treated 2 days and then processed for anti-MEKK-1 immunoblotting to assess MEKK-1 expression as described under “Experimental Procedures.” The anti-MEKK-1 antibody recognizes an epitope located within the last 50 amino acids of the rat MEKK-1 protein and is identical in sequence to the same region of the mouse MEKK-1 protein (see “Experimental Procedures”). B, cells were treated for either 1 or 2 days and then processed as in A. Vitamin D3-induced MEKK-1 up-regulation and N-terminal proteolysis were observed in at least four independent experiments.
undergoes caspase-3-mediated cleavage, resulting in the removal of the N-terminal regulatory domain and the production of a constitutively active C-terminal kinase domain (49). Thus, some or all of the lower molecular weight anti-MEKK-1-positive bands produced upon treatment with vitamin D₃ may represent MEKK-1 proteolytic products displaying constitutive kinase activity. Finally, as shown in Fig. 5B, MEKK-1 induction was observed by 22 h after treatment with vitamin D₃, a time at which vitamin D₃-induced apoptosis and cell detachment are not observed (data not shown), further suggesting a role for MEKK-1 in vitamin D₃-induced apoptosis. Induction of MEKK-1 by vitamin D₃ has not been previously reported.

MEK Cleavage and MEKK-1 Up-regulation Are Not Significantly Induced by Cisplatin—We next addressed whether the effects of vitamin D₃ on MEK and MEKK-1 described above were general phenomena of SCC cells that could be observed during apoptosis induced by other agents or if they were selectively induced by treatment with vitamin D₃. Cells were treated either with vitamin D₃ (10 nM) or with cisplatin (cDNA, 1 μg/ml) for 2 days, the attached and non-attached cell populations were separated, and lysates were prepared from both. Similar levels of apoptosis were observed for vitamin D₃ and cisplatin-treated cells at the time of harvest (data not shown). As shown in Fig. 6, vitamin D₃ and cisplatin both induced PARP cleavage in the floating cell populations but not in the attached cell populations, demonstrating that the floating cells from both treatments represent only those cells induced to undergo apoptosis. Significant loss of MEK expression and MEK cleavage, however, were only observed for lysates of apoptotic cells from vitamin D₃-treated cultures. Although the 33-kDa MEK fragment could be observed in lysates of cisplatin-treated floating cells, it was typically observed at levels less than those observed for lysates of vitamin D₃-treated floating cells (Fig. 6).

In addition, significant up-regulation of MEKK-1 was observed only for vitamin D₃-treated cells, particularly in the non-attached apoptotic population, and limited N-terminal MEKK-1 proteolysis was exclusively observed in the vitamin D₃-treated, detached cells (Fig. 6). Although a slight induction of MEKK-1 was observed for cisplatin-treated attached cells, this induction was no longer observed in the floating cells (Fig. 6). Thus, caspase-dependent MEK cleavage and MEKK-1 up-regulation/proteolysis are not general phenomena of apoptosis observed in cells but are selectively induced by vitamin D₃. Taken together, these results suggest that vitamin D₃ induces apoptosis in SCC cells by a mechanism that is distinct from that of genotoxic agents.

