[CANCER RESEARCH 63, 2483–2491, May 15, 2003]

RRR-α-Tocopheryl Succinate-induced Apoptosis of Human Breast Cancer Cells Involves Bax Translocation to Mitochondria[1]

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ABSTRACT

Previous studies have identified RRR-α-tocopheryl succinate (vitamin E succinate, VES) as a potential chemotherapeutic agent. VES induces human breast cancer cells to undergo apoptosis in a concentration- and time-dependent manner by restoring transforming growth factor β (TGF-β) and Fas (CD95) apoptotic signaling pathways, that contribute to the activation of c-Jun NH_2-terminal kinase (JNK)-mediated apoptosis. The objective of these studies was to clarify biochemical events involved in VES-induced apoptosis. Data show that VES-induced apoptosis involves: (*a*) translocation of Bax from the cytosol to the mitochondria and cytochrome *c* release from the mitochondria to the cytosol as determined by Western immunoblot analyses of mitochondrial- and cytosolic-enriched cellular fractions; (*b*) increased permeabilization of mitochondrial membranes as determined by confocal and fluorescence-activated cell sorting analyses of loss of a mitochondrial selective fluorescent dye; (*c*) processing of caspase-9 and -3 but not caspase-8 to active forms and cleavage of poly(ADP-ribose) polymerase (PARP) as determined by Western immunoblot analyses using antibodies capable of detecting both proenzyme and processed enzyme forms or the intact or cleaved forms of PARP. Transient transfection of cells with antisense oligonucleotides to Bax or transient overexpression of Bcl-2 prevented VES-induced mitochondrial permeability transition and apoptosis. The use of cell-permeable caspase inhibitors indicated that caspase-9 and -3 but not caspase-8 are involved in VES-induced apoptosis. JNK inhibitor II blocked VES-induced Bax conformational change, indicating a role for JNK in Bax translocation to the mitochondria. Taken together, these data suggest that the activation of JNK, translocation of Bax to the mitochondria, increased mitochondrial membrane permeability with release of cytochrome *c*, and activation of caspase-9 and -3 are critical events in VES-induced apoptosis of human MDA-MB-435 breast cancer cells.

INTRODUCTION

VES[3] is a potent growth inhibitor of various cancer cell types *in vitro* and *in vivo* (1–9). VES, which is an ester derivative of the naturally occurring RRR-form of α-tocopherol, has been demonstrated to have antitumor activity in animal xenograft and allograft models when administered i.p. (3, 6, 8, 9), suggesting a possible therapeutic potential. VES has also been shown to have inhibitory effects on carcinogen [benzo(*a*)pyrene]-induced forestomach carcinogenesis in mice, suggesting potential as an anticarcinogenic agent (7).

Previous *in vitro* studies have demonstrated that VES-induced cancer cell growth inhibition is produced by concentration- and time-dependent inhibition of DNA synthesis, induction of cellular differentiation, and induction of apoptosis (reviewed in Refs. 5 and 10–12). VES treatment of human breast cancer cells has multiple effects that have been demonstrated to be necessary, at least in part, to VES-induced apoptosis and include: activation of latent TGF-β and enhancement of cell surface membrane expression of TGF-β type II receptor (TGF-β RII) leading to increased cellular responsiveness to TGF-β1-induced apoptosis; translocation of cytosolic Fas/CD95 to the cell-surface membrane and restoration of Fas signaling of cell death; and prolonged activation of the ERK and JNK but not of p38 mitogen-activated protein kinases (12, 13–19). VES is noteworthy not only for its induction of DNA-synthesis arrest and prodifferentiation and proapoptotic effects on tumor cells but also for its lack of toxicity toward normal cells and tissues (1, 4, 5, 9).

Studies indicate that mitochondria may serve as death signal integrators in apoptosis triggered by a variety of apoptotic stimuli (20–22). Perturbation of mitochondrial membranes resulting in the release of cytochrome *c* appears to be a critical event (reviewed in Ref. 22). In this regard, Bax and Bak, two proapoptotic members of the Bcl-2 family, have been shown to play key roles in initiating mitochondrial dysfunction (23). Mitochondrial dysfunction leads to the release of cytochrome *c*, which forms a multiprotein/molecular complex with Apaf-1, pro-caspase-9, and dATP leading to the activation of caspase-9 and the induction of downstream effector (cell disassembly) caspases, such as caspases-3, -6, and -7, followed by proteolytic cleavage of a wide range of substrates that are responsible for the morphological and biochemical changes that are the hallmarks of apoptosis (24–27). Bax and Bcl-2 are involved in this apoptotic cascade by either promoting (Bax) or preventing (Bcl-2) mitochondrial-dependent apoptosis (26, 28–32). Bcl-2 is found associated with the nuclear membrane, endoplasmic reticulum, and the outer mitochondrial membrane, and has been shown to regulate cell survival, preventing MPT and apoptosis (26). In contrast, Bax acts as a dominant-negative inhibitor of Bcl-2, promoting MPT and apoptosis (31). There is evidence that Bax is translocated from the cytosol to the mitochondria, because studies show that Bax is in the cytosol of nonapoptotic cells and is associated with the mitochondria of apoptotic cells (23, 29, 30, 33). In mitochondrial-mediated apoptosis, Bax undergoes a conformational change leading to exposure of its NH_2 and COOH termini, which is thought to be required for the translocation of cytosolic Bax to the mitochondria (30, 34, 35).

This report characterizes for the first time the effects of VES treatment on the intrinsic death-signaling pathways in human breast cancer cells. Studies demonstrate that VES-induced apoptosis involves activation of JNK and induction of Bax conformational change, translocation of cytosolic Bax to the mitochondria followed by induction of MPT, release of cytochrome *c* from mitochondria to the cytosol, activation of caspases-9 and -3, and cleavage of PARP. MPT and the resulting cascade of events resulting in apoptosis can be prevented, at least in part, by either blockage of Bax or ectopic overexpression of Bcl-2. These findings provide additional insight into the proapoptotic signaling events critical to VES-induced apoptosis of human epithelial cancer cells.

Received 7/12/02; accepted 3/12/03.

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[1] Supported by Public Health Service Grant CA59739 awarded by the National Cancer Institute, by the Foundation for Research, and by the National Institute of Environmental Health Sciences Center Grant ES 07784.

