Role of Nongenomic Activation of Phosphatidylinositol 3-Kinase/Akt and Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase/Extracellular Signal-Regulated Kinase 1/2 Pathways in 1,25D3-Mediated Apoptosis in Squamous Cell Carcinoma Cells

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Abstract

Vitamin D is a steroid hormone that regulates calcium homeostasis and bone metabolism. The active form of vitamin D [1α,25-dihydroxyvitamin D3 (1,25D3)] acts through both genomic and nongenomic pathways. 1,25D3 has antitumor effects in a variety of cancers, including colorectal, prostate, breast, ovarian, and skin cancers. 1,25D3 exerts growth-inhibitory effects in cancer cells through the induction of apoptosis, cell cycle arrest, and differentiation. The mechanisms regulating 1,25D3-induced apoptosis remain unclear. We investigated the role of nongenomic signaling in 1,25D3-mediated apoptosis in squamous cell carcinoma (SCC) cells. 1,25D3 induced rapid and sustained activation of phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) 1/2 pathways in SCC cells. These effects were nongenomic; they occurred rapidly and were not inhibited by cycloheximide or actinomycin D. To examine whether the nongenomic activation of Akt and ERK1/2 plays a role in 1,25D3-mediated apoptosis, the expression of Akt or ERK1/2 was reduced by small interfering RNA (siRNA). siRNA-Akt significantly enhanced 1,25D3-induced apoptosis, the expression of Akt or ERK1/2 was reduced by addition, siRNA-Akt transfection followed by 1,25D3 treatment induced apoptosis much sooner than 1,25D3 alone. siRNA-Akt and 1,25D3 induced caspase-10 activation, suppressed the expression of c-IAP1 and XIAP, and promoted 1,25D3-induced caspase-3 activation. These results support a link between 1,25D3-induced nongenomic signaling and apoptosis. 1,25D3 induces the activation of phosphatidylinositol 3-kinase/Akt, which suppresses 1,25D3-mediated apoptosis and prolongs the survival of SCC cells. (Cancer Res 2006; 66(16): 8131-8)

Introduction

Vitamin D is a steroid hormone that regulates calcium homeostasis, bone metabolism, and many other physiologic responses in a variety of cell types (1). The active metabolite, 1α,25-dihydroxyvitamin D3 (1,25D3), acts through both genomic and nongenomic mechanisms (2). For genomic effects, 1,25D3 binds to intracellular vitamin D receptor (VDR), which subsequently forms a heterodimer with retinoid X receptor. The heterodimer binds to vitamin D response element in the promotor region of target genes and recruits multiple nuclear transcriptional components and coactivators to regulate gene transcription (1).

Nongenomic steroid actions are those that are not mediated directly through steroid receptor-ligand-DNA interaction, resulting in the modulation of gene expression. The characteristics of such actions are rapidity of onset and the lack of dependence on gene transcription and protein synthesis. Secondly, effects of nongenomic actions may indirectly affect gene transcription via the regulation of intracellular signaling pathways that target transcription factors (3). 1,25D3 induces several nongenomic responses, including rapid intestinal absorption of calcium (transcalcachia), release of calcium from intracellular stores, opening of voltage-gated calcium and chloride ion channels, and the activation of protein kinase C, protein kinase A, phosphatidylinositol 3-kinase (PI3K), and phospholipase C (4). There is no general agreement on how the nongenomic actions are initiated. There are data that suggest these effects begin at the plasma membrane and may involve classic or nonclassic VDR. Studies from functional nuclear VDR knockout mice indicate that classic VDR is required for both genomic and nongenomic actions of 1,25D3. The rapid increase of intracellular calcium concentration in response to 1,25D3 is absent in VDR knockout primary osteoblasts (5). 1,25D3 failed to modulate calcium and chloride ion channel activities in VDR knockout osteoblasts (6). VDR has been reported to exist in caveolae-enriched plasma membrane (7). Recently, a 1,25D3 membrane-binding protein, 1,25D3-membrane-associated, rapid response steroid binding, has been identified in chick intestinal basal lateral membranes (8, 9). 1,25D3 may also use G protein–coupled receptors to carry out the nongenomic actions (3). Once the signaling is initiated at the membrane, 1,25D3 delivers nongenomic signaling through kinase, cyclic AMP, or lipase (phospholipase C)–mediated pathways (3).

