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INTRODUCTION

Vitamin D₃ compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-D₃, the active form of vitamin D₃, acts through the nuclear vitamin D₃ receptor (VDR) and induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. We examined the 1,25-D₃ signaling pathway downstream of the VDR to identify intracellular events involved in 1,25-D₃ mediated apoptosis and to characterize events which are blocked in MCF-7^{D3Res} cells (a vitamin D₃ resistant variant) (Narvaez *et al.*, 1996). In particular, the effects of 1,25-D₃ mediated apoptosis on mitochondrial function and caspase activity were studied and compared to the effects of TNF α . TNF α was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by means of a well-defined pathway triggered by TNFR1, a cell surface death receptor whose signaling results in caspase activation and disruption of mitochondrial function (Budihardjo *et al.*, 1999). Caspases are a family of evolutionarily conserved cysteine proteases that become activated upon proteolytic cleavage, and are responsible for cell disassembly. Cleavage of specific substrates by caspases during apoptosis promotes the degradation of key structural proteins, including PARP, and leads to external display of phosphatidylserine, DNA fragmentation, and cellular condensation (Thornberry and Lazebnik, 1998). Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of cytochrome *c*, and production of reactive oxygen species (Green and Reed, 1998; Susin *et al.*, 1998). Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members, including Bax, can trigger these changes. For example, apoptotic signals induce conformational changes in Bax that lead to its translocation to the mitochondria (Tsujiyama and Shimizu, 2000). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspases and nuclease activators released from mitochondria leading to the ultimate destruction of the cell. While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase dependent pathways. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to determine the involvement of caspase-dependent proteolysis in 1,25-D₃ mediated apoptosis.

RESEARCH ACCOMPLISHMENTS

Previously, in the first Annual Report, I reported data demonstrating that 1,25-D₃ induces apoptosis in MCF-7 cells by disruption of mitochondrial function (a first observation) which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, I demonstrated that Bax translocation and mitochondrial disruption do not occur after 1,25-D₃ treatment of the vitamin D₃ resistant variant, MCF-7^{D3Res} cells. These mitochondrial effects of 1,25-D₃ do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor zVAD.fmk. Although caspase inhibition blocks 1,25-D₃ mediated events downstream of mitochondria (such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-D₃ mediated cell death is caspase-independent (submitted manuscript).

To expand on these findings, time course studies of cytochrome *c* release, ROS production, and phosphatidylserine exposure were performed to determine the timing of events that trigger apoptosis. Redistribution of cytochrome *c* from mitochondria to cytosol was detected within 48 hrs of 1,25-D₃

treatment in MCF-7 cells, before any morphological apoptotic features were detected. Long-term exclusion of cytochrome *c* from the electron transport chain can lead to impairment of proton flow and generation of ROS due to incomplete reduction of molecular oxygen. Time course studies have demonstrated that ROS production is enhanced within 72 hrs of 1,25-D₃ treatment in MCF-7 cells. Cytochrome *c* release into the cytosol triggers caspase activity downstream of mitochondria. External display of phosphatidylserine, an early morphological marker of apoptosis, has been shown by others to be provoked by caspases. Annexin V-FITC is a convenient probe for monitoring changes in the distribution of phosphatidylserine in the plasma membrane during apoptosis. The earliest detection of phosphatidylserine exposure by flow cytometry was 96 hrs after the initial treatment with 1,25-D₃ in MCF-7 cells. This demonstrates that mitochondrial disruption occurs prior to activation of downstream caspases.

Time course studies of Bax translocation by immunoblot analysis yielded inconclusive results. Although a time dependent decrease in cytosolic Bax was observed as early as 48 hrs, interpretation of immunoblots of membrane bound Bax which included separate subcellular fractions of non-nuclear (enriched in mitochondria) and nuclear membranes was complicated by the observation that the nuclear fraction was contaminated with mitochondrial proteins. Further studies will be undertaken using immunofluorescent detection with Bax antibody of cells grown and fixed on glass slides.

My observations indicate that 1,25-D₃ triggers both caspase-independent and caspase-dependent pathways in MCF-7 cells, and suggest that 1,25-D₃ can activate downstream effector caspases. Since cytochrome *c* release has been associated with autoactivation of procaspase-9, 1,25-D₃ may activate caspase-dependent pathways via cytochrome *c* release. However, no DEVDase activity was detected in 1,25-D₃ treated cytoplasmic extracts, suggesting that other, possibly unidentified, effector caspases may be activated by 1,25-D₃. Caspase-9 activity assays are currently underway to determine if this caspase is responsible for 1,25-D₃ mediated caspase dependent events. Other caspases will also be examined i.e. caspase-2 and -6.

A role for the pro-apoptotic protein Bax in 1,25-D₃ mediated apoptosis is consistent with previous studies that support a role for Bcl-2, the anti-apoptotic antagonistic partner of Bax, in mediating the effects of 1,25-D₃ on both breast and prostate cancer cells. Thus, 1,25-D₃ down-regulates Bcl-2 (James *et al.*, 1995; Simboli-Campbell *et al.*, 1997) and overexpression of Bcl-2 blocks 1,25-D₃ induced apoptosis (Blutt *et al.*, 2000; Mathiasen *et al.*, 1999). Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data suggest that down-regulation of Bcl-2 in conjunction with translocation of Bax may be necessary for 1,25-D₃ mediated apoptosis. Signals generated by 1,25-D₃ that induce Bax translocation to mitochondria are currently unknown. Since events upstream of Bax translocation to mitochondria in response to 1,25-D₃ are abrogated in the vitamin D₃ resistant MCF-7 variant, comparison of early events in VDR signaling in these cells will be an important subject for future studies.

The original scope of this study was to examine the role phosphorylation plays in vitamin D₃ induced apoptosis. Previous studies have shown that the VDR can be phosphorylated by protein kinase C (PKC) and casein kinase II (Hilliard *et al.*, 1994; Jurutka *et al.*, 1993). Our observation that the phorbol ester TPA, a PKC activator, can potentiate the effects of 1,25-D₃ on induction of apoptosis in breast cancer cells, and more importantly, that TPA can partially sensitize the vitamin D₃ resistant variant to the effects of 1,25-D₃ suggests an important role for phosphorylation in dictating sensitivity to vitamin D₃ mediated apoptosis (Narvaez and Welsh, 1997). Now in addition to looking at phosphorylation, the role of mitochondria and caspase activity in vitamin D₃ mediated apoptosis was also examined. I demonstrated that mitochondrial disruption and caspase activity are abrogated in response to 1,25-D₃ treatment in the MCF-7^{D₃Res} cells. However, when the MCF-7^{D₃Res} cells are treated with 1,25-D₃ in the presence of TPA, activation of caspases is observed as assessed by phosphatidylserine exposure. This indicates that the effects of 1,25-D₃ on mitochondrial disruption and caspase activity

might be sensitized through activators of PKC. By utilizing this vitamin D₃ resistant cell line, we may be able to identify the signals induced by 1,25-D₃ upstream of Bax translocation.

STATEMENT OF WORK

Task 1: *Compare phosphorylation state of the VDR in MCF-7 and MCF-7^{D3res} cells following treatment with ethanol, 1,25-D₃ and TPA. Conduct time courses (up to 24 hrs) and dose responses (1-100 nM).*

Task 2: *Conduct 2D peptide mapping and phosphoamino acid analysis to determine if distinct sites are phosphorylated after treatment with 1,25-D₃ or TPA and determine whether differences correlate to sensitivity to apoptosis.*

Due to technical difficulties as outlined in the first Annual Report, Tasks 1 & 2 were set aside for the time being. However, if TPA alters 1,25-D₃ mediated indices of apoptosis or VDR transactivation (Tasks 3-6), we will work backwards to determine if altered phosphorylation of VDR in MCF-7^{D3Res} cells can be linked to cellular sensitivity to 1,25-D₃ mediated apoptosis.

Task 3: *Monitor biochemical and morphological indices of apoptosis to determine whether differences in phosphorylation patterns correlate to sensitivity to apoptosis.*

This task was expanded to include the effects of 1,25-D₃ on mitochondria and caspase activity. Work on this Task yielded a first observation that 1,25-D₃ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. In addition, TPA potentiated the effects of 1,25-D₃ on MCF-7^{D3Res} cells by activating downstream caspases, thus indicating that the effects of 1,25-D₃ on mitochondrial disruption and caspase activity might be sensitized through activators of PKC.

Time course of Bax translocation to mitochondria will be conducted using immunocyto fluorescence techniques.

To identify downstream effector caspases of 1,25-D₃ actions, caspase-9 activity assays are currently underway to determine if this caspase is responsible for 1,25-D₃ mediated caspase activation. Other caspases will also be examined i.e. caspase-2 and -6.

Task 4: *Conduct gel shift assays to determine whether phosphorylation affects binding of the VDR to selective VDREs and whether VDR binding is altered in MCF-7 and MCF-7^{D3res} cells after treatment with 1,25-D₃ or TPA ±1,25-D₃.*

Will be completed after Task 6.

Task 5: *Subclone multiple copies of chosen VDREs into Luciferase reporter plasmid.*

We have obtained 24-hydroxylase luciferase promoter construct, which will be used as a representative 1,25-D₃ stimulated target gene (see Task 6). Completed.

Task 6: *Conduct transient transfections and Luciferase assays on MCF-7 and MCF-7^{D3Res} cells treated with 1,25-D₃ or TPA ±1,25-D₃ to determine if differences in VDR phosphorylation and sequence specific DNA binding translate to alterations in VDR mediated transactivation in the whole cell system.*

Preliminary studies indicate that basal 24-hydroxylase activity is similar in MCF-7 and MCF-7^{D3Res} cell lines, however, 1,25-D₃ stimulated 24-hydroxylase luciferase activity is blunted in MCF-7^{D3Res} cells compared to parental MCF-7. Effects of TPA pretreatment will be studied next.