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[3] The abbreviations used are: VES, vitamin E succinate (RRR-α-tocopheryl succinate); Cox IV, cytochrome *c* oxidase subunit IV; DAPI, 4′,6-diamidino-2-phenylindole; DAXX, death-domain-associated protein; ECL, enhanced chemiluminescence; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-jun NH_2-terminal kinase; MPT, mitochondrial permeability transition; PARP, poly-(ADP-ribose) polymerase; VEH, vehicle; TGF-β, transforming growth factor β; ERK, extracellular signal-regulated kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

MATERIALS AND METHODS

Cell Culture and VES Treatment. MDA-MB-435 human breast cancer cells (originally obtained from Dr. Janet E. Price, Department of Cell Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX) are an estrogen-receptor-negative/estrogen-nonresponsive epithelial cell line isolated from the pleural effusions of a human with breast cancer (36). MCF-7 cell line (originally provided by Dr. Suzanne Fuqua, Baylor College of Medicine, Houston, TX) is an estrogen-receptor-positive/estrogen-responsive human breast cancer cell line (19). Human MCF-10A breast epithelial cells (purchased from American Type Culture Collection, Manassas, VA) are an immortalized, nontumorigenic cell line (37). Cells were maintained and routinely examined to verify the absence of *Mycoplasma* contamination as described previously (19).

For experiments, the percentage of fetal bovine serum (HyClone Laboratories, Logan, UT) was reduced to 2%, and exponentially growing cells were plated at 3×10^5 cells/well in 6-well plates for the measurement of MPT, at 1.6×10^6 cells per T-25 flasks for Western immunoblotting assays, or at 1.5×10^5 cells/well in 12-well plates for apoptosis analyses. Cells were allowed to attach overnight, then were incubated with VES [20 μg/ml in a final concentration of 0.2% (vol/vol) ethanol] or VEH control (amounts of succinic acid and ethanol equivalent to amounts in VES-treatment). VES (RRR-α-tocopheryl succinate) and sodium succinate were purchased from Sigma (St. Louis, MO).

Measurement of MPT. MPT was characterized by the retention of the cell-permeable mitochondrial-selective dye Mito Tracker Red CMX (Molecular Probes, Inc., Eugene, OR) as determined by confocal microscopy or FACS. Nuclei were visualized using the fluorescent DNA-binding dye DAPI (Boehringer Mannheim, Indianapolis, IN).

Briefly, MDA-MB-435 or MCF-7 cells at 3×10^5 cells/well were cultured overnight in 6-well plates for FACS analyses or in 6-well plates containing poly-L-lysine (0.01% solution; Sigma)-coated coverslips for confocal microscopic analyses. Cells were treated with 20 μg/ml VES for 6, 12, and 24 h or with VEH for 24 h. Cells were stained for 30 min with Mito Tracker Red CMX dye at 20 ng/ml final concentration in medium prepared from a 20-μg/ml Mito Tracker Red CMX-dye stock solution solubilized in DMSO. For FACS analyses, attached cells were washed two times with PBS and then were resuspended in PBS. Samples were analyzed for cell number and for the mitochondrial selective dye (FL2 488Ex/585Em) using a Becton Dickinson flow cytometer. For confocal analyses, the Mito Tracker Red CMX dye-stained cells were washed twice with PBS, followed by DAPI staining (2 μg/ml of DAPI in 100% methanol) for 20 min at 37°C. Cells attached to the coverslips were analyzed using a confocal microscope (Leica TCS 4D; Heidelburg, Germany) using 568 Ex/599Em for the visualization of the Mito Tracker Red CMX stain and 356 Ex/518Em for the visualization of DAPI.

Apoptosis Assay (Morphological Analyses of DAPI-stained Cells). Because previous studies (15, 16) have characterized VES-induced apoptosis in human breast cancer cells by the use of detection of DNA fragmentation by gel electrophoresis and terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL), detection of hypodiploid cells by flow cytometry, and analyses of nuclear morphology using DNA dye DAPI, we limited apoptosis assessment in these studies to the last-named technique. Assessment of apoptosis based on nuclear morphology using DAPI has been described previously (19). Briefly, cells in which the nucleus contained clearly condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Apoptotic data are reported as percentage apoptosis, obtained by determining the numbers of apoptotic cells *versus* the total number of cells. For each sample, a minimum of 3 counts involving a minimum of 100–200 cells/count were scored. Apoptotic data are presented as the mean ± SD for three independently performed experiments. Reagents for morphological analyses of apoptosis were purchased from Boehringer Mannheim.

Cell Fractionation. Cytosolic and mitochondria-enriched fractions were isolated following the methods of Pastorino *et al.* (31), and whole cell protein extracts were prepared as we have described previously (14). For cytosolic and mitochondrial preparations, VES- or VEH-treated MDA-MB-435 cells at 5×10^6 cells/T-75 flask were washed twice with cold PBS and were resuspended in 200 μl of extraction buffer [20 mM HEPES-KCl (pH 7.4), 10 mM KCl, 250 mM sucrose, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DDT, 10 mM phenylmethylsulfonylfluoride, 10 μM leupeptin, and 10 μM aprotinin] and incubated for 10 min on ice. Next, cells were homogenized by 10 passages through a 26-gauge needle. Homogenates were centrifuged at $1,000 \times g$ for 5 min to remove unbroken cells and nuclei. The supernatant fraction was centrifuged at $12,000 \times g$ for 30 min at 4°C. The resulting supernatant contained the cytosolic fraction, and the pellet contained the enriched mitochondria fraction. Pellets containing mitochondria were treated with lysis buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM DTT, 2 mM sodium orthovanadate, and 10 μg/ml phenylmethylsulfonyl fluoride) and were incubated on ice for 20 min; then the lysate was centrifuged at $15,000 \times g$ for 5 min at 4°C, and the resulting supernatant was kept as the solubilized enriched mitochondria fraction. Cell fractions were assayed for protein concentration using the Bio-Rad Dye Binding protein assay (Bio-Rad Laboratories, Hercules, CA); then equivalent amounts of protein were analyzed by Western/ECL analyses. Antibodies to the mitochondrial protein, Cox IV (Molecular Probes) and the cytosolic protein actin (sc-1616; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used in Western immunoblotting procedures to assess the success of fractionation procedure for degree of enrichment of mitochondrial and cytosolic fractions.

Western Immunoblot Analyses. Western immunoblot analyses were used to detect Bax (sc-526; Santa Cruz Biotechnology, Inc.); active, conformationally changed Bax (B-8429, 6A7 monoclonal antibody; Sigma); Bak (PharMingen, San Diego, CA); phosphorylated c-Jun (sc-822, p-Jun; Santa Cruz Biotechnology, Inc.); cytochrome *c* (sc-7159; Santa Cruz); pro- and cleaved caspase 3 (sc-7148; Santa Cruz); pro-and cleaved caspase 8 (Cell Signaling Technology, Beverly, MA); pro- and cleaved caspase 9 (Cell Signaling Technology); PARP (sc-7150; Santa Cruz); Bcl-2 (M0887; DAKO Corporation, Carpinteria, CA); and GAPDH (made in-house). GAPDH was used for monitoring lane loads as described previously (19). Briefly, 50–100 μg protein were loaded per lane, proteins were separated by using 15% SDS-PAGE under reducing conditions and were electro-blotted onto a nitrocellulose membrane. Immunoblotting was performed using primary rabbit antibodies and peroxidase-conjugated goat antirabbit as the secondary antibody (Jackson Immunoresearch Laboratory, West Grove, PA), followed by detection with ECL (Pierce, Rockford, IL). Quantitation of band intensity was performed using Scion Image Software (Scion Corporation, Frederick, MD).