An increasing body of evidence shows that 1,25D3 inhibits the growth of a variety of cancer cells, including colorectal, prostate, breast, ovarian, and skin cancers. 1,25D3 exerts growth-inhibitory effects through the induction of apoptosis, cell cycle arrest, and differentiation in cancer cells. We showed that 1,25D3 inhibits the growth of murine squamous cell carcinoma (SCC) cell line SCCVII/SF in vitro and in vivo (10–12). 1,25D3 induces caspase-3 activation and poly(ADP-ribose) polymerase cleavage in SCC cells (12). This apoptosis is associated with reduced levels of phosphorylation of Akt and extracellular signal-regulated kinase (ERK) 1/2. In addition, the apoptosis is enhanced by the combination of 1,25D3 and dexamethasone (12). Additionally, we showed that pretreatment...
with 1,25D3 enhances paclitaxel-, cisplatin-, or carboplatin-mediated antitumor activities (13, 14).

The mechanisms regulating 1,25D3-induced apoptosis are unclear. In the current study, we investigate the role of non-genomic signaling in 1,25D3-mediated apoptosis in SCC cells. We examine the activation of PI3K/Akt and mitogen-activated protein kinase (MAPK)/ERK kinases (MEK)/ERK1/2 pathways in SCC cells and the potential roles of these pathways in 1,25D3-mediated apoptosis.

Materials and Methods

Materials
1,25D3 (Hoffmann-La Roche, Nutley, NJ) was reconstituted in 100% ethanol and stored, protected from light, under nitrogen gas at −70°C. All handling of 1,25D3 was done with indirect lighting. 1,25D3 was diluted in cell culture medium just before use. RPMI 1640, fetal bovine serum, PBS, penicillin, and streptomycin were obtained from Life Technologies, Inc. (Gaithersburg, MD). Cycloheximide, actinomycin D, and propidium iodide (PI) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). LY294002, Wortmannin, PD98059, and U0126 were purchased from Calbiochem (San Diego, CA). Annexin V-phycoerythrin and 7AAD were from BD Biosciences. Wortmannin, PD98059, and U0126 were purchased from Calbiochem (San Diego, CA). Cell Death Detection ELISA kit was from Roche Applied Science (Indianapolis, IN). Anti-Akt antibody (9272), anti-phosphorylated Akt (Ser473, 9271), anti-ERK1/2 (9102), anti-caspase-3 (9662), anti-caspase-9 (9504), anti-caspase-10 (9752), and anti-XIAP (2042) were from Cell Signaling Technology (Beverly, MA). Anti-phosphorylated ERK1/2 (sc-7383), anti-VDR (sc-1008), anti-Bcl-2 (sc-492), anti-Bcl-XL (sc-634), anti-Bax (sc-493), anti-Mcl-1 (sc-819), anti-c-IAP1 (sc-7943), and anti-c-IAP2 (sc-7944) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8 (552038) was from BD Biosciences. Anti-actin (CP-01) was from Calbiochem.

Cell Culture
Murine SCC cell (SCCVII/SF) tumor model was used as described previously (15). SCCVII/SF cells were maintained in 6- to 10-week-old female C3H/HeJ mice purchased from The Jackson Laboratory (Bar Harbor, ME). SCC cells were cultured in RPMI 1640 supplemented with 12% fetal bovine serum and 1% penicillin/streptomycin sulfate and incubated at 37°C in a humidified incubator containing 5% CO2. SCC cells were used within two passages. Cells were adhered to the plates overnight and then treated as described below. The vehicle control ethanol never exceeded a final concentration of 0.0004%.

Immunoblot Analysis
Cell lysates were prepared as described previously (11) from SCC cells treated as indicated. Lysates were electrophoresed on SDS-PAGE (10% polyacrylamide gels) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) by semidy blotting. Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in TBS containing 0.05% Tween 20. The membranes were then incubated overnight at 4°C in nonfat dry milk in TBS containing 0.05% Tween 20 with the indicated antibodies. Membranes were washed in nonfat dry milk in TBS containing 0.05% Tween 20 and incubated with either donkey anti-rabbit (1:5,000) or anti-mouse (1:5,000) secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Visualization of the protein bands was done using the enhanced chemiluminescence plus kit as recommended by the manufacturer (Perkin-Elmer, Boston, MA).