KEY RESEARCH ACCOMPLISHMENTS

First Year

- ✓ First observation implicating a role for mitochondrial events in 1,25-D₃ mediated apoptosis.
- ✓ **Caspase independent events** involved in 1,25-D₃ mediated apoptosis.
 - Translocation of Bax
 - Release of cytochrome *c*
 - Production of reactive oxygen species
- ✓ **Caspase dependent events** involved in 1,25-D₃ mediated apoptosis.
 - PARP cleavage
 - External display of phosphatidylserine
 - DNA fragmentation
- ✓ The observation that 1,25-D₃ mediated cell death is caspase independent.
- ✓ Techniques acquired so far in the course of this study
 - Flow Cytometry
 - Immunoprecipitation
 - Electromobility Shift Assay

Second Year

- ✓ Time course studies of cytochrome *c* release, ROS production, and phosphatidylserine exposure suggests that mitochondrial disruption occurs prior to activation of downstream caspases.
- ✓ The observation that TPA potentiates the effects of 1,25-D₃ on MCF-7^{D3Res} cells by activating downstream caspases, thus indicating that the effects of 1,25-D₃ on mitochondrial disruption and caspase activity might be sensitized through activators of PKC.
- ✓ The observation that 1,25-D₃ stimulated 24-hydroxylase luciferase activity is blunted in MCF-7^{D3Res} cells compared to parental MCF-7.

REPORTABLE OUTCOMES

- ✓ Narvaez CJ and Welsh J (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. Manuscript submitted to a peer-reviewed journal.
- ✓ Narvaez CJ, Waterfall T, Welsh J (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. *Proceedings of the Eleventh Workshop on Vitamin D*. In Press.
- ✓ Abstract promoted to a 10 min presentation at the **Eleventh Workshop on Vitamin D** in Nashville, TN, May 27-June 1, 2000. Abstract Title: “Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells.”
- ✓ Poster presented at the ***Era of Hope* Department of Defense Breast Cancer Research Program Meeting** in Atlanta, GA, June 8-11, 2000. Abstract Title: “Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells.”

CONCLUSION

1,25-(OH)₂D₃ mediates apoptosis of MCF-7 cells through mitochondrial signaling which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1,25-(OH)₂D₃ mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-(OH)₂D₃. Our data suggest distinct differences in the mechanisms of apoptosis induced by 1,25-(OH)₂D₃ and TNF α , since inhibition of caspases was able to rescue MCF-7 cells from TNF α , but not 1,25-(OH)₂D₃, mediated cell death. Although caspase inhibition blocked biochemical changes associated with caspase activation downstream of mitochondrial perturbations and loss of cytochrome *c*, the commitment of MCF-7 cells to 1,25-(OH)₂D₃ mediated apoptosis is clearly caspase-independent.

By understanding the effects of 1,25-D₃ on mitochondria and caspase activity, one can determine how TPA (and PKC pathway) interact with vitamin D signaling in potentiating apoptosis in D₃-resistant MCF-7 cells. This project has important implications for breast cancer since a breast cancer cell that is resistant to the apoptosis-inducing effects of 1,25-D₃ might be sensitized through activators of PKC. This basic knowledge could lead to new therapeutics for treatment of certain forms of breast cancer.

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**ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS OF MCF-7
BREAST CANCER CELLS**

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Running Title: Caspase-independent apoptosis mediated by vitamin D₃

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SUMMARY

Vitamin D₃ compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃), the active form of vitamin D₃, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-(OH)₂D₃ mediated apoptosis in MCF-7 breast cancer cells *in vitro*. The effect of 1,25-(OH)₂D₃ on MCF-7 cells was compared to that of tumor necrosis factor α (TNF α), which induces apoptosis via a caspase-dependent pathway. Our major findings are that 1,25-(OH)₂D₃ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-(OH)₂D₃ treatment of a MCF-7 cell clone selected for resistance to 1,25-(OH)₂D₃ mediated apoptosis. These mitochondrial effects of 1,25-(OH)₂D₃ do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD.fmk). Although caspase inhibition blocks 1,25-(OH)₂D₃ mediated events downstream of mitochondria (such as poly (ADP-ribose) polymerase (PARP) cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-(OH)₂D₃ mediated cell death is caspase-independent.

INTRODUCTION

1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃), the active form of vitamin D₃, acts through the nuclear vitamin D₃ receptor (VDR) and is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo* (1). A variety of synthetic vitamin D₃ analogs that induce mammary tumor regression in animals are now undergoing clinical trials in human patients (2,3). Our lab has shown that 1,25-(OH)₂D₃ induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, and DNA fragmentation) in MCF-7 breast cancer cells (4,5), however, the precise mechanism by which 1,25-(OH)₂D₃ and the VDR mediate apoptosis is poorly understood.

To characterize the mechanisms of 1,25-(OH)₂D₃ mediated apoptosis in breast cancer cells, we compared specific intracellular events in MCF-7 cells after treatment with 1,25-(OH)₂D₃ or TNF α . TNF α was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by a well-defined pathway triggered by TNFR1, a cell surface death receptor. Death receptors contain homologous cytoplasmic regions termed "death domains" which transmit apoptotic signals through recruitment of adaptor molecules that activate caspases, a family of cysteine proteases involved in cell disassembly. The best-characterized death receptors (Fas, TNFR1) use FADD and TRADD adaptors to recruit and activate caspase-8 (6). Cleavage of specific substrates by caspases during apoptosis promotes the degradation of key structural proteins, including poly (ADP-ribose) polymerase (PARP), and lead to external display of phosphatidylserine (PS), DNA fragmentation and cellular condensation (7).

Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of cytochrome *c* and apoptosis-inducing factor (AIF), and production of reactive oxygen species (ROS) (8,9). Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members, including Bax, can trigger these changes. For example, apoptotic signals induce conformational changes in Bax, which lead to exposure of the pro-apoptotic BH3 domain, and translocation to the mitochondria (10). The effects of pro-apoptotic Bcl-2 family members are achieved by both caspase-dependent and caspase-independent mechanisms (11,12).

While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase-dependent pathways. To probe the mechanisms whereby vitamin D₃ signaling modulates apoptosis in MCF-7 cells, we studied the effects of 1,25-(OH)₂D₃ on mitochondrial function and caspase activity using a cell permeable inhibitor of caspase-related proteases (zVAD.fmk). In addition, the effects of 1,25-(OH)₂D₃ and TNF α on a vitamin D₃ resistant variant of MCF-7 cells (MCF-7^{D3Res} cells) were examined to identify events that contribute to vitamin D₃ resistance (13,14). The MCF-7^{D3Res} cells do not undergo cell cycle arrest or apoptosis in response to 1,25-(OH)₂D₃, however, these cells retain sensitivity to other inducers of apoptosis such as TNF α and antiestrogens (13).

Our major findings are that 1,25-(OH)₂D₃ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of ROS. These mitochondrial effects of 1,25-(OH)₂D₃ do not require caspase activation, since they are not blocked by the caspase inhibitor zVAD.fmk. Although caspase inhibition blocks 1,25-(OH)₂D₃ mediated events downstream of mitochondria (such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-(OH)₂D₃ mediated cell death is caspase-independent.

EXPERIMENTAL PROCEDURES

Cells and cell culture

MCF-7 cells (originally obtained from ATCC) were used to generate the vitamin D₃ resistant variant (MCF-7^{D3Res}) that has been previously described (13). Both cell lines were cultured in α -MEM medium (Life Technologies, Inc., Gaithersburg, MD) containing 25 mM HEPES and 5% FBS (Life Technologies). Cells were routinely plated at 5000 cells/cm² and passaged every 3-4

days. Stock cultures of MCF-7^{D3Res} cells were routinely grown in medium containing 100 nM 1,25-(OH)₂D₃ (kindly provided by LEO Pharmaceuticals, Ballerup, Denmark). For experiments, MCF-7 and MCF-7^{D3Res} cells were plated in α -MEM containing 5% FBS plus antibiotics, and treated with 1,25-(OH)₂D₃ or ethanol vehicle 2 days after plating. Parallel cultures were treated with TNF α (Sigma, St Louis, MO) 2-3 days before scheduled harvest of 1,25-(OH)₂D₃ treated dishes. Caspase inhibitors, zVAD.fmk or zDEVD.fmk (Enzyme Systems Products, Livermore, CA) were added at the same time (1,25-(OH)₂D₃ or TNF α) or two days after (1,25-(OH)₂D₃) initial treatment. For cell growth assays, cells were seeded at 1000 cells/well in 24-well plates, treated for the indicated times and analyzed by crystal violet assay. Briefly, cells were fixed with 1% glutaraldehyde for 15 min, incubated with 0.1% crystal violet (Fisher Scientific, Pittsburgh, PA) for 30 min, destained with H₂O and solubilized with 0.2% Triton X-100. Absorbance at 562 nm (minus background at 630 nm) was determined on a microtiter plate reader.

Clonogenicity Assay

MCF-7 cells were incubated with 1,25-(OH)₂D₃ for 6 days or TNF α for 1 day in the presence or absence of 25 μ M zVAD.fmk. For 1,25-(OH)₂D₃ treatment, medium was replaced every two days. After treatments, cells were trypsinized, media and washes were pooled, cells were pelleted by centrifugation, and resuspended in fresh medium. The cells were seeded in 96-well plates at 5, 50, 500, and 2500 cells/well in 24 replicates. After 14 days, the cells were fixed and stained with crystal violet as described above, and clonogenic potential was estimated by counting positive wells (15).