Detection of Active Conformationally Changed Bax. Procedures described by Yamaguchi *et al.* (35) were followed to detect Bax conformational change. Briefly, cells were lysed with CHAPS lysis buffer [10 mM HEPES (pH 7.5), 150 mM NaCl, 1% CHAPS] in the presence of protease inhibitors for 20 min. A total of 500 μg of protein in 0.5 ml of CHAPS lysis buffer was incubated overnight at 4°C with 2 μg of anti-Bax 6A7 monoclonal antibody. Next, 20 μl of protein G-agarose beads were added to the reaction and were incubated for 2 h at 4°C and then were washed three times in CHAPS lysis buffer. Beads were boiled in Laemmli buffer, and immunoprecipitated conformationally changed Bax protein was analyzed by SDS-PAGE and detected by Western immunoblotting using anti-Bax polyclonal antibody (sc526).

Caspase Inhibitors. MDA-MB-435 and MCF-10A cells at 1.5×10^5 cells/well in 12-well plates were pretreated with the following caspase inhibitors each at 2 μM (all purchased from BioVision Research Products, Palo Alto, CA): caspase 3 inhibitor Z-DEVD-FMK, caspase 9 inhibitor Z-LEHD-FMK, or caspase 8 inhibitor Z-IETD-FMK or with VEH (DMSO at 0.1% concentration) for 2 h followed by treatment with 20 μg/ml of VES for 2 days for MDA-MB-435 cells or 100 ng/ml agonistic anti-Fas antibody (antihuman Fas mouse monoclonal IgM antibody; clone CH-11; Upstate Biotechnology, Lake Placid, NY) for 1 day for MCF-10A cells. The percentage of cells undergoing apoptosis in caspase inhibitor-treated *versus* control cultures was determined by analyses of DAPI-stained cells.

JNK Inhibitor. MDA-MB-435 cells were pretreated with 40 nM of JNK inhibitor II (Calbiochem-Novabiochem Corp. San Diego, CA) or DMSO control for 2 h, followed by treatment with VES for 12 h. Cell lysates were analyzed by Western immunoblotting to verify the inhibition of JNK activity as determined by the inhibition of JNK phosphorylation of c-Jun and to verify the role for JNK in Bax involvement in MPT and apoptosis induced by VES through the inhibition of Bax conformational change and through reduced mitochondrial cytochrome *c* release and reduced PARP cleavage.

Antisense Blockage Assay for Assessing Bax Involvement. MDA-MB-435 cells were plated at 3×10^5 cells/well in 6-well plates for FACS analyses of mitochondria permeability, at 1.5×10^5 cells/well in 12-well plates for

apoptotic analyses, and at 1.6×10^6/T-25 flask for Western immunoblot analyses. Cells were permitted to attach overnight and then were transiently transfected with antisense (CTG CTC CCC GGA CCC GTC CAT) or sense (ATG GAC GGG TCC GGG GAG CAG) oligomers to Bax using LipofectAMINE, following the company's instructions (LipofectAMINE Reagent; Life Technologies, Inc., Rockville, MD). Briefly, cells were washed two times with serum-free medium (MEM) and were incubated with 0.5 ml of serum-free medium (MEM-Option) containing 100 μl of DNA/LipofectAMINE complex for apoptosis studies, 1 ml of serum-free media (MEM-Option) containing 200 μl of DNA LipofectAMINE complex for MPT analyses, and 3 ml of serum-free medium (MEM-Option) containing 800 μl of DNA/LipofectAMINE complex for Western immunoblot. DNA/LipofectAMINE complex is made by mixing 2 μg of DNA/50 μl serum-free medium with 4 μl of LipofectAMINE Reagent/50 μl serum-free medium, followed by a 45-min incubation at room temperature. After a 3–4-h incubation with the DNA/LipofectAMINE complex, the transfected cells were washed with culture medium and were treated with 20 μg/ml VES for 2 days before analyses for apoptosis by DAPI staining or for 12 h before MPT analyses by FACS or cytochrome *c*, Bax, caspase 3, and PARP protein expression by Western immunoblot analyses.

Overexpression of Wild-Type Bcl-2. MDA-MB-435 cells were plated as described above before being transiently transfected with either a wild-type Bcl-2-expressing plasmid (gift of Dr. David Hockenbery, Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 38) or with a vector control. Transfections were performed as described above for Bax transfections with the following modification. DNA/LipofectAMINE/Plus reagent complex was made by first mixing 0.7 μg of DNA/50 μl of serum-free medium with 5 μl of Plus reagent followed by 15-min incubation, and then mixing the DNA/Plus reagent with 2 μl of LipofectAMINE reagent/50 μl of serum-free medium followed by 15 min-incubation. After 3–4 h transfection, the cells were incubated with culture medium overnight [using this technique, we routinely obtain ~50% transfected cells (12)]. Next, the transfected cells were treated with 20 μg/ml VES for 2 days before analyses for apoptosis or for 12 h before MPT or Western immunoblot analyses.

RESULTS

VES Induces MPT. VES treatment of MDA-MB-435 human breast cancer cells induces a time-dependent loss of Mito Tracker Red CMX fluorescence, detected by confocal microscopy or FACS analyses (Fig. 1, *A* and *B*), which is correlated with a loss of mitochondria membrane potential $\Delta\psi_m$, because mitochondrial accumulation and retention of Mito Tracker Red CMX is dependent on mitochondria membrane potential $\Delta\psi_m$ (39). After treatment with 20 μg/ml VES for 6, 12, or 24 h, the intensity of the Mito Tracker Red CMX dye was reduced in comparison with the intensity after the 24-h VEH-control treatment of cells, as determined by confocal microscopy (Fig. 1*A*). The nuclei of treated cells were intact as determined by analyses of DAPI-stained nuclei (Fig. 1*A*). FACS (Fig. 1*B*) analyses of cells containing mitochondria dye, Mito Tracker Red CMX, showed a loss of the dye at 12 and 24 h after treatment with VES. Taken together, the confocal and FACS analyses show that MPT occurs in VES-treated MDA-MB-435 cells. To determine whether VES-induced MPT was observed in other human breast cancer cells, human estrogen receptor-positive/estrogen-responsive MCF-7 cells were treated in an identical fashion as the MDA-MB-435 cells and were analyzed for MPT. The intensity of the Mito Tracker Red CMX dye was reduced at 6 and 12 h after treatment with 20 μg/ml VES (Fig. 1*C*). FACS analyses of MCF-7 cells containing Mito Tracker Red CMX dye showed a loss of dye after 12 h of treatment with 20 μg/ml VES (Fig. 1*D*).