Small Interfering RNA Transfection
Synthetic small interfering RNA (siRNA) siGENOME siRNAs specific for Akt, ERK1, and ERK2, siCONTROL-nonspecific siRNA (siRNA-NS), and DharmaFECT 1 transfection reagent were purchased from Dharmacon (Lafayette, CO). SCC cells were plated at equal densities in six-well plates (3.3 × 10^4 per well) overnight. Cells were transfected with 50 nmol/L siRNA-NS or 50 nmol/L siRNA against Akt or ERK1/2 for 24 hours using DharmaFECT 1 transfection reagent following the manufacturer’s protocol. Following transfection, SCC cells were further treated with 1,25D3 for 24 or 48 hours and harvested for experiments as indicated.

Figure 1. 1,25D3 induces rapid and sustained non-genomic activation of Akt and ERK1/2. A, SCC cells were treated with 0.1 to 1,000 nmol/L 1,25D3 for 5 minutes, and the levels of phosphorylated Akt (P-Akt) and ERK1/2 (P-ERK1/2) were evaluated using phosphorylated specific antibody to Akt (Ser473) or ERK1/2 by immunoblot analysis. Total Akt or ERK1/2 level was assessed as the loading control. B, SCC cells were treated with 10 nmol/L 1,25D3 for 5 to 240 minutes, and the levels of phosphorylated Akt and ERK1/2 were evaluated by immunoblot analysis. C, SCC cells were treated with vehicle control DMSO, 10 μg/mL cycloheximide (CHX), or 10 μg/mL actinomycin D (ActD) for 1 hour followed by 10 nmol/L 1,25D3 for 5, 30, or 240 minutes. The levels of phosphorylated Akt and ERK1/2 were evaluated by immunoblot analysis. D, SCC cells were pre-treated with vehicle control DMSO, 10 μg/mL cycloheximide, or 10 μg/mL actinomycin D for 1 hour followed by 10 nmol/L 1,25D3 for 4 or 24 hours. VDR expression was assessed by immunoblot analysis. Actin was the loading control. Results are representative of three independent experiments.
Apoptosis Assays

Annexin V/7AAD staining. SCC cells were collected and double stained with phycoerythrin-conjugated Annexin V and 7AAD according to the manufacturer’s instructions. Annexin V-positive cells were considered apoptotic, and their percentage of the total number of cells was calculated. Ten thousand events were collected for each sample using a Becton Dickinson FACScan (Flow Cytometry Facility, Roswell Park Cancer Institute, Buffalo, NY), and data were analyzed using the Winlist program (Verity Software House, Topsham, ME).

Determination of subdiploid DNA content. SCC cells were harvested, fixed in 70% ethanol, and stained with PI (50 μg/mL) as described previously (16). The apoptotic profile was determined by flow cytometry using Becton Dickinson FACScan and Winlist software. The subdiploid DNA peak (<2N DNA), immediately adjacent to the G0-G1 peak (2N DNA), represented apoptotic cells and was quantified by histogram analyses. Objects with minimal light scatter representing debris were excluded so that quantitation of the subdiploid population would not be inappropriately skewed (17, 18).

DNA fragmentation ELISA. SCC cells were harvested and lysed, and DNA fragmentation was quantitatively evaluated by Cell Death Detection ELISA Plus™ according to the manufacturer’s instructions. The enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm was calculated as the ratio of the absorbance of the sample cells/absorbance of control cells. The enrichment factor was used as a variable of apoptosis and shown on the Y axis as mean ± SE of triplicate experiments. An enrichment factor of 1 represents background or spontaneous apoptosis.

Statistics

Statistical significances between groups were determined by two-tailed Student’s t test.