Subcellular Fractionation

Cells were trypsinized, pooled together with media and washes containing floating cells, and pelleted by centrifugation at 500xg for 3 min at 4°C. Pellets were resuspended with 3 volumes of Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM DTT, 250 mM sucrose plus protease and phosphatase inhibitors), lysed with a Dounce homogenizer, and fractionated by differential centrifugation (16). Briefly, homogenates were centrifuged twice at 500xg for 5 min at 4°C, and the nuclear pellet was resuspended in Buffer A, sonicated 2x10 s, and stored at -80°C in multiple aliquots. The supernatants were combined and further centrifuged at 10,000xg for 30 min at 4°C, and the resultant mitochondrial pellets were resuspended in Buffer A, sonicated, and stored at -80°C in multiple aliquots. The supernatant from the 10,000xg spin was further centrifuged at 100,000xg for 1 h at 4°C. The resulting supernatant was designated S100 (containing cytosol) and stored at -80°C in multiple aliquots. Protein concentrations were determined by the Micro BCA protein assay (Pierce, Rockford, IL).

Immunoblot analysis

Subcellular fractions isolated as described above were solubilized in Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. Proteins derived from mitochondria and/or S100 extracts were immunoblotted with Bax rabbit polyclonal (13666E; PharMingen, San Diego, CA) or cytochrome *c* mouse monoclonal antibodies (7H8.2C12; PharMingen) diluted 1:500 or 1:250, respectively, in PBS/5% skim milk. S100 extracts were also probed with cytochrome oxidase subunit II mouse monoclonal antibody (clone 12C4-F12, Molecular Probes, Eugene, OR) to exclude mitochondrial contamination. Nuclear extracts were probed with PARP mouse monoclonal antibody (C2.10, Enzyme Systems Products) diluted 1:1000. Specific antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:5000 in PBS/5% skim milk/0.1% Tween 20 and autoradiography with enhanced chemiluminescence (Pierce).

Immunocytochemistry

MCF-7 cells grown on Lab-Tek II chamber slides (Fisher Scientific) were treated with ethanol vehicle, 100 nM 1,25-(OH)₂D₃, or 2.5 ng/ml TNF α for 96 h (ethanol, 1,25-(OH)₂D₃) or 48 h (TNF α) in the presence or absence of 25 μ M zVAD.fmk. The cells were fixed in 4% formaldehyde in PBS for 5 min at room temperature, permeabilized in methanol at -20°C for 5 min, and blocked overnight with PBS/1% BSA containing 0.02% sodium azide. The slides were then incubated with cytochrome *c* mouse monoclonal antibody (6H2.B4; PharMingen), diluted 1:100 in blocking buffer, for 3 h at 37°C in a humidified chamber. Slides were washed 3x5 min with PBS followed by incubation for 1 h at room temperature with anti-mouse secondary antibody conjugated to ALEXA-488 (a photostable dye with spectral properties similar to fluorescein; Molecular Probes) diluted 1:50 in blocking buffer. Slides were washed 3x5 min with PBS, incubated for 15 min at room temperature with 1 μ g/ml Hoechst 33258 (Sigma), washed 5x5 min with PBS, rinsed with dH₂O, and coverslips were applied with an antifade reagent. Fluorescence was detected using Olympus AX70 microscope equipped with a Spot RT digital camera.

Flow cytometry

For analysis of mitochondrial membrane potential and reactive oxygen species, cells harvested by trypsinization were pooled with media plus washes and pelleted by centrifugation. Cell suspensions (1x10⁶ cells) were incubated with 1 μ M tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) in PBS containing 130 mM KCl to abolish the plasma membrane potential. After incubation for 10 min at 37°C, cells were washed once in PBS and then analysed for TMRE red fluorescence by flow cytometry. Live cells rapidly and reversibly take up TMRE, and accumulation of the dye in mitochondria has been shown to be potential driven (17). For analysis of ROS, cell suspensions (5x10⁵ cells) were incubated with 4 μ M hydroethidine (HE, Molecular Probes) in PBS for 15 min at 37°C, and conversion of HE to ethidium by superoxide anion was analyzed by flow cytometry.

For analysis of DNA fragmentation and phosphatidylserine externalization, MCF-7 cells were harvested by trypsinization, collected by centrifugation, fixed in 2% formaldehyde in PBS, and permeabilized in 70% EtOH at -20°C. DNA strand breaks in cells undergoing apoptosis were indirectly labeled with bromodeoxyuridine (Br-dUTP) by terminal transferase (Boehringer Mannheim,

Indianapolis, IN) and detected by FITC-conjugated monoclonal antibody to Br-dUTP using the APO-BRDU kit according to manufacturer's protocol (Phoenix Flow Systems, San Diego, CA). Cells were counterstained with 5 µg/ml propidium iodide (PI; Sigma) containing RNase A (Boehringer Mannheim) for detection of total DNA, and two color analysis of DNA strand breaks and cell cycle was achieved by flow cytometry.

For detection of phosphatidylserine (PS) externalization, 1×10^6 cells were incubated in the presence of 10 µg/ml annexin V-FITC (BioWhittaker, Walkersville, MD) and 5 µg/ml PI in binding buffer (10 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 2.5 mM CaCl_2) for 15 min at 37°C. Cells were washed twice in binding buffer, fixed in 2% formaldehyde in PBS for 15 min on ice, and then washed two more times in PBS/0.2% BSA. Pellets were resuspended in PBS/0.2% BSA and analyzed by flow cytometry. There were less than 1% PI^+ cells in the population and were therefore excluded from analysis.

All flow cytometric analyses were performed on an Epics XL Flow Cytometer (Coulter Corp, Miami, FL) equipped with an argon laser. TMRE and HE were analyzed on FL3 using a 620 nm band pass filter. For DNA fragmentation analysis, FITC was analyzed on FL1 using a 520 nm band pass filter and PI was analyzed on FL3 with no color compensation. For PS externalization, annexin V-FITC was analyzed on FL1 and PI was analyzed on FL2 (580 nm band pass filter) using software color compensation. Data was modeled with the MULTIPLUS AV software (Phoenix Flow Systems).

Caspase Activity Assay

Caspase activity was analyzed with the ApoAlert CPP32/Caspase-3 assay kit according to manufacturer's protocol (CLONTECH, Palo Alto, CA). Briefly, after harvesting by trypsinization, 2×10^6 cells were pelleted and stored at -20°C. For analysis, cell pellets were lysed, re-pelleted to remove cell debris, and supernatants were incubated with 50 µM DEVD-AFC for 1 h at 37°C. The samples were analyzed using a fluorescence spectrophotometer with $\text{Ex}=380$ nm and $\text{Em}=508$ nm.

Statistical Evaluation

Data are expressed as mean \pm SEM. One-way ANOVA was used to assess statistical significance between means. Differences between means were considered significant if p values less than 0.05 were obtained with the Bonferroni method using GraphPad InStat software (GraphPad Software, San Diego, CA).

RESULTS

Disruption of mitochondrial function, as determined by subcellular localization of Bax and cytochrome *c*, and ROS generation, by 1,25-(OH)₂D₃.

To identify specific intracellular events involved in 1,25-(OH)₂D₃ mediated apoptosis, we examined the signaling pathway downstream of the VDR in MCF-7 cells. Since disruption of mitochondrial function is a primary event in apoptosis that can be triggered by translocation of Bax to mitochondrial outer membrane, we first examined the subcellular distribution of Bax after 1,25-(OH)₂D₃ treatment of MCF-7 cells. As demonstrated in Fig. 1, Bax redistribution from the cytosolic to the mitochondrial fraction occurred after treatment with 1,25-(OH)₂D₃ or TNFα in MCF-7 cells (Fig. 1, *Top*). Not only was Bax translocated to mitochondria, but both 1,25-(OH)₂D₃ and TNFα treated cells exhibited cleavage of Bax from the intact 21 kDa protein to an 18 kDa fragment, an observation which is consistent with reports of Bax cleavage during drug-induced apoptosis (18). In both 1,25-(OH)₂D₃ and TNFα treated cells, the Bax cleavage product was detected in mitochondrial, but not cytosolic, fractions, and others have proposed that Bax cleavage enhances homodimerization and its pro-apoptotic properties (19). These are the first data to implicate translocation and cleavage of Bax during 1,25-(OH)₂D₃ induced apoptosis. To determine the relationship between Bax translocation and sensitivity to 1,25-(OH)₂D₃ induced apoptosis, we examined the subcellular distribution of Bax in a vitamin D₃ resistant variant of MCF-7 cells which does not undergo apoptosis after treatment with 1,25-(OH)₂D₃ but retains sensitivity to other triggers, including TNFα (Narvaez and Welsh, unpublished data). In the MCF-7^{D3Res} cells, 1,25-(OH)₂D₃ did not induce translocation or cleavage of Bax (Fig. 1, *Bottom*). However, in these cells, Bax translocation and cleavage was triggered by TNFα, indicating that Bax functions appropriately in MCF-7^{D3Res} cells during apoptosis induced by agents other than 1,25-(OH)₂D₃. The inability of Bax to redistribute to mitochondria in response to 1,25-(OH)₂D₃ in the vitamin D₃ resistant variant suggests that Bax translocation may be a critical initiating event in 1,25-(OH)₂D₃ mediated apoptosis of MCF-7 cells.

Translocation of Bax to mitochondria has been associated with release of cytochrome *c*, an event that is considered a commitment point for activation of apoptosis. As expected for viable cultures, no cytochrome *c* was detected in cytosolic fractions from MCF-7 cells treated with ethanol vehicle for up to 120 h (Fig. 2A). In contrast, redistribution of cytochrome *c* from mitochondria to cytosol was detected within 48 h of 1,25-(OH)₂D₃ treatment in MCF-7 cells, before any morphological apoptotic features were detected. The absence of cytochrome oxidase in cytosolic fractions confirmed that extracts were free of mitochondrial contamination (data not shown). In MCF-7^{D3Res} cells, 1,25-(OH)₂D₃ did not trigger release of cytochrome *c*, however, cytochrome *c* was detected in cytosolic fractions after TNFα treatment of both MCF-7 and MCF-7^{D3Res} cell lines (Fig. 2B, see also Fig. 7).