VES Induces Translocation of Bax from the Cytosol to the Mitochondria, Resulting in a Release of Cytochrome *c* from the Mitochondria to the Cytosol. One consequence of MPT is the release of cytochrome *c* into the cytosol (40, 41). Western immunoblotting analyses of mitochondrial-enriched cell fractions, obtained after cells were treated with 20 μg/ml of VES for 3, 6, 12, or 24 h,

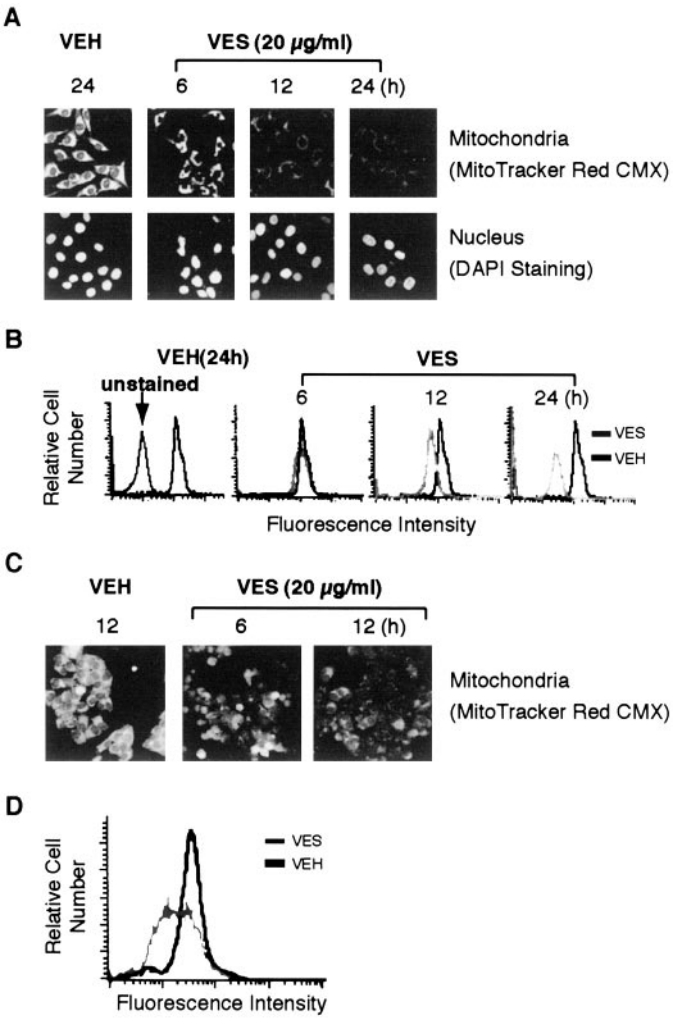

Fig. 1. Effects of VES on loss of MPT as measured by loss of retention of a cell-permeable, mitochondrial-selective red fluorescent dye by confocal microscopy or by FACS. MDA-MB-435 (*A* and *B*) and MCF-7 (*C* and *D*) cells were treated with VES at 20 μg/ml for various time intervals as indicated before staining with Mito Tracker Red CMX dye (*A, top panel*, and *B–D*). MDA-MB-435 cells were counterstained with DAPI for the detection of DNA (*A, bottom panel*). Data for *A–D* are representative of a minimum of two independent experiments each.

showed a time-dependent increase in Bax protein in the mitochondrial-enriched fraction and a decrease in mitochondrial-associated cytochrome *c* protein levels (Fig. 2*A*). Densitometric analyses showed mitochondrial-associated Bax protein levels to increase 1.3-, 1.9-, 2.2-, and 1.5-fold in comparison with VEH-treated cells after 3, 6, 12, and 24 h, respectively. In contrast, the same mitochondrial-enriched cell fractions exhibited 0.9-, 1.2-, 2.5-, and 9-fold decreases in cytochrome *c* in comparison with VEH-treated cells after 3-, 6-, 12-, and 24-h VES treatment, respectively. Comparisons of Bax and cytochrome *c* protein levels in mitochondrial-enriched and cytosolic-enriched fractions from MDA-MB-435 cells treated with 20 μg/ml VES or VEH control for 12 h are shown in Fig. 2*B*. The mitochondrial-enriched fraction from VES-treated cells showed Bax levels to increase and cytochrome *c* levels to decrease (Fig. 2*B*); whereas, the cytosolic-enriched fraction from VES-treated cells showed Bax levels to decrease and cytochrome *c* levels to increase (Fig. 2*B*). Mitochondrial- and cytosolic- enriched fractions were assayed with antibodies to the mitochondrial protein Cox IV, as a control for the quality of the fractionation procedure (35, 42), and were assayed with antibodies to actin, as a marker for cytosolic fraction. Cox IV protein was detected in the mitochondrial-enriched fraction but not in the cytosolic-