Results

1,25D3 induces nongenomic activation of Akt and ERK1/2. We showed previously that 1,25D3 induced apoptosis in SCC cells (11, 15). Akt and MAPK/ERK pathways play important roles in controlling cell survival and apoptosis. To investigate whether Akt and ERK1/2 pathways regulate 1,25D3-mediated apoptosis, we examined whether 1,25D3 induces activation of Akt and ERK1/2. SCC cells were treated with variable concentrations of 1,25D3 (0.1–1,000 nmol/L) for 5 minutes, and the activation of Akt and ERK1/2 was assessed using phosphorylation specific antibodies by immunoblot analysis. 1,25D3 induced rapid activation of both Akt and ERK1/2 (Fig. 1A) even at the lowest concentration. Because our previous studies showed that 10 nmol/L 1,25D3 was able to induce apoptosis in SCC cells, this concentration was used in the following experiments. Treatment with 10 nmol/L 1,25D3 also caused sustained activation of Akt and ERK1/2 (Fig. 1B). To evaluate whether the 1,25D3 induces the activation of Akt and ERK1/2 through a nongenomic action, SCC cells were pretreated with 10 μg/mL cycloheximide or 10 μg/mL actinomycin D for 1 hour and then treated with 10 nmol/L 1,25D3 for variable times. Neither cycloheximide nor actinomycin D inhibited 1,25D3-induced activation of Akt and ERK1/2 (Fig. 1C), indicating a nongenomic mechanism because the synthesis of mRNA or protein was not involved. In contrast, pretreatment with cycloheximide or actinomycin D readily inhibited 1,25D3-induced overexpression of VDR at 4 and 24 hours (Fig. 1D).

1,25D3 induces Akt activation through a PI3K-dependent pathway. Akt may be activated by several pathways, including PI3K, protein kinase A, and calcium/calmodulin-dependent kinase (19). To investigate which pathway contributes to 1,25D3-induced Akt activation, pharmacologic inhibitors of PI3K were used. Pretreatment with either LY294002 or Wortmannin for 1 hour inhibited 1,25D3-induced rapid Akt phosphorylation in a dose-dependent manner (Fig. 2A). Time-course study showed that 50 μmol/L LY294002 or 1 μmol/L Wortmannin abrogated both rapid and sustained Akt activation (Fig. 2A). These results indicate that 1,25D3 induces Akt activation through a PI3K-dependent pathway.

1,25D3 induces ERK1/2 activation through a MEK1/2-dependent pathway. To investigate the upstream activator of ERK1/2 activation, MEK1/2 inhibitors U0126 and PD98059 were used. Pretreatment with either U0126 or PD98059 suppressed 1,25D3-induced ERK1/2 phosphorylation in a dose-dependent manner (Fig. 2B). Time-course study showed that 10 μmol/L...
U0126 or 50 μmol/L PD98059 abrogated both rapid and sustained ERK1/2 activation (Fig. 2B), indicating the involvement of MEK1/2 in ERK1/2 activation.

siRNA-Akt promotes 1,25D3-induced apoptosis. To investigate whether the nongenomic activation of PI3K/Akt pathway regulates 1,25D3-induced apoptosis, siRNA specific to Akt was used to knockdown the expression of Akt (Fig. 3A). Following the transfection with siRNA, SCC cells were further treated with 10 nmol/L 1,25D3 for 48 hours. Apoptosis was first assessed by Annexin/7AAD staining using flow cytometry. Compared with untransfected and control siRNA-NS-transfected cells, transfection with siRNA-Akt significantly increased 1,25D3-induced Annexin V-positive cells (Fig. 3B). To further characterize apoptosis, SCC cells were subjected to PI staining and apoptosis was defined by the sub-G1 population (<2N DNA). Likewise, siRNA-Akt significantly increased 1,25D3-induced sub-G1 population at 48 hours (Fig. 3C). We next evaluated apoptosis by a third method, Cell Death Detection ELISA, which detects and quantitates DNA fragmentation (mononucleosomes and oligonucleosomes). Transfection with siRNA-Akt markedly increased 1,25D3-induced DNA fragmentation at 48 hours (Fig. 3D). Taken together, these data indicate that Akt pathway protects SCC cells from 1,25D3-mediated apoptosis.