Vitamin D₃ and Genotoxic Agents Display Differential Effects on the Expression and Activity of Various Anti-apoptotic and Pro-apoptotic Proteins—In an effort to more fully understand the molecular basis of vitamin D₃-induced apoptosis and how it compares with that of standard chemotherapeutic drugs, cells were treated with either vitamin D₃, cisplatin, or etoposide for 2 days, and the expression/phosphorylation of various members of the survival and stress signaling pathways were examined. Apoptosis in the floating cells was confirmed by assessing loss of intact PARP as an indicator of caspase activation (Fig. 7). In addition, actin expression was assessed to confirm equal loading of lysates to SDS-PAGE and to demonstrate selectivity of protein degradation. As shown in Fig. 7, in the attached cells, phosphorylation of MEK and ERK1/2 was modestly inhibited by cisplatin and etoposide but was not affected by vitamin D₃. In the floating cells, the phosphorylation of these proteins was still moderately inhibited by the genotoxic agents but was strongly decreased to virtually undetectable levels in lysates of vitamin D₃-treated cells (Fig. 7). Moreover, cisplatin and etoposide had little effect on MEK expression in both the attached and the non-attached cell populations, whereas vitamin D₃ reduced MEK expression to nearly undetectable levels in non-attached cells (Fig. 7). ERK1/2 expression, while again being unaffected by cisplatin or etoposide, was only modestly reduced by vitamin D₃ in detached cells (Fig. 7). Expression of the pro-survival kinase Akt, on the other hand, was inhibited by all three agents in the non-attached cell populations, albeit to different extents (Fig. 7). Loss of Akt expression during apoptosis was expected, because Akt was previously reported to undergo caspase-dependent cleavage in human Jurkat leukemia cells after treatment with various cytotoxic agents (52). It is interesting to note that a greater loss in Akt expression was observed by vitamin D₃ than by either cisplatin or etoposide, suggesting that vitamin D₃ induces greater activation of caspase(s) capable of cleaving Akt than either genotoxic agent. These results support the idea that inhibition of the MEK-ERK and Akt survival pathways may be a common mechanistic theme for agents that induce apoptosis (33, 34, 52). However, as might be expected, the molecular basis of inhibition of sur-
D3 promotes apoptosis is distinct from that of genotoxic drugs. It should
activate c-Jun, and p38 MAPK, as described under “Experimental Procedures.”
Actin, as well as the phosphorylation/activity of MEK, ERK1/2,
expression of PARP, MEK, ERK1/2, Akt, MEKK-1, SEK-1, p38 MAPK,
mentally Procedures.” Immunoblotting was then carried out to assess the
expression of various signaling proteins. SCC cells were treated 2 days with either
vehicle, 10 nM vitamin D3, 10 μM etoposide (VP16), or 1 μg/ml cisplatin (cDDP), harvested, and lysates prepared as described under “Experimental Procedures.”

**Fig. 7.** Vitamin D3 and genotoxic agents exhibit similar and
divergent effects on the expression and phosphorylation of various
signaling proteins. SCC cells were treated 2 days with either
vehicle, 10 nM vitamin D3, 10 μM etoposide (VP16), or 1 μg/ml cisplatin (cDDP), harvested, and lysates prepared as described under “Experimental Procedures.” Immunoblotting was then carried out to assess the expression of PARP, MEK, ERK1/2, Akt, MEKK-1, SEK-1, p38 MAPK, and actin, as well as the phosphorylation/activity of MEK, ERK1/2, c-Jun, and p38 MAPK, as described under “Experimental Procedures.”

These results suggest that the molecular mechanism by which vitamin D3 promotes apoptosis is distinct from that of genotoxic drugs. It should
be noted that, although genotoxic drugs consistently increased (or
maintained) levels of phosphorylation for p38 MAPK and c-Jun in the
attached cell population, as shown here, their effects on the levels of these phospho-proteins in the floating cell population were variable.

Apart from the JNK pathway, other stress signaling pathways were
also observed on the stress-signaling proteins examined. First,
similar to the results presented in Fig. 6, only vitamin D3 significantly induced MEKK-1 expression, formation of the
MEKK-1 doublet, and production of high molecular weight
MEKK-1 proteolytic species (Fig. 7 and data not shown). Expression of SEK-1, a major downstream effector of MEKK-1,
exhibited similar behavior to MEK, with strong down-regulation
induced by vitamin D3 in apoptotic cells but little effect
exhibited by the DNA-damaging agents (Fig. 7). Although

**DISCUSSION**

Taken together, the above results strongly suggest that vitamin D3 induces programmed cell death in SCC cells via the induction of the pro-apoptotic signaling molecule MEKK-1 while blocking pro-survival signals from the MEK-ERK and Akt pathways. Based on these findings, we propose that, prior
to commitment to apoptosis, vitamin D3 up-regulates MEKK-1 in non-apoptotic, attached cells (Fig. 5, A and B), but at levels that are insufficient to overcome the opposing effects of the MEK-ERK and Akt survival pathways. Unknown factors then trigger limited activation of caspases, including an unidentified
caspase (or caspase-dependent protease; discussed below) that is selectively activated in vitamin D3-treated cells. This caspase activity promotes MEK cleavage and removal of the
MEK-ERK pro-survival signal (Figs. 2 and 3). Furthermore, caspase-dependent proteolysis of Akt kinase occurs (52) and
results in abrogation of this survival signal as well (Fig. 4).