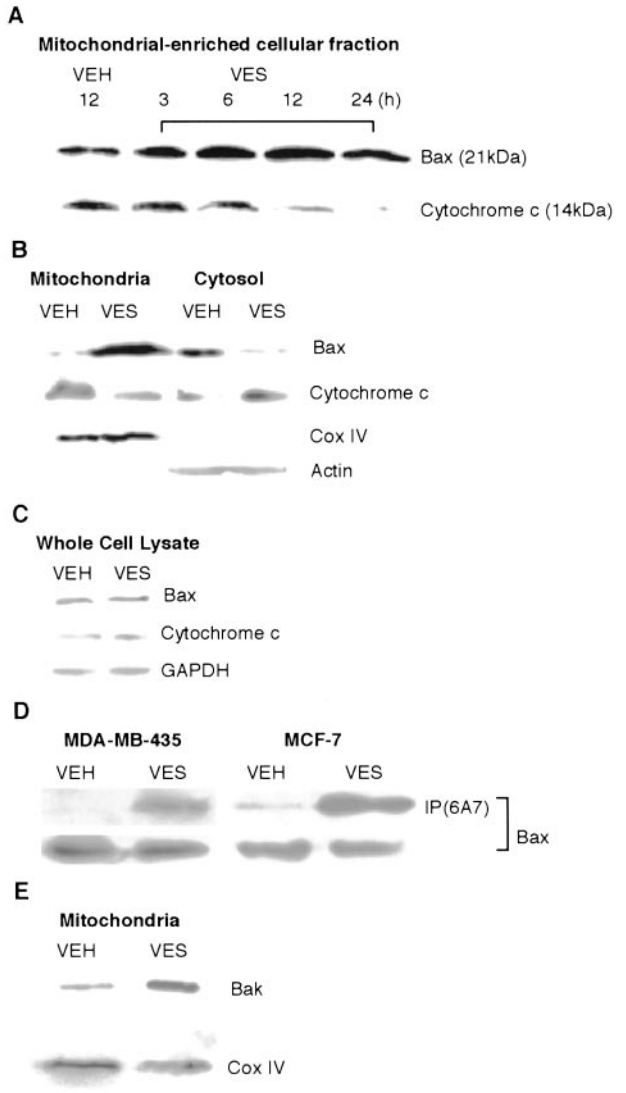

Fig. 2. VES (20 μg/ml) treatment of MDA-MB-435 cells induces the translocation of Bax to the mitochondria and the release of cytochrome *c* into the cytosol. Western immunoblot analyses of Bax and cytochrome *c* protein expression in mitochondrial-enriched fractions after treatment of MDA-MB-435 cells with VES for 3, 6, 12, and 24 h or VEH for 12 h (*A*). Western immunoblot analyses of Bax and cytochrome *c* expression in cytosolic- and mitochondrial-enriched fractions after treatment of MDA-MB-435 cells with VES or VEH for 12 h (*B*). Mitochondrial protein Cox IV and cytosolic actin were used as controls to verify enrichment of mitochondrial and cytosolic fractions. *B*, Western immunoblot analyses of Bax and cytochrome *c* in whole cell lysates from MDA-MB-435 cells treated with VES and VEH for 12 h (*C*). MDA-MB-435 and MCF-7 cells were cultured with VES or VEH for 12 h, and Bax was analyzed. Bax was immunoprecipitated (*IP*) with a monoclonal antibody (*6A7*), which binds an epitope specific for conformationally changed Bax. The immunoprecipitated Bax (*D, top panel*) and whole cell lysates (*D, bottom panel*) were analyzed by Western immunoblotting using a polyclonal antibody to Bax that detects denatured Bax protein. Western immunoblot analyses of Bak and Cox IV levels in mitochondrial-enriched fractions after treatment of MDA-MB-435 cells with VES or VEH for 12 h (*E*). Data are representative of two independent experiments. *kDa*, M_r in thousands.

enriched fraction (Fig. 2*B, third panel*). The mitochondrial-enriched fraction contained less than 11% of the amount of actin found in the cytosolic-enriched fraction (Fig. 2*B, fourth panel*). Whole cell extracts from VES- and VEH-treated cells showed similar levels of Bax and cytochrome *c* (Fig. 2*C*).

Further support for Bax involvement in VES-induced MPT come from studies showing that Bax from MDA-MB-435 and MCF-7 cells undergoes conformational change after treatment with VES (Fig. 2*D, top panel*). Although VES causes an increase in conformationally altered Bax, the overall cellular level of Bax was similar in whole cell extracts from VEH- and VES-treated cells, which showed that VES did not cause an increase in the total cellular level of Bax (Fig. 2*D, second panel*). A comparison of Bak protein levels in mitochondrial-enriched fractions from VES- and VEH-treated cells showed that VES also induced the translocation of Bak into the mitochondria (Fig. 2*E, top panel*). Cox IV protein was monitored as a control for the quality and quantity of the fractionation procedure (Fig. 2*E, second panel*).

VES Induces Activation of Caspase-9 and -3 but not of Caspase-8, Followed by Cleavage of PARP. A consequence of cytochrome *c* release is the formation of a protein complex called the apoptosome composed of cytochrome *c*, Apaf-1, dATP, and several molecules of pro-caspase-9 (24, 38, 39). Activation of caspase-9 can lead to activation of "executioner" caspases (caspases-3, -6, and -7) which carry out proteolytic events leading to cellular protein and nuclear DNA breakdown (43). Among a large number of substrates that are broken down during apoptosis, PARP is recognized as a useful indicator of apoptosis (43).

Western immunoblotting was used to analyze the time-dependent cleavage of caspases-3, -8, and -9 and of PARP after VES treatment. Evidence that VES treatment resulted in the cleavage of caspase-9 is presented in Fig. 3 (*top panel*), with the appearance of a M_r 37,000 fraction of caspase-9 appearing by 3 h after VES treatment. Activated caspase-3 was detected 12 and 24 h after VES treatment as M_r 17,000/20,000 cleaved fragments (Fig. 3, *second panel*). As demonstrated by data in Fig. 3-third panel, PARP cleavage from the M_r 116,000 intact form to a M_r 84,000 cleavage product served as an additional indicator of caspase activity in MDA-MB-435 cells at 12 and 24 h after the initiation of VES treatment.

Because VES-mediated apoptosis involves restoration of Fas signaling (16, 19), the possibility that caspase-8 might be involved in VES-induced apoptosis was investigated. No evidence for VES activation of caspase-8 (absence of caspase-8 fragments) was observed (Fig. 3, *bottom panel*). MCF-10A immortalized but nontumorigenic human mammary epithelial cells, treated with agonistic anti-Fas an-

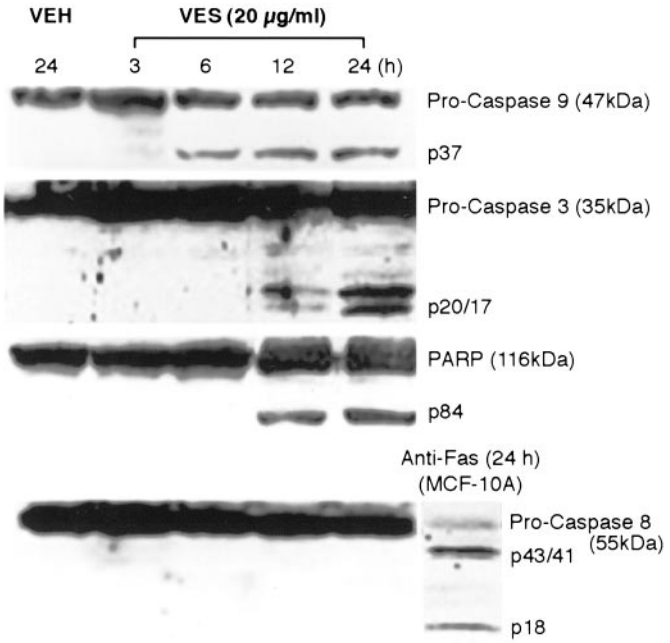

Fig. 3. Western immunoblot analyses demonstrate that VES induces cleavage of caspase-9 and -3 and of PARP but fails to induce processing of pro-caspase 8. MDA-MB 435 cells were treated with 20 μg/ml VES for 3, 6, 12, and 24 h or VEH for 24 h. *Bottom panel, inset*, a positive control for Western immunoblot detection of processed caspase-8 in which MCF-10A cells were treated with 100 ng/ml agonistic anti-Fas antibody for 24 h. Data are representative of two independent experiments. *kDa*, M_r in thousands.

tibody for 24 h, served as a positive control for caspase-8 activation (Fig. 3, *bottom panel*).