ERK1/2 pathway is not involved in 1,25D3-induced apoptosis. To investigate whether the activation of ERK1/2 pathway regulates 1,25D3-induced apoptosis, ERK1/2 expression was knocked down by siRNAs specific to ERK1 and ERK2 (Fig. 5A). siRNA-ERK1/2

Figure 3. siRNA-Akt promotes 1,25D3-induced apoptosis. SCC cells were transfected with siRNA-NS, siRNA-Akt, or left untransfected for 24 hours followed by the treatment with 10 nmol/L 1,25D3 for 48 hours. A. Akt expression and the level of phosphorylated Akt were evaluated by immunoblot analysis. Actin was the loading control. B. SCC cells were harvested, stained with Annexin V-phycoerythrin and 7AAD, and analyzed by flow cytometry. The populations of Annexin V+/7AAD−, Annexin V+/7AAD+ and Annexin V+/7AAD− corresponded to live cells, early apoptotic cells, and late apoptotic cells. Columns, mean of Annexin V-positive cells in triplicate experiments; bars, SE. C. SCC cells were harvested, fixed, and stained with PI, and the sub-G1 population (<2N DNA) was analyzed by flow cytometry. The results were summarized in a bar graph. D. SCC cells were harvested and lysed, and DNA fragmentation was evaluated by Cell Death Detection ELISAPLUS according to the manufacturer’s protocol. Y axis, enrichment factor used as a variable of apoptosis. Columns, mean of triplicate experiments; bars, SE. Results are representative of three independent experiments.
reduced the level of ERK1/2 phosphorylation in 1,25D3-treated cells (Fig. 5A). In contrast to the results with siRNA-Akt, transfection with siRNA-ERK1/2 had no effects on 1,25D3-mediated apoptosis at 48 hours, as assessed by Annexin/7AAD staining (data not shown) and sub-G1 population analysis (Fig. 5B), indicating that ERK1/2 pathway does not contribute to 1,25D3-induced apoptosis.

Akt enhances 1,25D3-induced activation of caspase-10 and caspase-3. We further investigated the mechanisms of Akt regulating 1,25D3-induced apoptosis. Caspases are the executioners of apoptosis. The caspases related to apoptosis can be divided into two major groups, initiator caspases, including caspase-8, caspase-10, and caspase-9, and effector caspases, such as caspase-3, caspase-6, and caspase-7. When initiator caspases are activated by oligomerization, they cleave the effector caspases, which subsequently target and cleave specific cellular substrates and lead to apoptosis. To investigate what caspases are involved in 1,25D3-induced apoptosis, the activation of the major caspases were studied by immunoblot analysis. Compared with control transfections, siRNA-Akt markedly promoted the cleavage of procaspase-10 and procaspase-3 following the treatment with 1,25D3 for 48 hours (Fig. 6A), whereas the levels of procaspase-8 and procaspase-9 were unaffected (Fig. 6A). These results indicate that the knockdown of Akt promoted 1,25D3-induced apoptosis through the caspase-10-caspase-3 pathway.

Akt regulates the expression of c-IAP1 and XIAP. Bcl-2 and inhibitor of apoptosis (IAP) family members are the major regulators of apoptosis and many of them are regulated by PI3K/Akt pathway (23, 24). Therefore, to explore the target of Akt in the regulation of 1,25D3-mediated apoptosis, the expression levels of Bcl-2 and IAP family members were examined by immunoblot analysis. Akt had no effects on the protein levels of antiapoptotic Bcl-2, Bcl-XL, and Mcl-1 and the proapoptotic Bax (Fig. 6B). Among IAP proteins, the expression levels of c-IAP1 and XIAP were reduced by siRNA-Akt transfection followed by 1,25D3 treatment (Fig. 6C), whereas 1,25D3 treatment alone did not down-regulate c-IAP1 and XIAP. c-IAP2 level was not affected by siRNA-Akt transfection (Fig. 6C). Taken together, these data suggest that the knockdown of Akt may promote 1,25D3-induced apoptosis through the down-regulation of antiapoptotic IAP proteins.

Discussion

In this study, we have identified a novel link between 1,25D3-induced nongenomic signaling and apoptosis. 1,25D3 induces rapid and sustained activation of both PI3K/Akt and MEK/ERK1/2 pathways in SCC cells. siRNA-mediated knockdown of Akt promotes an earlier onset of 1,25D3-induced apoptosis and enhances apoptosis, whereas ERK1/2 is not involved in this apoptosis.

1,25D3 is widely recognized for its role in calcium and phosphate homeostasis and bone development and maintenance (1).
Bax levels were evaluated by immunoblot analysis. Actin was the loading control. Procaspase-10, procaspase-9, and procaspase-3 levels were evaluated by immunoblot analysis. Results are representative of three independent experiments.