Long-term exclusion of cytochrome *c* from the electron transport chain can lead to impairment of proton flow and generation of ROS due to incomplete reduction of molecular oxygen. Hence, mitochondrial generation of ROS in response to 1,25-(OH)₂D₃ and TNFα was examined by flow cytometry. Production of superoxide anion was indirectly assessed as oxidation of hydroethidine to ethidium, which fluoresces red upon DNA intercalation. As presented in Fig. 3, ROS production was enhanced by 1,25-(OH)₂D₃ in

MCF-7, but not MCF-7^{D3Res} cells, whereas TNF α increased ROS production comparably in both cell lines. Time course studies have demonstrated that ROS production is enhanced within 72 h of 1,25-(OH)₂D₃ treatment in MCF-7 cells (data not shown).

1,25-(OH)₂D₃ mediates PARP cleavage, PS externalization, and DNA fragmentation in a caspase-dependent manner.

Cytochrome *c* released into the cytosol is thought to trigger caspase activation downstream of mitochondria through binding to Apaf-1 and autoactivation of procaspase-9. Activated caspase-9 can activate additional effector caspases responsible for cell disassembly and events such as PS externalization, PARP cleavage and DNA fragmentation. To determine the involvement of caspase-dependent proteolysis in 1,25-(OH)₂D₃ mediated apoptosis, we examined whether a broad-spectrum cell permeable caspase inhibitor (zVAD.fmk) could abrogate the effects of 1,25-(OH)₂D₃ in MCF-7 cells.

Proteolytic activity associated with caspase activation was analyzed by three distinct methods: cleavage of an endogenous caspase substrate (PARP), and flow cytometric analysis of PS exposure and DNA fragmentation, which others have shown are provoked by caspases (7). As demonstrated in Fig. 4A, PARP was cleaved after treatment of MCF-7 cells with either 1,25-(OH)₂D₃ or TNF α , and in both cases, cleavage was blocked by zVAD.fmk. Furthermore, both 1,25-(OH)₂D₃ and TNF α induced PS externalization, which was also completely blocked by zVAD.fmk (Fig. 4B). Finally, the effects of 1,25-(OH)₂D₃ and TNF α on DNA fragmentation was assessed as terminal transferase mediated incorporation of bromodeoxyuridine, detected by FITC-conjugated anti-bromodeoxyuridine antibody by flow cytometry (Fig. 5). 1,25-(OH)₂D₃ treatment of MCF-7 cells induced DNA fragmentation primarily in the G₁ phase of the cell cycle, with only a small population of cells (5%) accumulating in sub-G₁. TNF α treatment induced extensive DNA fragmentation in the G₁ phase of the cell cycle, with accumulation of 29% of the population in sub-G₁. Despite the differences in the magnitude and profiles of DNA fragmentation between 1,25-(OH)₂D₃ and TNF α treated cells, zVAD.fmk completely blocked DNA fragmentation induced by both agents.

Caspase activity induced by 1,25-(OH)₂D₃ is not DEVDase.

DEVDase cleavage activity was measured with the fluorogenic substrate DEVD-AFC, which can detect activity of caspases-3 and -7. Since MCF-7 cells do not express functional caspase-3 (data not shown, ref. (20)), any DEVDase activity detected in these cells would most likely correspond to caspase-7. For these experiments, MCF-7 cells were pretreated with 1,25-(OH)₂D₃ for 2 days before cytosolic extracts were collected for DEVDase activity assays at the indicated time points. As demonstrated in Fig. 6, TNF α , but not 1,25-(OH)₂D₃, induced DEVDase cleavage activity in MCF-7 cells. Even with extended treatment times in additional studies, no DEVDase cleavage activity could be detected after 1,25-(OH)₂D₃ treatment of MCF-7 cells (data not shown). This observation indicates that other known or as yet unidentified effector caspases may be activated by 1,25-(OH)₂D₃, which mediate PARP cleavage, PS exposure, or DNA fragmentation.

Caspase inhibition does not block 1,25-(OH)₂D₃ mediated cytochrome *c* release, mitochondrial dysfunction or cell death.

Since zVAD.fmk blocked 1,25-(OH)₂D₃ mediated caspase-dependent events downstream of mitochondria, we examined the effects of the caspase inhibitor on cytochrome *c* release and mitochondrial function. As shown in Fig. 7, zVAD.fmk did not abrogate 1,25-(OH)₂D₃ mediated cytochrome *c* release or ROS production under the same conditions where PS exposure, PARP cleavage and DNA fragmentation were blocked. In contrast, the caspase inhibitor effectively blocked cytochrome *c* release and ROS generation triggered by TNF α (Fig. 7A, 7C).

To further probe mitochondrial function, the membrane potential sensitive probe TMRE was used to detect mitochondrial membrane potential by flow cytometry. 1,25-(OH)₂D₃ treatment significantly enhanced the percentage of cells with reduced mitochondrial membrane potential, and zVAD.fmk did not block the decrease in mitochondrial membrane potential induced by 1,25-(OH)₂D₃. TNF α treatment also enhanced the percentage of cells with decreased mitochondrial membrane potential, however, in contrast to 1,25-(OH)₂D₃, the effect of TNF α was completely blocked by zVAD.fmk (Fig. 7B).

Subcellular localization of cytochrome *c* protein was examined by fluorescence microscopy to confirm the finding that cytochrome *c* release can proceed independently of caspase activation after 1,25-(OH)₂D₃ treatment. In Fig. 8, cytochrome *c* fluorescence (middle panels) is presented alongside phase contrast (top panels) and Hoechst nuclear staining (bottom panels) to compare cytochrome *c* localization in individual viable and apoptotic cells. In vehicle treated control cells, apoptotic morphology was not present, and cytochrome *c* staining was restricted to punctate cytoplasmic regions, consistent with mitochondrial localization (Fig. 8). After treatment with 1,25-(OH)₂D₃ or TNF α , apoptotic cells identified by phase contrast and Hoechst nuclear staining exhibited chromatin condensation, nuclear fragmentation, and cytosolic vacuolization. In these apoptotic cells, diffuse cytoplasmic cytochrome *c* staining was detected throughout the cell, which obscured the nuclei, consistent with redistribution of cytochrome *c* from mitochondria to cytoplasm (21). Consistent with the immunoblotting data (Fig. 7A), treatment with zVAD.fmk failed to prevent 1,25-(OH)₂D₃ mediated cytochrome *c* release, as demonstrated by persistence of diffuse cytoplasmic cytochrome *c* staining in 1,25-(OH)₂D₃ plus zVAD.fmk treated cells. However, zVAD.fmk did prevent the morphological signs of apoptosis, including chromatin condensation and nuclear fragmentation, consistent with its ability to block PS redistribution, PARP cleavage, and DNA fragmentation induced by 1,25-(OH)₂D₃.

Since zVAD.fmk did not block cytochrome *c* release or mitochondrial dysfunction induced by 1,25-(OH)₂D₃, but did protect MCF-7 cells from morphological signs of apoptosis, including DNA fragmentation, we examined whether zVAD.fmk treated cells actually remained viable and/or maintained clonogenic potential. As shown in Fig. 9, both zVAD.fmk and zDEVD.fmk caspase inhibitors rescued MCF-7 cells from TNF α mediated cell death, as demonstrated by total cell numbers, with zVAD.fmk offering the greater protection. However, neither zVAD.fmk nor zDEVD.fmk caspase inhibitors could protect MCF-7 cells from 1,25-(OH)₂D₃

mediated apoptosis, since the reduction in total cell number was not abrogated by either inhibitor (Fig. 9). Finally, the clonogenic potential was determined after treatment of cells with 1,25-(OH)₂D₃ or TNF α in the presence or absence of zVAD.fmk followed by replating at limiting dilutions in fresh medium. In vehicle control treated cultures, at least 1 out of 5 cells had the ability to produce clones. In TNF α treated cultures, clonogenicity was less than 1 out of 500 cells ($f < 0.002$) but in the presence of zVAD.fmk, the frequency of cells with clonogenic potential was significantly increased ($f \geq 0.2$). In 1,25-(OH)₂D₃ treated cultures, clonogenicity was less than 1 out of 50 cells ($f < 0.02$), and this was not enhanced in the presence of zVAD.fmk.

DISCUSSION

Here we report for the first time that 1,25-(OH)₂D₃ induces apoptosis in MCF-7 cells by disruption of mitochondrial function, which is accomplished by translocation of Bax to mitochondria, and increased permeability of the outer mitochondrial membrane. Of particular interest, neither Bax translocation nor the mitochondrial disruption is induced by 1,25-(OH)₂D₃ in a variant line of MCF-7 cells selected for resistance to 1,25-(OH)₂D₃ mediated apoptosis (13). Collectively, these data implicate an essential role for mitochondrial signaling in the induction of apoptosis by 1,25-(OH)₂D₃ and identify the pro-apoptotic protein Bax as an important downstream target of the VDR in MCF-7 cells.

In addition to Bax translocation, we report that treatment of MCF-7 cells with 1,25-(OH)₂D₃ induces cytochrome *c* release and ROS generation, events which have been observed in cells induced to undergo apoptosis by overexpression of Bax (22,23). These data further support the concept that 1,25-(OH)₂D₃ mediated apoptosis may be driven by Bax translocation. A role for the pro-apoptotic protein Bax in 1,25-(OH)₂D₃ mediated apoptosis is consistent with previous studies that support a role for Bcl-2, the anti-apoptotic antagonistic partner of Bax, in mediating the effects of 1,25-(OH)₂D₃ on both breast and prostate cancer cells. Thus, 1,25-(OH)₂D₃ down-regulates Bcl-2 (24,25) and overexpression of Bcl-2 blocks 1,25-(OH)₂D₃ induced apoptosis (26,27). Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data suggest that down-regulation of Bcl-2 in conjunction with translocation of Bax may be necessary for 1,25-(OH)₂D₃ mediated apoptosis. Further studies will be necessary to identify the signals generated by 1,25-(OH)₂D₃ that induce Bax translocation to mitochondria. Since events upstream of Bax translocation to mitochondria in response to 1,25-(OH)₂D₃ are abrogated in the vitamin D₃ resistant MCF-7 variant, comparison of early events in VDR signaling in these cells will be an important subject for future studies.