As an alternate approach to verify the involvement of caspases-3, and -9 but not caspase-8 in VES-mediated apoptosis, inhibitors of caspase-9 (Z-LEHD-FMK; Ref. 44), caspase-3 (Z-DEVD-FMK; Ref. 45), and caspase-8 (Z-IETD-FMK; Ref. 45) were used. As illustrated in Fig. 4*A*, the inhibition of caspase-9 and caspase-3 resulted in a 43% (42 ± 4 control + VES *versus* 24 ± 3 inhibitor + VES) and a 49% (45 ± 5 control + VES *versus* 23 ± 3 inhibitor + VES) blockage of VES-induced apoptosis, respectively. Further proof that caspase-8 is not involved in VES-induced apoptosis was obtained when the caspase-8 inhibitor produced no effect (Fig. 4*B, right panel*). As a positive control for the blocking ability of the caspase-8 inhibitor used in these experiments, agonistic anti-Fas antibody-triggered apoptosis of MCF-10A cells was demonstrated to be blocked by 51% (68 ± control *versus* 33 ± 3 caspase-8 inhibitor; Fig. 4*B, left panel*).

Functional Knockout of Bax Using Antisense Oligonucleotides Demonstrates Role for Bax in VES-induced Apoptosis. MDA-MB-435 cells were transiently transfected with Bax antisense and sense oligomers to further verify a role for Bax in VES-induced apoptosis. Data show that antisense oligomers to Bax block VES (20 μg/ml VES for 2 days) mediated apoptosis by 46% (30% ± 4 apoptosis in cells transfected with antisense oligomers *versus* 56% ± 5.9 apoptosis for cells transfected with sense oligomers; Fig. 5*A*).

FACS analyses of VES-treated cells loaded with mitochondrial Mito tracker red dye show increased levels of fluorescence intensity in cells transfected with antisense (“*A*”) oligomers to Bax in comparison with cells transfected with Bax sense (“*S*”) oligomers (Fig. 5*B*), which suggests that blockage of Bax retains mitochondrial membrane integrity in VES-treated cells.

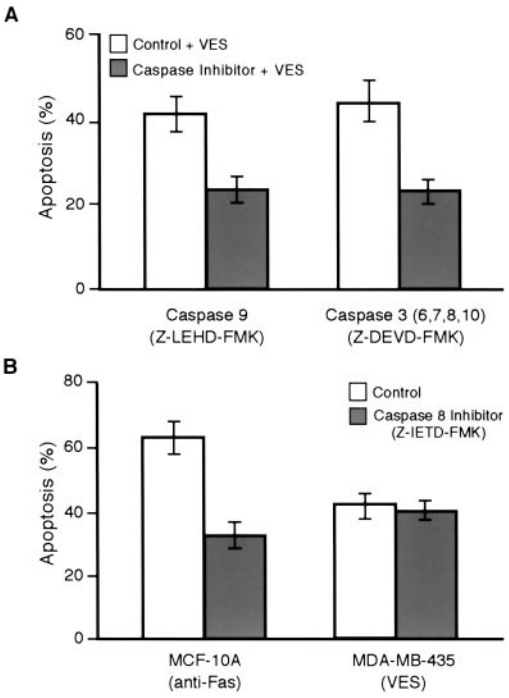

Fig. 4. Cell-permeable inhibitors to caspase-9 (*Z-LEHD-fmk*) and caspases-3, -6, -7, -8, and -10 (*Z-DEVD-fmk*) block VES-induced apoptosis (*A*); but the inhibitor of caspase-8 (*Z-IETD-fmk*) fails to block VES-induced apoptosis (*B, right panel*). MDA-MB-435 cells were pretreated with the caspase inhibitors (at 2 μM) for 2 h before treatment with 20 μg/ml VES for two days. Percentage of cells undergoing apoptosis was determined by counting apoptotic nuclei of DAPI-stained cells. *B, left panel*, ability of caspase-8 inhibitor Z-IETD-fmk to block apoptosis initiated by agonistic anti-Fas antibody in MCF-10A cells. Data are representative of two independent experiments.

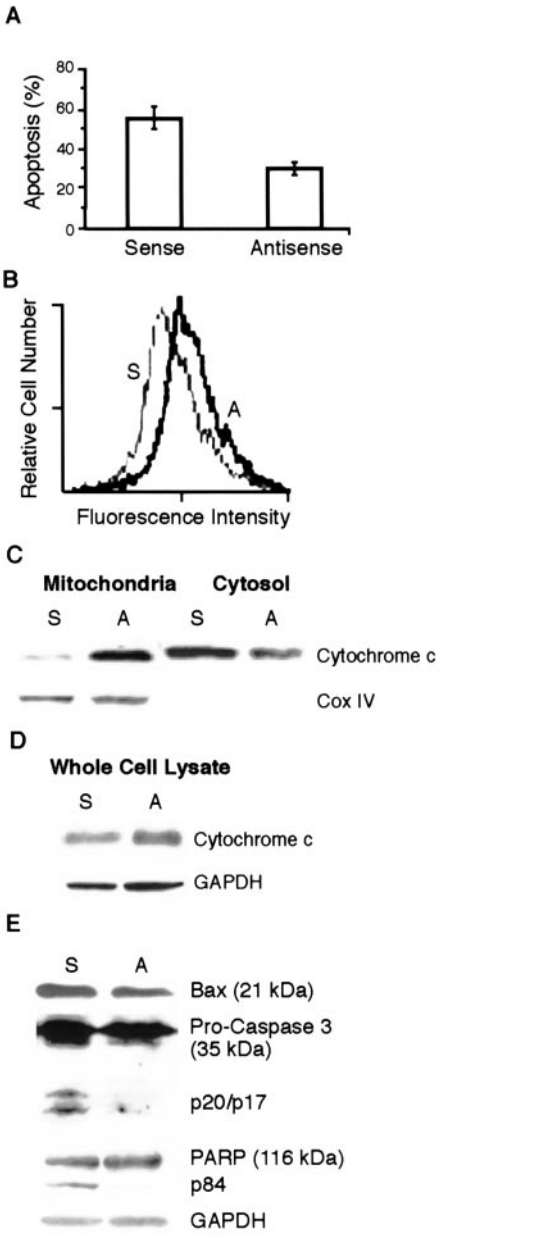

Fig. 5. Evidence that Bax plays a necessary role in VES-induced apoptosis, MPT, cytochrome *c* redistribution, downstream caspase activation, and PARP cleavage. MDA-MB-435 cells transiently transfected with Bax antisense or Bax sense oligomers were treated with 20 μg/ml VES for 2 days before analysis for apoptosis or for 12 h before FACS or Western immunoblotting analyses. *A*, ability of antisense oligomers to Bax to block VES-induced apoptosis; *B*, FACS analyses show greater retention of mitochondrial selective red fluorescent dye after VES treatment by antisense(*A*)-*treated* in comparison with sense(*S*)-treated cells; *C*, Western immunoblot analyses of cytochrome *c* expression in cytosolic- and mitochondrial-enriched fractions. Cox IV was assessed as a mitochondrial-specific marker; *D*, Western immunoblot analyses of cytochrome *c* expression in whole cell lysates. GAPDH levels were determined to verify lane loads; *E*, Western immunoblot analyses of Bax, caspase-3 cleavage, PARP cleavage, and GAPDH levels (lane load control) in whole cell lysates. All data are representative of two independent experiments. *kDa*, M_r in thousands.