More recently, numerous epidemiologic and experimental studies have shown that 1,25D3 also has antitumor effects, which mostly are based on the inhibition of proliferation and the induction of differentiation and apoptosis of cancer cells. Clinical trials of either 1,25D3 alone or in combination with dexamethasone or cytotoxic agents, carboplatin and taxanes, are under way in patients with prostate cancer and advanced cancer (20). The results show that 1,25D3 potentiates the cytotoxic effects of carboplatin. In addition, clinical responses are observed with 1,25D3 in combination with dexamethasone in androgen-independent prostate cancer (20).

1,25D3 acts through both genomic and nongenomic mechanisms. The classic genomic action involves 1,25D3 binding to the VDR and translocating to the nucleus to regulate the transcription of target genes. In addition, 1,25D3 has effects that occur too rapidly to be dependent on the regulation of transcription. These effects are defined as nongenomic effects and are related to the activation of various protein kinase cascades (3).

Because 1,25D3 may act through a variety of nongenomic mechanisms, we investigated whether these mechanisms may also contribute to apoptosis induced by 1,25D3 in SCC cells. We show that 1,25D3 induces rapid and sustained activation of PI3K/Akt and MEK/ERK1/2 pathways. Because the specificity of pharmacologic inhibitors may be compromised at higher dose, for instance, LY294002 may also inhibit DNA-dependent protein kinase at high dose (25), two inhibitors with different mechanisms for inhibiting each kinase were used to confirm the results. The activation of these two pathways seems to be nongenomic because they occur rapidly and do not require the synthesis of mRNA or protein. Two other MAPK kinases, c-Jun NH2-terminal kinase and p38, are not activated (data not shown). Effects on Akt and ERK1/2 were not limited to SCC cells and were also observed in the human prostate cell line DU145. Effects on the human androgen-dependent prostate cancer cell line LNCaP were limited to ERK1/2 with no effects observed on the human androgen-independent prostate cancer cell line PC3. Studies are ongoing to determine the mechanisms involved in the cell line differences observed in these pathways. We further show that PI3K/Akt pathway promotes and prolongs SCC cell survival on 1,25D3 treatment using the RNA interference approach. When Akt expression is knocked down, 1,25D3 induces more apoptosis in SCC cells. The apoptosis also occurs much earlier, indicating that 1,25D3-induced PI3K/Akt activation may be one of the reasons why 1,25D3-mediated apoptosis is delayed after treatment. In contrast, ERK1/2 activation does not contribute to 1,25D3-mediated cell survival.

1,25D3 has been reported to activate PI3K and ERK1/2 via nongenomic mechanisms. For instance, it induces the activation of PI3K, which subsequently leads to the phospholipase Cγ activation in muscle cells (26). 1,25D3-induced rapid activation of PI3K also contributes to the rat vascular smooth muscle cell migration (27). Rapid activation of PI3K and c-Jun NH2-terminal kinase, but not p38 kinase, has been reported to contribute to the protection role of 1,25D3 in etoposide-induced apoptosis in osteoblasts (28). 1,25D3 regulates some of the physiologic functions, such as proteoglycan sulfation, of growth zone chondrocytes through nongenomic activation of ERK1/2 signaling pathway, which requires PKC, phospholipase C, and the production of prostaglandin (29). In line with our study, ERK1/2 activation is not involved in the proliferation of growth zone chondrocytes (29).

We further show that siRNA-Akt promotes 1,25D3-induced apoptosis in SCC cells through the caspase-10-caspase-3 pathway, whereas caspase-8 and caspase-9 are not involved. Akt may regulate apoptosis through several different mechanisms depending on the apoptotic stimuli and cell types, which involve the regulation of phosphorylation and protein expression (24, 30). Akt may phosphorylate proapoptotic Bcl-2 family member BAD and prevent its binding and inhibition of Bcl-XL. Akt may also inhibit the activity of caspase-9 by phosphorylation. Akt has also been shown to phosphorylate XIAP at Ser87 and thus inhibiting its degradation (31). On the other hand, Akt may regulate the expression of Bcl-2 and IAP family members (24, 32, 33). In SCC cells, we show that siRNA-Akt down-regulates the protein levels of c-IAP1 and XIAP on 1,25D3