Examination of events downstream of mitochondria indicated that 1,25-(OH)₂D₃ induced features of apoptosis associated with caspase activation, such as PARP cleavage, PS exposure and DNA fragmentation. To determine whether caspase activation was required for 1,25-(OH)₂D₃ mediated apoptosis, we used the broad spectrum, cell permeable caspase inhibitor zVAD.fmk. We observed that 1,25-(OH)₂D₃ signaling on mitochondria does not require caspase activation, since zVAD.fmk was unable to block 1,25-(OH)₂D₃ induced cytochrome *c* release, decrease in mitochondrial membrane potential, or ROS production. Again, this is consistent with apoptosis driven by Bax translocation, which promotes cytochrome *c* release via caspase-independent pathways (28-31). Our data also complement that of Mathiasen *et al* (27) who reported that inhibition of caspase activity by overexpression of CrmA, a cowpox-derived caspase inhibitor, or caspase inhibitory peptides (Ac-DEVD-CHO, Ac-IETD-CHO, and zVAD.fmk) did not block vitamin D₃ mediated growth arrest or apoptosis.

Although caspase inhibition did not block mitochondrial events induced by 1,25-(OH)₂D₃, zVAD.fmk did block events downstream of mitochondria such as PARP cleavage, external display of PS, and DNA fragmentation. These findings are similar to reports of Bax induced apoptosis, where caspase inhibitors had no effect on Bax-induced cytochrome *c* release or mitochondrial disruption, but prevented cleavage of nuclear and cytosolic substrates and DNA degradation (28-31). However, our data conflict with that of Mathiasen *et al*, who observed that zVAD.fmk did not block 1,25-(OH)₂D₃ mediated DNA fragmentation in MCF-7 cells (27). This discrepancy may reflect differences in doses (1 μ M vs. 25 μ M) or experimental design between the two studies. The lower dose of zVAD.fmk (1 μ M) used by Mathiasen *et al* may have been insufficient to block mitochondrial-initiated caspases (caspase-9) (32).

The data presented in this paper indicates that 1,25-(OH)₂D₃ triggers both caspase-independent and caspase-dependent pathways in MCF-7 cells, and suggests that 1,25-(OH)₂D₃ can activate downstream effector caspases. Since cytochrome *c* release has been associated with autoactivation of procaspase-9, 1,25-(OH)₂D₃ may activate caspase-dependent pathways via cytochrome *c* release. However, no DEVDase activity was detected in 1,25-(OH)₂D₃ treated cytoplasmic extracts, suggesting that other, possibly unidentified, effector caspases may be activated by 1,25-(OH)₂D₃, or that caspase-dependent events occur at later stages in the apoptotic program. Although blocking caspase activation prevented some of the morphological aspects of 1,25-(OH)₂D₃ mediated apoptosis, MCF-7 cells were not rescued from death by zVAD.fmk. This finding is consistent with reports that many cell types eventually die by a slower, non-apoptotic cell death if caspases are inactivated (8). These data support the concept that mitochondrial damage represents a cell death commitment step in the course of apoptosis induced by many stimuli (33), including 1,25-(OH)₂D₃.

In summary, 1,25-(OH)₂D₃ mediates apoptosis of MCF-7 cells through mitochondrial signaling which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1,25-(OH)₂D₃ mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-(OH)₂D₃. Our data suggest distinct differences in the mechanisms of apoptosis induced by 1,25-(OH)₂D₃ and TNF α , since inhibition of caspases was able to rescue MCF-7 cells from TNF α , but not 1,25-(OH)₂D₃ mediated cell death. Although caspase inhibition blocked biochemical changes associated with caspase activation downstream of mitochondrial perturbations and loss of cytochrome *c*, the commitment of MCF-7 cells to 1,25-(OH)₂D₃ mediated apoptosis is clearly caspase-independent.

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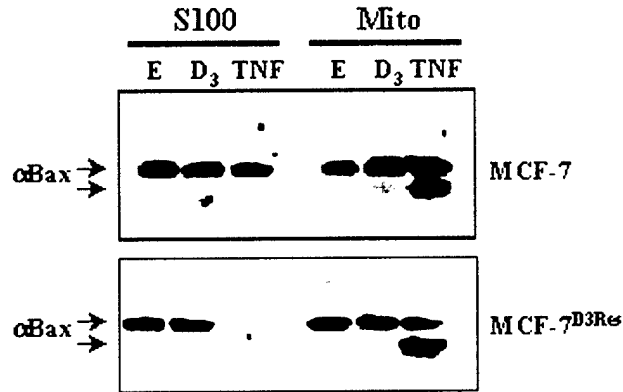


FIG. 1. Subcellular distribution of Bax after treatment with 1,25-(OH)₂D₃ or TNF α in parental MCF-7 or MCF-7^{D3Res} cells. MCF-7 or MCF-7^{D3Res} cells were plated at a density of 2×10^5 cells/150-mm dish. Two days after plating, cells were treated with vehicle control (ethanol) or 100 nM 1,25-(OH)₂D₃ for 96 h, or with 2.5 ng/ml TNF α for 48 h. Mitochondria and S100 isolated as described in *Experimental Procedures* were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal antibody to Bax. The results are representative of at least three independent experiments.

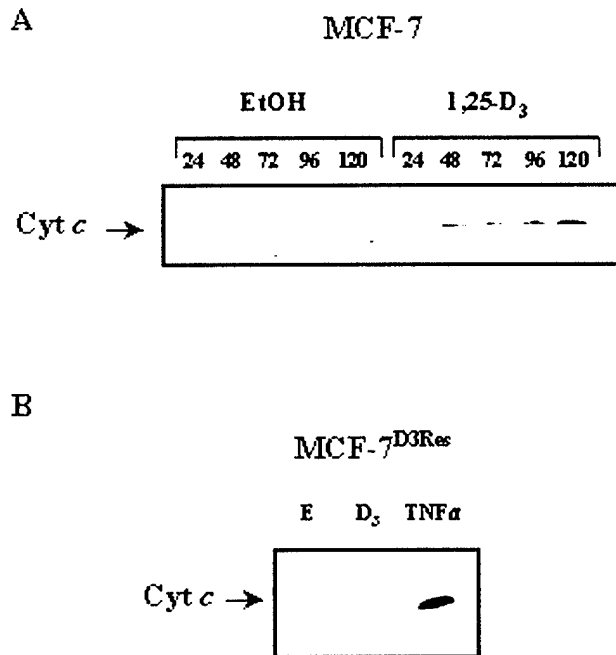


FIG. 2. Cytosolic localization of cytochrome *c* after treatment with 1,25-(OH)₂D₃ or TNF α . **A.** Time course of cytosolic cytochrome *c* after treatment with vehicle control (ethanol) or 100 nM 1,25-(OH)₂D₃ in MCF-7 cells. Cells were plated at density of 1×10^5 cells/150-mm dish. Two days after plating, the cells were treated with ethanol or 1,25-(OH)₂D₃ and re-fed two days later. S100 fractions prepared at the indicated time points as described in *Experimental Procedures* were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with cytochrome *c* (7H8.2C12) antibody. **B.** Cytosolic cytochrome *c* in MCF-7^{D3Res} cells. Cells were plated and treated with ethanol or 1,25-(OH)₂D₃ as described above and 2.5 ng/ml TNF α was added 3 days before harvest. All the dishes were harvested on day 5 of treatment, and S100 fractions were prepared and immunoblotted as described above. The results are representative of at least three independent experiments.

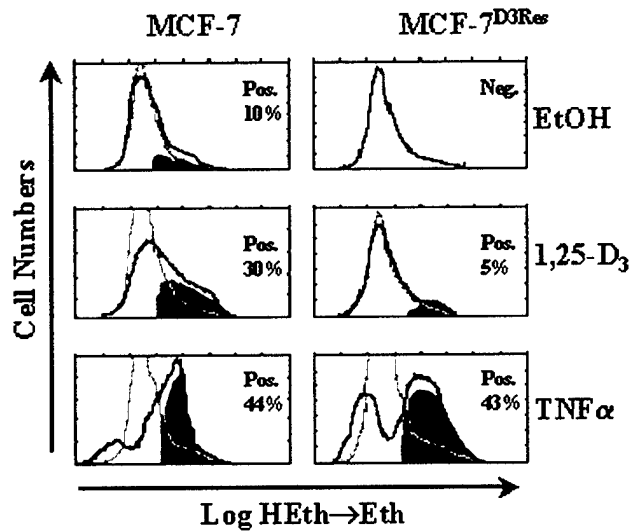


FIG. 3. ROS production after treatment with 1,25-(OH)₂D₃ or TNFα in parental MCF-7 or MCF-7^{D3Res} cells. Cells were plated at a density of 1x10⁵ cells/150-mm dish. Two days after plating, the cells were treated with vehicle control (ethanol), 100 nM 1,25-(OH)₂D₃, or media only, re-fed two days later, and 2.5 ng/ml TNFα was added to dishes containing media only. All dishes were harvested on day 5 when ROS generation was assessed by flow cytometry as described in *Experimental Procedures*. Data are expressed as the percentage of cells positive for ROS after negative subtraction of data derived from vehicle treated MCF-7^{D3Res} cells. The results are representative of at least three independent experiments.