VES-treated MDA-MB-435 cells, transiently transfected with antisense oligomers to Bax, showed reduced levels of cytochrome *c* in the cytosol-enriched fraction, and increased levels of cytochrome *c* in the mitochondrial-enriched fraction in comparison with cells transiently transfected with sense oligomers to Bax (Fig. 5*C*). Mitochondrial- and cytosolic-enriched fractions were verified by the presence of Cox IV protein in the mitochondrial fraction and by the absence of Cox IV protein in the cytosolic fraction (Fig. 5*C*). Cytochrome *c*

levels in whole cell extracts from VES-treated cells transiently transfected with sense and antisense oligomers showed cytochrome *c* levels to be essentially the same (Fig. 5*D*). GAPDH protein levels were used to verify lane loads (Fig. 5*D*). Western immunoblot analyses show that biochemical measures of apoptosis, namely, activation of caspase-3 (presence of M_r 17,000/20,000 fragments) and cleavage of PARP (p84 fragment), were reduced in cells transfected with Bax antisense oligomers and treated with VES in comparison with cells transfected with Bax sense oligomers and treated with VES (Fig. 5*E*). Confirmation that the Bax antisense oligonucleotide treatment reduced Bax protein expression is provided in the top panel of Fig. 5*E*. Densitometric comparisons of Bax protein expression in antisense *versus* sense oligonucleotide-treated cells show a 42% reduction. GAPDH was monitored to verify lane load equivalency (Fig. 5*E, bottom panel*).

Ectopic Expression of Bcl-2 Blocks VES-induced Apoptosis and MPT. Because pro-apoptotic effects of Bax can be blocked by Bcl-2, and because the ratio of antiapoptotic Bcl-2 and pro-apoptotic Bax in mitochondria is reported to dictate whether a cell responds to a proximal apoptotic stimulus (46), we characterized the effects of transient transfection of wild-type Bcl-2 on VES-induced apoptotic events. Transient overexpression of Bcl-2 (61% increase) in MDA-MB-435 cells blocked VES (20 μg/ml VES for 2 days)-induced apoptosis by 43% (28 $\pm$ 3% apoptosis in cells transfected with Bcl-2 *versus* 49 $\pm$ 5% apoptosis in vector control cells; Fig. 6*A*).

VES-treated MDA-MB-435 cells, loaded with mitochondrial Mito tracker Red dye and transiently transfected with wild-type Bcl-2, exhibited enhanced fluorescence as detected by FACS analyses, which is indicative of reduced MPT in comparison with VES-treated cells, loaded with Mito tracker Red dye but transiently transfected with vector control (Fig. 6*B*).

Western immunoblot analyses showed that cytochrome *c* levels from the mitochondrial-enriched fraction were increased in cells transiently transfected with Bcl-2 in comparison with vector control cells (Fig. 6*C, left panel*). Levels of cytochrome *c* in the cytosolic-enriched fraction in the vector control cells were elevated in comparison with cytochrome *c* levels from Bcl-2-transfected cells (Fig. 6*C, right panel*). Mitochondrial enrichment was verified by the presence of Cox IV protein in the mitochondrial-enriched fraction and the absence in the cytosolic-enriched fraction (Fig. 6*C*). Cytochrome *c* levels in whole cell lysates from cells overexpressing Bcl-2 or from vector control did not change (Fig. 6*D*). GAPDH protein levels were used as lane load controls (Fig. 6*D*).

As expected, Western immunoblot analyses showed that cells transfected with the wild-type Bcl-2 plasmid exhibited increased levels of Bcl-2 (61% increase) in comparison with vector control cells, verifying elevated Bcl-2 expression (Fig. 6*E, top panel)*. Levels of the M_r 17,000 cleavage fragment of caspase-3 and a M_r 84,000 cleavage fragment of PARP induced by VES treatment were reduced in cells transiently overexpressing Bcl-2 in comparison with vector control cells (Fig. 6*E, second* and *third panels*, respectively). GAPDH levels were used to monitor the equivalency of lane loads (Fig. 6*E, bottom panel*).

Inhibition of JNK Prevents Bax Conformational Change. Previous studies in our laboratory showed VES-induced apoptosis to involve JNK activation and the phosphorylation of c-Jun protein (12, 17, 18). The use of a chemical inhibitor of JNK suggests that JNK is involved upstream of the Bax conformational change. When MDA-MB-435 cells were treated with JNK-inhibitor II for 2 h before treatment with 20 μg/ml VES, analyses of whole cell extracts showed that the JNK inhibitor reduced the VES induction of c-Jun phosphorylation (Fig. 7*A*) and Bax conformational change (Fig. 7*B, top panel*). Bax protein levels in whole cell extracts from control (cells not treated with the JNK inhibitor) and cells treated with the JNK inhibitor were essentially the same (Fig. 7*B, bottom panel*). Furthermore, MDA-MB-435 cells treated with the JNK inhibitor showed reduced levels of cytochrome *c* in the cytosol (Fig. 7*C*), and reduced levels of PARP cleavage (Fig. 7*D*).