Finally, MEKK-1 undergoes partial proteolysis at its N-terminal
regulatory domain (Figs. 5A and 6), producing species that exhibit constitutive activity, further activating caspases and
significantly enhancing the pro-apoptotic signal (49). Activation of the MEK-1-mediated stress pathway(s), without the presence of offsetting anti-apoptotic signals, is proposed to be sufficient for committing cells to enter apoptosis.

Caspase-dependent cleavage of MEK has not been previously
reported. This may, in part, be explained by the fact that significant MEK cleavage was only observed for vitamin D3-induced apoptosis and not for apoptosis induced by more commonly used cytotoxic agents (Figs. 6 and 7). Widmann et al. (52) have recently performed an extensive screening of more than
30 signaling proteins, including MEK, for their ability to undergo caspase-dependent proteolysis after induction of apopto-
sis by either treatment with etoposide, exposure to ultraviolet-C, or Fas ligation. They reported that, although all of the
treatments induced apoptosis and the processing of caspase-3,
as well as Akt cleavage, they had little effect on the expression of MEK (52). These results indicate that MEK cleavage is not a
general phenomenon of the apoptotic program and suggest that a protease capable of cleaving MEK is selectively induced by
vitamin D3. It is important to note that, although MEK cleavage
is blocked by the pan-caspase inhibitor zVAD (Fig. 3), this
does not necessarily indicate that caspases directly cleave
MEK. The pro-apoptotic molecule Bax, for example, has been demonstrated to undergo caspase-dependent cleavage but is
not directly cleaved by caspases. Instead, caspase-3 first mediates the cleavage and consequent activation of calpain, which then acts to cleave Bax (53, 54). Identification of the caspase(s)/
protease(s) responsible for MEK cleavage is an area of current research.
It is somewhat surprising that vitamin D₃ would also strongly down-regulate (in caspase-dependent fashion; data not shown) the stress signaling molecule SEK-1 in apoptotic cells, similar to MEK (Fig. 7). However, other models have shown a lack of correlation to exist between activation of the SEK-JNK pathway and induction of apoptosis (55, 56). In fact, for some cells, signaling in this pathway has been linked to cell survival (57) and even to cell proliferation (58). Thus, the possibility exists that elimination of SEK-1 signaling may actually assist in commitment to the cell death program. As with MEK cleavage, caspase-dependent SEK-1 proteolysis has not been previously described. It is interesting to note that MEK and SEK-1 have analogous central positions in their respective three-kinase signaling modules (reviewed in Ref. 59) and that both should be efficiently targeted for destruction in a vitamin D₃-selective fashion. Thus, the possibility exists that a single, vitamin D₃-induced, caspase/protease activity is capable of cleaving these critical signaling molecules and irreversibly blocking their respective pathways.

As mentioned above, Akt, in contrast to MEK and SEK-1, appears to be down-regulated in vitamin D₃-treated apoptotic cells as part of the general apoptotic program, because it was also observed after treatment with genotoxic agents (Fig. 7). Co-treatment with zVAD blocked vitamin D₃-induced loss of Akt expression, indicating that it is also caspase-dependent (Fig. 4B). These findings support previous work that demonstrates caspase-dependent cleavage of Akt upon induction of apoptosis by DNA-damaging agents or Fas ligation (52). It is presently not known whether vitamin D₃-induced Akt down-regulation is mediated by the same caspase(s) activated by genotoxic agents or if distinct activities may be involved.