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Figure 6. Effects of siRNA-Akt and 1,25D3 treatment on caspase activation and the expression levels of Bcl-2 and IAP family members. SCC cells were transfected with siRNA-NS, siRNA-Akt, or left untransfected for 24 hours followed by the treatment with 1,25D3 for 48 hours. A, procaspase-8, procaspase-10, procaspase-9, and procaspase-3 levels were evaluated by immunoblot analysis. Actin was the loading control. B, Bcl-2, Bcl-XL, Mcl-1, and Bax levels were evaluated by immunoblot analysis. C, c-IAP1, c-IAP2, and XIAP levels were evaluated by immunoblot analysis. Results are representative of three independent experiments.

3 Y. Ma and C.S. Johnson, unpublished data.
treatment. Besides inhibiting caspases, XIAP and c-IAP1 can also be substrates of caspases and cleaved into fragments in apoptosis induced by certain stimuli (34, 35). Therefore, down-regulation of their levels may also be mediated by caspase activation. The expression of several Bcl-2 family members and c-IAP2 are not affected by siRNA-Akt. BAD and caspase-9 are not phosphorylated by these treatments (data not shown).

The mechanisms for 1,25D3-induced apoptosis remain to be fully elucidated and they seem to be cell type and tumor type dependent. 1,25D3 induces apoptosis in breast cancer cell line MCF-7 through the interruption of mitochondrial function, which is related to Bax translocation and the production of reactive oxygen species. This apoptosis is independent of caspase activation (21). 1,25D3 and its low calcemic analogue EB1089 have also been shown to promote apoptosis in MCF-7 cells through the increase of intracellular calcium level and the subsequent activation of the calcium-dependent cysteine protease m-calpain (36). In prostate cancer LNCaP and ALVA-31 cells, 1,25D3 suppresses the expression of antiapoptotic Bcl-2 family member proteins and IAP proteins. It also activates mitochondrial apoptotic pathway and induces the activation of caspase-9 and caspase-3 (37). 1,25D3 induces apoptosis in SCC cells via mechanisms involving the increased protein level of MAPK kinase kinase-1 and caspase-dependent MEK cleavage (11). In another study, 1,25D3 induces apoptosis in ovarian cancer OVCAR3 cells through the down-regulation of telomerase by decreasing the stability of the human telomerase reverse transcriptase mRNA (22). EB1089 promotes apoptosis through the induction of p38 MAPK activation, suppression of ERK activation, and the reduction of Bcl-2 and Mcl-1 in peripheral blood lymphocytes isolated from B-cell chronic lymphocytic leukemia patients (38). This apoptosis is partially dependent on caspase activation and independent of p53 (38). 1,25D3 and EB1089 also induce apoptosis that is associated with increased levels of the proapoptotic Bak in five colorectal adenoma and carcinoma cell lines (39).

In contrast to the proapoptotic effects on cancer cells, 1,25D3 protects normal cells from apoptosis. 1,25D3 inhibits UV-B–induced apoptosis through the blockage of cytochrome c release from mitochondria in primary human keratinocytes (40). 1,25D3 up-regulates Bcl-2 protein expression in normal human thryocytes and protects them from apoptotic cell death (41). 1,25D3 also protects human pancreatic islet cells from tumor necrosis factor/interleukin-1β/IFN-γ-induced apoptosis via the induction of antiapoptotic protein A20 and suppression of Fas expression (42, 43). 1,25D3 suppresses death receptor–mediated apoptosis by inhibiting caspase-8 activation and increasing Bcl-2/Bax ratio in human osteoblasts (44). These studies support the concept that 1,25D3 selectively targets cancer cells for apoptotic cell death. This may have significant chemopreventive and chemotherapeutic applications.

Our findings document a novel link between nongenomic signaling of 1,25D3 and apoptosis in a SCC model system. We show that 1,25D3 induces nongenomic activation of PI3K/Akt and MEK/ERK1/2 pathways in SCC cells. Down-regulation of Akt expression by RNA interference enhances 1,25D3-induced apoptosis and also promotes an earlier onset of apoptosis in SCC cells, whereas ERK1/2 does not contribute to this apoptosis.

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References


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