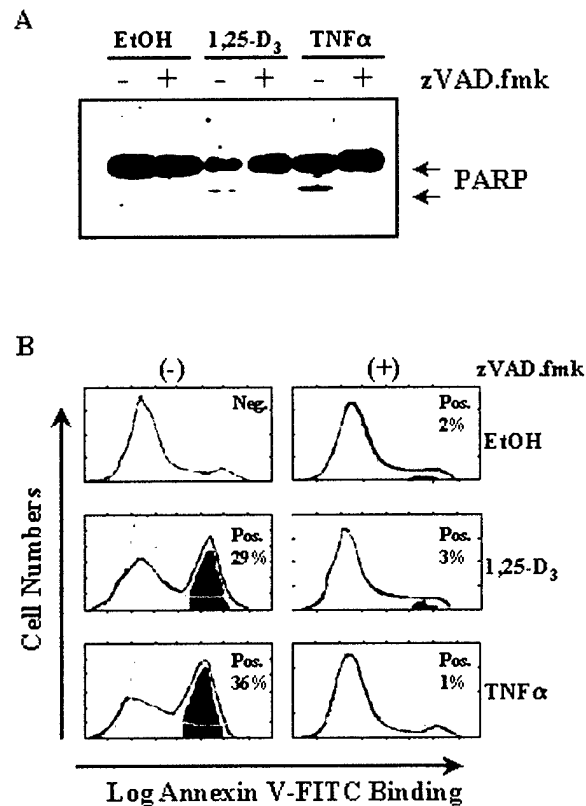


FIG. 4. Effects of caspase inhibitor on PARP cleavage and PS exposure after treatment with 1,25-(OH)₂D₃ or TNFα in MCF-7 cells. **A.** PARP cleavage. Cells were plated and treated as described in FIG. 3 in the presence or absence of 25 μM zVAD.fmk. All the dishes were harvested on Day 5. Nuclear extracts prepared as described in *Experimental Procedures* were separated on SDS-PAGE and immunoblotted with mouse monoclonal antibody to PARP. **B.** PS externalization. Cells plated and treated as described above were incubated with annexin V-FITC and PI as described in *Experimental Procedures*. Data are expressed as the percentage of annexin V-FITC positive cells after negative subtraction of data generated with vehicle treated cells. The results are representative of at least three independent experiments.

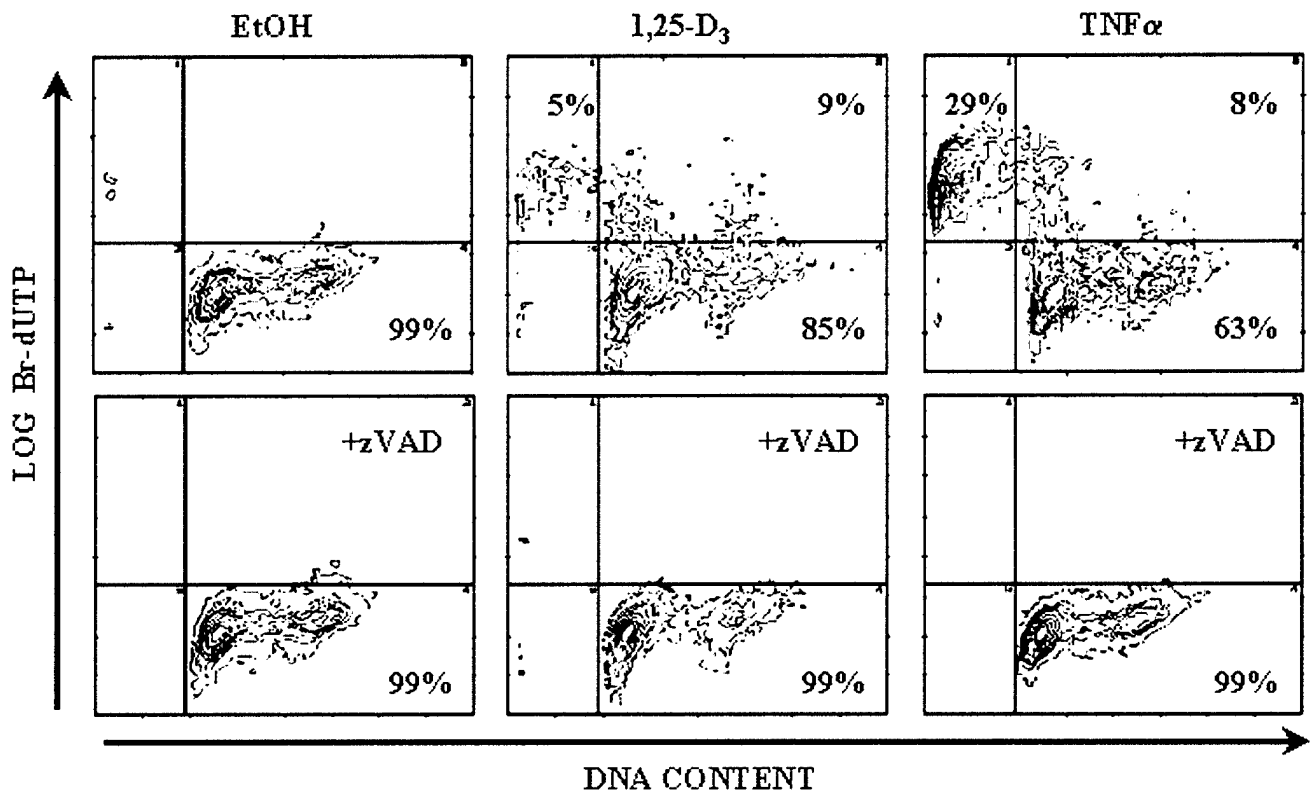


FIG. 5. Effect of caspase inhibitor on DNA fragmentation after treatment with 1,25-(OH)₂D₃ or TNF α in MCF-7 cells. Cells were plated and treated as described in FIG. 4, and DNA fragmentation was determined by flow cytometry as described in *Experimental Procedures*. The results are representative of at least three independent experiments.

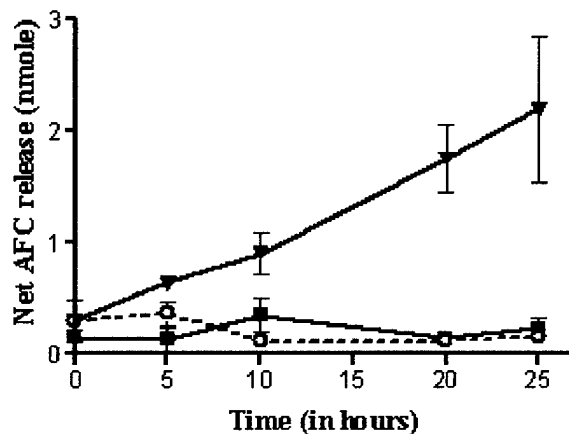


FIG. 6. Time course of DEVDase cleavage activity after treatment with 1,25-(OH)₂D₃ or TNF α in MCF-7 cells. Cells were plated at a density of 2×10^5 cells/150-mm dish. Two days after plating, the cells were pretreated with ethanol, 100 nM 1,25-(OH)₂D₃ or media only. Two days later, medium was replaced with ethanol (o), 100 nM 1,25-(OH)₂D₃ (■), or 10 ng/ml TNF α (▼) (T=0). Cytosolic extracts of cells harvested at the indicated time points were incubated with DEVD-AFC for 1 h at 37°C, and analyzed on a fluorescence spectrophotometer. Data represent mean \pm SEM of two independent experiments performed in duplicate.

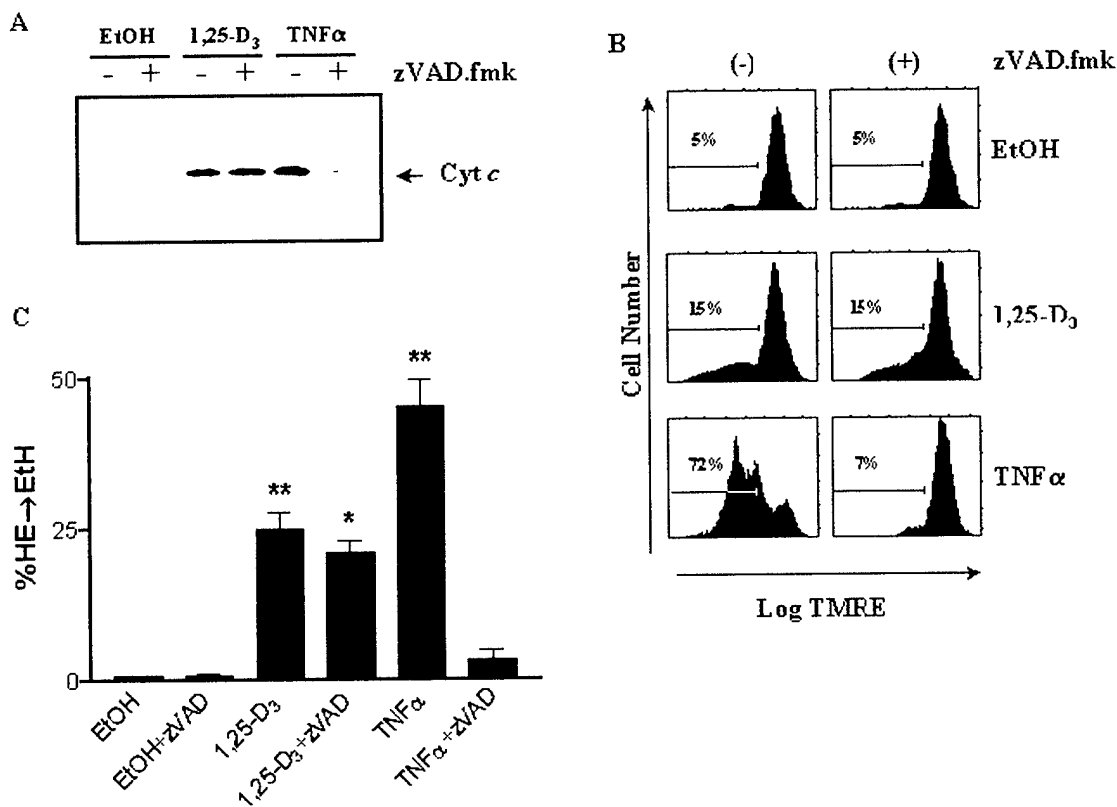


FIG. 7. Effects of caspase inhibitor on cytochrome *c* release, mitochondrial membrane potential, and ROS production after treatment with 1,25-(OH)₂D₃ or TNF α in MCF-7 cells. **A.** Cytochrome *c* release. S100 extracts prepared from cells treated as described in FIG. 4 were separated on SDS-PAGE and immunoblotted with cytochrome *c* (7H8.2C12) mouse monoclonal antibody. **B.** Mitochondrial membrane potential. Cells plated and treated as described above were incubated with 1 μ M TMRE as described in *Experimental Procedures* and analyzed by flow cytometry. Data are expressed as the percentage of cells with reduced mitochondrial membrane potential. **C.** ROS production. Cells treated as described above were incubated with 4 μ M HE as described in *Experimental Procedures* and analyzed by flow cytometry. The data represent the percentage of cells positive for ROS. Each bar represents the mean \pm SEM of 2-4 independent experiments. **, $p < 0.001$; *, $p < 0.01$; treated vs. ethanol control as evaluated by ANOVA. The results (A & B) are representative of at least three independent experiments.