MEKK-1 has been demonstrated to play a pivotal role in mediating apoptosis induced by various cytotoxic treatments, including genotoxins (49) and Fas ligation (50). MEKK-1 is known to be activated by phosphorylation (autophosphorylation or via a distinct kinase (60, 61)) and/or caspase-3-mediated cleavage (49). Treatment of cells with DNA-damaging agents results initially in phosphorylation-dependent activation of MEKK-1 and, subsequently, in its caspase-mediated cleavage, leading to nearly complete loss of the 196-kDa intact protein and the appearance of a 91-kDa fragment with constitutive kinase activity (49, 52). Interestingly, this pattern is not maintained in cells induced to undergo apoptosis by vitamin D₃. Instead, intact MEKK-1 expression is further induced in apoptotic cells concurrent with the expression of a slightly lower molecular mass MEKK-1 species (about 190 kDa) as well as multiple smaller MEKK-1 fragments representing N-terminal proteolytic species (Fig. 5). The cellular scaffolding protein 14-3-3 has been shown to associate with the N-terminal portion of MEKK-1 and is thought to regulate its biological function by sequestering the protein to membranes, enabling it to respond to growth factor and cytokine stimulation (62). Whereas endogenous MEKK-1 has been shown to be exclusively associated with membranes, MEKK-1 overexpression leads to significant accumulation of the protein within the cytosol (about half of the expressed protein), uncontrolled MEKK-1 activation, and cell death (49). Similarly, caspase-3-mediated cleavage not only renders the C-terminal kinase domain to be constitutively active but also liberates it from membranes (50, 62). Thus, deregulated localization of MEKK-1 within the cell appears to play a role in the induction of apoptosis via MEKK-1. At present, it is not known whether the intact MEKK-1 protein and/or the N-terminal proteolytic fragments induced by vitamin D₃ are still associated with 14-3-3 or whether they are expressed in the cytosol.

The results presented herein shed light on the molecular events involved in vitamin D₃-induced apoptosis and provide a biochemical basis for the use of vitamin D₃ (and vitamin D₃ analogues) in the treatment of cancer. Indeed, clinical trials are currently underway to evaluate the efficacy of vitamin D₃-based compounds both as a single agent (63, 64) as well as in combination with traditional chemotherapeutic agents (65). Several studies have demonstrated that vitamin D₃ or its analogues can enhance the in vitro cytotoxicity of various anti-neoplastic agents, including tumor necrosis factor α (66), radiation (67), cisplatin (51, 68, 69), adriamycin (69, 70), and paclitaxel (70). In all but the earliest of these studies (68), enhancement of cytotoxicity required pretreatment of the cells with vitamin D₃. It is intriguing to speculate, based on the results described above, that vitamin D₃ may be priming the cells to exhibit heightened sensitivity to the cytotoxic regimen by either up-regulating MEKK-1 expression/activity, inducing a vitamin D₃-selective caspase/protease, or both. This notion gains support from studies demonstrating that acute expression of activated MEKK-1 potentiates apoptosis induced by low doses of DNA-damaging agents or tumor necrosis factor-α (71). Moreover, MCF-7 cells, which do not express caspase-3 but require its expression for cisplatin-induced apoptosis (72), show enhanced cisplatin cytotoxicity when pretreated with vitamin D₃ (68), suggesting that vitamin D₃ may selectively induce a caspase-3-like activity in these cells that renders them susceptible to cisplatin. In a similar fashion, vitamin D₃ may alleviate drug resistance that is based on caspase-3 deficiency as in cisplatin-resistant human ovarian adenocarcinoma 2008/C13 cells (73). Furthermore, vitamin D₃ may attenuate drug resistance that is based on the MEK-ERK (reviewed in Ref. 34) or Akt (74) survival pathways, via induction of a caspase/protease activity that interferes with these signaling mechanisms.

Acknowledgments—We wish to express our gratitude to Dr. Pamela A. Hershberger and Dr. Daniel Johnson for their careful review of this manuscript. We also thank Robert M. Rueger for his technical assistance in maintaining the murine squamous cell carcinoma cell line.

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*J. Biol. Chem.* 2001, 276:26365-26373.
doi: 10.1074/jbc.M010101200 originally published online April 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010101200

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