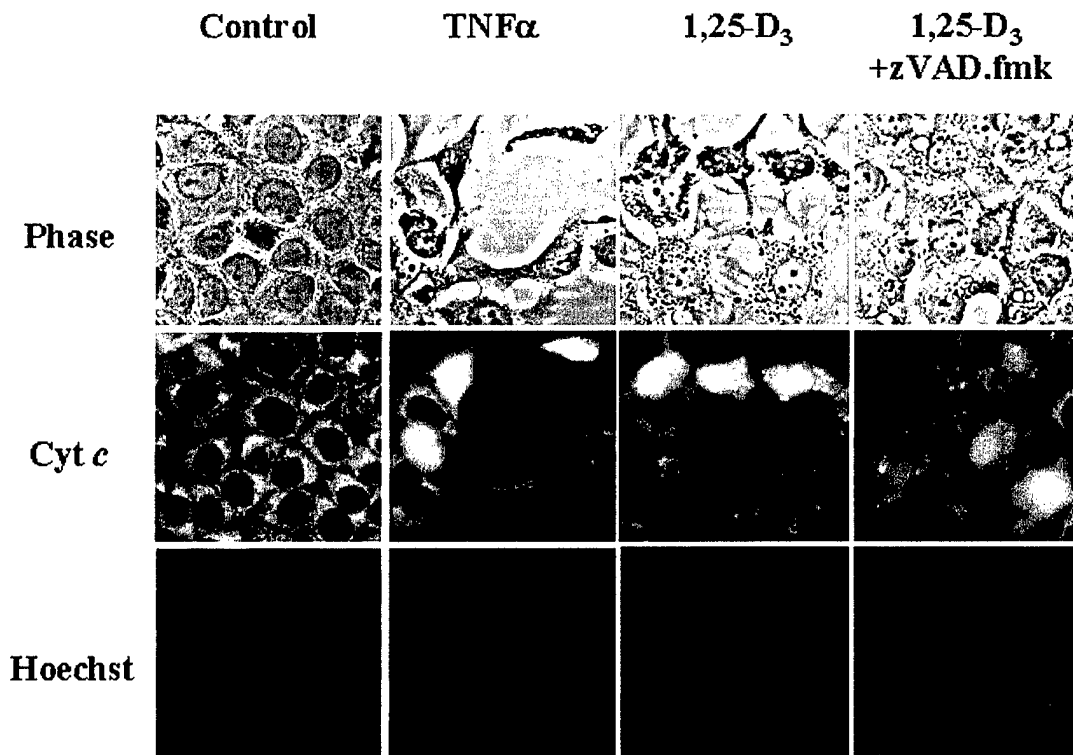


FIG. 8. Effects of 1,25-(OH)₂D₃ or TNF α on morphology and cytochrome *c* release in MCF-7 cells. Cells grown on Lab-Tek II chamber slides were treated with ethanol, 100 nM 1,25-(OH)₂D₃, or 2.5 ng/ml TNF α \pm 25 μ M zVAD.fmk, fixed after 96 h (ethanol, 1,25-(OH)₂D₃) or 48 h (TNF α), immunostained with cytochrome *c* (6H2.B4) mouse monoclonal antibody and visualized with ALEXA-488 conjugated secondary antibody. Nuclei were counterstained with Hoechst 33258. The images were taken with an Olympus AX70 fluorescence microscope at 400x magnification. *Top*, phase contrast; *Middle*, cytochrome *c* (green); *Bottom*, Hoechst (blue). The results are representative of at least two independent experiments.

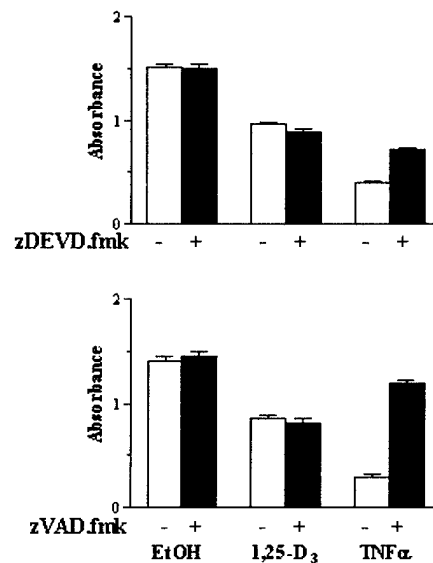


FIG. 9. Effect of caspase inhibitors on MCF-7 cell number after treatment with 1,25-(OH)₂D₃ or TNF α . Cells were seeded at a density of 1000 cells/well in 24-well plates. Two days after plating, cells were treated with ethanol, 100 nM 1,25-(OH)₂D₃ or 10 ng/ml TNF α for 5 days \pm 25 μ M zVAD.fmk (broad spectrum, *Bottom*) or zDEVD.fmk (caspase-3/7 specific, *Top*). Total cell number was determined by crystal violet assay as described in *Experimental Procedures*. Data represent mean \pm SEM of four replicate determinations. The results are representative of at least two independent experiments.

Title: ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS

Authors: Carmen J. Narvaez, Thomas Waterfall, JoEllen Welsh

Key Words: *breast cancer cells, apoptosis, vitamin D resistance, mitochondria, cytochrome c*

Cell Lines: *MCF-7, MCF-7^{D3Res}*

ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS

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Introduction 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃), the active form of vitamin D₃, acts through the nuclear vitamin D receptor (VDR) and is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo*. Our lab has shown that 1,25-(OH)₂D₃ induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, and DNA fragmentation) in MCF-7 breast cancer cells (1). The precise mechanism of how 1,25-(OH)₂D₃ and its nuclear receptor, the VDR, mediate apoptosis is poorly understood.

We examined the 1,25-(OH)₂D₃ signaling pathway downstream of the VDR in order to identify specific intracellular events involved in 1,25-(OH)₂D₃ mediated apoptosis and to characterize events which are blocked in MCF-7^{D3Res} cells (a vitamin D₃-resistant variant)(2). In particular, the effects of 1,25-(OH)₂D₃ mediated apoptosis on mitochondrial function and caspase activity were studied and compared to the effects of TNF α . TNF α was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by means of a well-defined pathway triggered by TNFR1, a cell surface death receptor whose signaling results in caspase activation and disruption of mitochondrial function (3). Caspases are a family of evolutionarily conserved cysteine proteases that become activated upon proteolytic cleavage, and are responsible for cell disassembly. Mitochondria play a central role in controlling cell death. Translocation of Bax from cytosol to mitochondria, release of cytochrome c, and activation of caspases may initiate disruption of mitochondrial function (4,5). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspases and nuclease activators released from mitochondria leading to the ultimate destruction of the cell.

While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase dependent pathways. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to determine the involvement of caspase-dependent proteolysis in 1,25-(OH)₂D₃ mediated apoptosis.

Results and Discussion Disruption of mitochondrial function is one of the primary events that occur during apoptosis. Translocation of Bax to the mitochondrial outer membrane has been implicated in induction of apoptosis. Bax

redistribution to mitochondria occurs in the presence of both 1,25-(OH)₂D₃ and TNF α in MCF-7 cells (Figure 1). Not only was Bax translocated to mitochondria, but it was also cleaved from 21 kDa to 18 kDa. This observation is consistent with other reports of Bax cleavage during drug-induced apoptosis (6). Bax translocation to mitochondria and apoptosis in response to TNF α can be triggered in MCF-7^{D3Res} cells indicating that Bax functions appropriately during apoptosis induced by agents other than 1,25-(OH)₂D₃ (Figure 1).

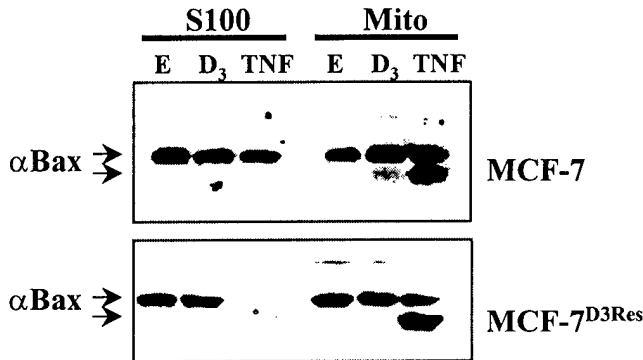


Figure 1 Subcellular distribution of Bax in MCF-7 and MCF-7^{D3Res} cells after treatment with 1,25-D₃ or TNF α .

Translocation of Bax to mitochondria is associated with subsequent release of cytochrome c, events that are considered to be commitment points for activating apoptosis. Cytochrome c normally resides within the intermembrane space of live cells. Cytochrome c is not detected in the cytosolic fraction of vehicle treated control cells. However, 1,25-(OH)₂D₃ induces redistribution of cytochrome c from mitochondria to cytosol as early as 48 hrs in MCF-7 cells, before

any morphological apoptotic events are detected. By contrast, TNF α , but not 1,25-(OH)₂D₃, induces release of cytochrome c in MCF-7^{D3Res} cells.

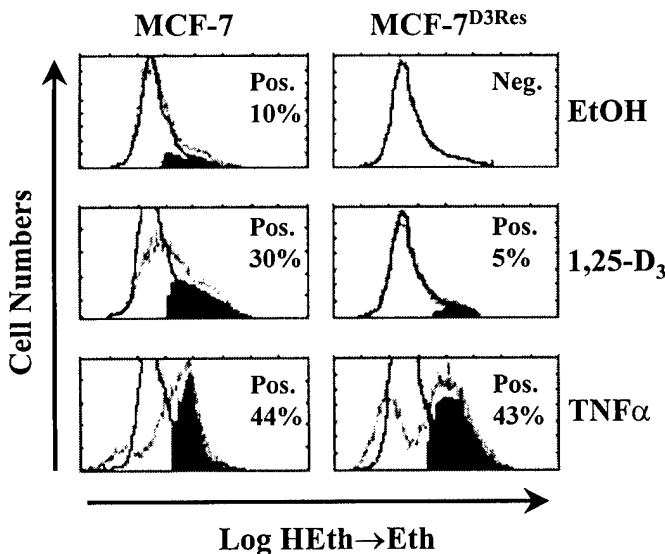


Figure 2 ROS production in MCF-7 or MCF-7^{D3Res} cells after treatment with 1,25-D₃ or TNF α .

Long term exclusion of cytochrome c from the electron transport chain can lead to impairment of proton flow, and generation of reactive oxygen species (ROS) due to incomplete reduction of molecular oxygen (7). Hence, mitochondrial generation of ROS in response to apoptotic stimuli was examined. By using flow cytometric techniques, production of superoxide anion was assessed by the degree of oxidation of hydroethidine to ethidium, a DNA stain that fluoresces red upon DNA intercalation.

MCF-7 cells, but not MCF-7^{D3Res}, produced ROS in the presence of 1,25-(OH)₂D₃, whereas TNF α induced ROS production in both cell lines (Figure 2).

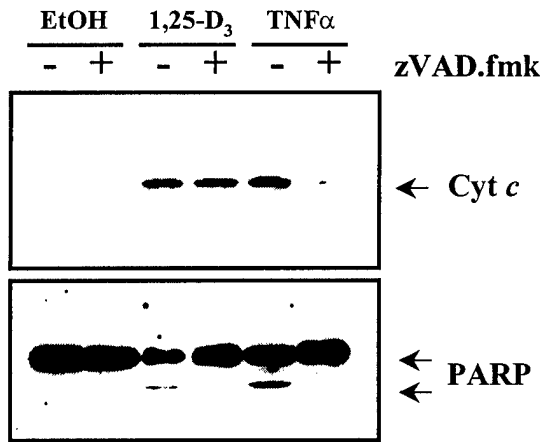


Figure 3 Expression of cytochrome c and PARP in MCF-7 cells (in the presence or absence of zVAD.fmk).

Cytochrome c release into the cytosol triggers caspase activity downstream of mitochondria. In order to determine the involvement of caspase-dependent proteolysis in 1,25-(OH)₂D₃ mediated apoptosis, we used a broad-spectrum cell permeable inhibitor (zVAD.fmk) in MCF-7 cells. Proteolytic activity associated with caspases was analyzed by three different methods. First, caspase activity was detected by immunoblot analysis of the cleavage of an endogenous caspase substrate, poly (ADP-ribose) polymerase (PARP). PARP was cleaved in the presence of both 1,25-(OH)₂D₃ and

TNF α . The cleavage was blocked by zVAD.fmk (Figure 3). The second and third methods utilized flow cytometry to analyze phosphatidylserine (PS) exposure and DNA fragmentation, respectively, which others have shown can be provoked by caspases (8). Annexin V-FITC is a convenient probe for monitoring changes in the distribution of PS in the plasma membrane during apoptosis. Both 1,25-(OH)₂D₃ and TNF α induced PS exposure in MCF-7 cells, which was blocked by zVAD.fmk treatment. DNA fragmentation was assessed by incorporation of bromodeoxyuridine by terminal transferase and detection by anti-bromodeoxyuridine antibody conjugated to FITC. Both 1,25-(OH)₂D₃ and TNF α induced DNA fragmentation, which was completely blocked by zVAD.fmk. However, TNF α , but not 1,25-(OH)₂D₃, induced DEVDase (caspase-3/7) cleavage activity in MCF-7 cells. This observation indicates that other, or as yet unidentified, effector caspases may be responsible for 1,25-(OH)₂D₃ mediated PARP cleavage, PS exposure, or DNA fragmentation.

Since zVAD.fmk blocked caspase activity downstream of mitochondria, we examined the effects of the caspase inhibitor on cytochrome c release and mitochondrial activity. The caspase inhibitor had no effect on 1,25-(OH)₂D₃ induced cytochrome c release (Figure 3), decrease in mitochondrial membrane potential, or ROS production. However, caspase inhibitor was able to block all mitochondrial events in response to TNF α . This demonstrates that TNF α induced cell death occurs by a caspase-dependent mechanism consistent with a role for caspase-8 in triggering cytochrome c release.

Since zVAD.fmk was unable to block cytochrome c release and mitochondrial dysfunction in response to 1,25-(OH)₂D₃, we determined the effect of caspase inhibitor on cell death and clonogenic potential. Both zVAD.fmk and zDEVD.fmk

caspase inhibitors protected MCF-7 from TNF α cell death with zVAD.fmk exhibiting the greatest response. However, neither caspase inhibitor could protect MCF-7 cells from 1,25-(OH) $_2$ D $_3$ mediated apoptosis since the reduction in cell number was not blocked by these inhibitors. In addition, MCF-7 cells treated with 1,25-(OH) $_2$ D $_3$ lost their clonogenic potential even when they were treated in the presence of zVAD.fmk. This suggests that the activation of caspases by 1,25-(OH) $_2$ D $_3$ occurs subsequent to the events that commits the cells to die.

Conclusion This study demonstrates for the first time that 1,25-(OH) $_2$ D $_3$ induces apoptosis in MCF-7 cells by disrupting mitochondrial function, which is accomplished by translocation of Bax to mitochondria, release of cytochrome *c*, production of ROS, and decrease in mitochondrial membrane potential. This is the first data that demonstrates that 1,25-(OH) $_2$ D $_3$ signaling on mitochondria does not require caspase activation, since broad-spectrum caspase inhibitor zVAD.fmk was unable to block these mitochondrial events. The failure of 1,25-(OH) $_2$ D $_3$ to disrupt mitochondrial function in MCF-7^{D $_3$ Res} cells suggests that 1,25-(OH) $_2$ D $_3$ signaling on mitochondria is a complex event requiring more than a functional VDR. Events upstream of Bax translocation to mitochondria in response to 1,25-(OH) $_2$ D $_3$ are abrogated in the vitamin D $_3$ -resistant cells, and contributed to resistance to 1,25-(OH) $_2$ D $_3$ mediated apoptosis. Caspases act solely as executioners by facilitating 1,25-(OH) $_2$ D $_3$ mediated apoptosis, but caspase activation is not required for induction of cell death by 1,25-(OH) $_2$ D $_3$ in MCF-7 cells. Although caspase inhibitor blocked all the biochemical changes associated with caspase activation occurring following perturbation of mitochondria and loss of cytochrome *c*, the commitment of MCF-7 cells to 1,25-(OH) $_2$ D $_3$ mediated apoptosis is caspase independent.

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Abstract promoted to a 10 min presentation at the **Eleventh Workshop on Vitamin D** in Nashville, TN, May 27-June 1, 2000.

ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS. CJ Narvaez and J Welsh Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556

Vitamin D compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D₃ (1,25-D₃), the active form of vitamin D₃, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-D₃ mediated apoptosis in MCF-7 breast cancer cells *in vitro*. Treatment of MCF-7 cells with 1,25-D₃ induced DNA fragmentation, external display of phosphatidylserine (PS), and cleavage of the caspase substrate, poly (ADP-ribose) polymerase (PARP). Mitochondrial events associated with 1,25-D₃ mediated apoptosis included translocation of Bax, disruption of mitochondrial membrane potential, generation of reactive oxygen species (ROS), and release of cytochrome *c*. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (z-Val-Ala-Asp-fluoromethylketone, zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D₃ mediated apoptosis. The effect of 1,25-D₃ on MCF-7 cells was compared to that of tumor necrosis factor α (TNF α), which induces apoptosis via a caspase-dependent pathway. zVAD.fmk prevented 1,25-D₃ mediated PARP cleavage, PS exposure, and DNA fragmentation. In contrast, zVAD.fmk did not prevent 1,25-D₃ mediated redistribution of Bax from cytosol to mitochondria, loss of mitochondrial membrane potential, generation of ROS, or release of cytochrome *c*. In addition, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-D₃ treatment of a MCF-7 cell clone selected for resistance to 1,25-D₃ mediated apoptosis. Most significantly, zVAD.fmk did not protect MCF-7 cells from 1,25-D₃ induced apoptosis, indicating that the commitment of MCF-7 cells to 1,25-D₃ mediated cell death is caspase independent. The sensitivity of 1,25-D₃ induced apoptosis to caspase inhibition was found to be distinct from that of TNF α induced apoptosis, which was completely prevented by zVAD.fmk. In summary, these data clearly indicate that although caspase inhibition can block some of the late stages of 1,25-D₃ mediated apoptosis in MCF-7 cells; the commitment to cell death is caspase independent. Our data implicate Bax distribution and mitochondrial disruption as critical caspase independent events in 1,25-D₃ mediated apoptosis of breast cancer cells. *Supported by NIH CA69700 & DAMD17-97-1-7183.*

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