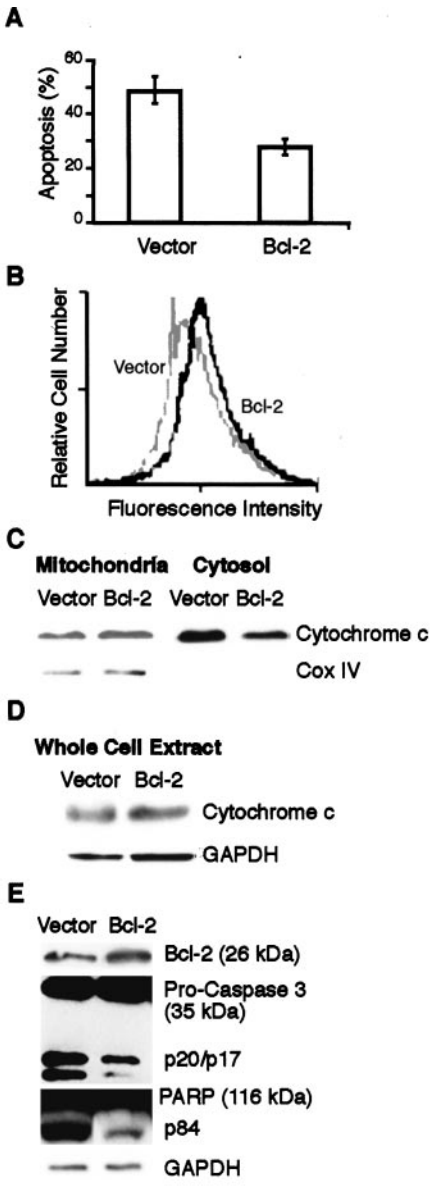

Fig. 6. Evidence that overexpression of Bcl-2 can block VES-induced apoptosis, MPT, cytochrome *c* redistribution, downstream activation of caspase-3, and PARP cleavage. MDA-MB-435 cells were transiently transfected with wild-type Bcl-2 or empty vector (*Vector*), then treated with 20 μg/ml VES for 2 days before analyses for apoptosis, or 12 h before FACS or Western immunoblotting analyses. *A*, ability of elevated levels of Bcl-2 to block VES-induced apoptosis; *B*, FACS analyses show greater retention of mitochondrial selective red fluorescent dye after VES treatment by cells overexpressing Bcl-2 in comparison with VES-treated cells transfected with vector control; *C*, Western immunoblot analyses of cytochrome *c* expression in mitochondrial- and cytosolic-enriched fractions. Cox IV serves as a mitochondrial-specific marker. *D*, Western immunoblot analyses of cytochrome *c* expression in whole cell lysates with GAPDH as a lane load control; *E*, Western immunoblot analyses of Bcl-2 expression, caspase-3 cleavage, PARP cleavage, and GAPDH levels (lane load control) in whole cell lysates. All of the data are representative of two independent experiments. *kDa*, M_r in thousands.

DISCUSSION

Studies reported here demonstrate that the time course of VES-induced apoptosis in human breast cancer cells involves the activation

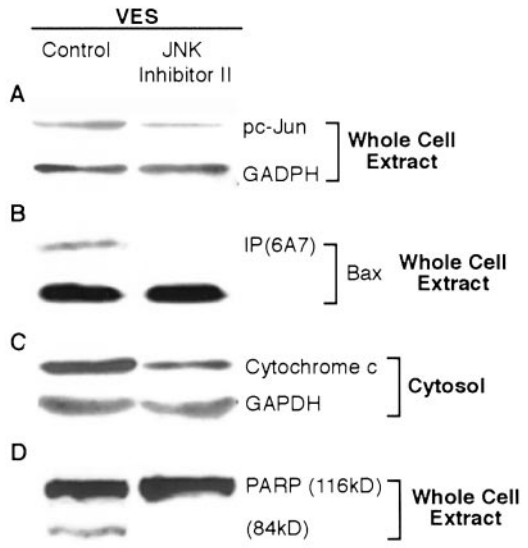

Fig. 7. Evidence that VES-induced JNK activation is upstream of Bax conformational change. MDA-MB-435 cells were pretreated with 40 nM JNK inhibitor II or DMSO (control) for 2 h, followed by treatment with 20 μg/ml VES for 12 h. Western immunoblots of whole cell extracts were performed to determine the effects of pretreating MDA-MB-435 cells with JNK inhibitor II on phosphorylation of JNK substrate c-Jun (*pc-Jun*, *A*), Bax conformational change (*B*), and PARP cleavage (*D*). Western immunoblots of cytosolic-enriched fractions were performed to determine the effects of JNK inhibitor II on cytochrome *c* release into the cytosol (*C*). All of the data are representative of two or more independent experiments. *kDa*, M_r in thousands.

of JNK, translocation of cytosolic Bax to the mitochondria followed by alterations in mitochondrial permeability, release of cytochrome *c* from mitochondria to the cytosol, activation of caspases-9 and-3, and cleavage of PARP. MPT and the resulting cascade of events resulting in apoptosis can be prevented, at least in part, in VES-treated MDA-MB-435 cells either by blockage of Bax expression using antisense oligomers or by ectopic overexpression of Bcl-2, suggesting that mitochondrial events play a critical role in VES-induced apoptosis. VES induced the relocation of Bax and Bak to the mitochondria and MPT in both estrogen receptor-negative/estrogen-nonresponsive MDA-MB-435 and estrogen receptor-positive/estrogen-responsive MCF-7 human breast cancer cells. Furthermore, VES induced a conformational change in Bax that is thought to be required for translocation of cytosolic Bax to the mitochondria (30, 34, 35). This conformational change was observed in both MDA-MB-435 and MCF-7 cells.

VES is a derivative of the RRR-α-tocopherol form of vitamin E produced by the addition of a succinic acid moiety via an ester linkage to the C-6 hydroxyl of RRR-α-tocopherol (47). Although VES can be enzymatically hydrolyzed to free RRR-α-tocopherol and succinic acid *in vivo* and by a few cell types *in vitro* via cleavage by esterases, intact VES is a redox-inactive compound, and it is the intact VES compound, not free RRR-α-tocopherol, that exhibits the unique antiproliferative properties (47).

VES is being characterized for its chemotherapeutic and anticarcinogenic potential (1, 4–8, 47). VES, administered by i.p. injections, suppresses xenografted human breast and colon and allografted murine melanoma tumor growth (3, 6, 8, 9), as well as chemical carcinogen-induced forestomach cancer (7), in animal models. VES exhibits potent antiproliferative effects on human tumor cells in culture (2, 4, 5) and selectively induces apoptosis in tumor cells, but not normal cells, both *in vitro* and *in vivo* (4, 6, 19, 48). Previous studies have shown VES to inhibit tumor cell growth via a variety of cell fates, including induction of DNA synthesis arrest, cellular differentiation, and apoptosis (1, 3, 5, 6, 11, 12, 15, 17–19, 48–51).

Because most human malignancies are of epithelial origin, it is of interest to assess VES-induced apoptosis in cancer cells of epithelial origin such as human breast cancer cells. VES induces apoptosis of human breast cancer cells in a concentration- and time-dependent manner (15, 17). For example, treatment of MDA-MB-435 cells with VES at 5, 10, and 20 μg/ml for 3 days induces 10, 48, and 70% apoptosis, respectively; and treatment of MDA-MB-435 cells with VES at 10 μg/ml for 1, 2, 3, and 4 days induces 8, 19, 50, and 75% apoptosis, respectively (2). Previous investigations by our laboratory into cellular events that are involved in VES-initiated apoptosis of human breast cancer cells have documented the involvement of several signaling pathways including: TGF-β, Fas (CD95), and the mitogen-activated protein kinases ERK and JNK (12, 15, 17–19), in that down-regulation of these signaling pathways with dominant-negative mutants, blocking antibodies, and chemical inhibitors blocks VES-initiated apoptosis. We have also demonstrated that Fas signaling plays a role in VES induction of apoptosis in human LNCaP prostate carcinoma cells, which are of epithelial origin (48).

It appears that VES-induced apoptosis exhibits cell type-specific events. For example, VES-induced apoptosis of human promyelocytic leukemia HL-60 cells is reported to not involve ERKs because the inhibition of ERK activity by the chemical inhibitor PD98059 showed no significant effect on VES-induced apoptosis (52). In contrast, we have shown that expression of dominant-negative mutants ERK1 or MEK-1 (mitogen-activated protein kinase kinase/ERK activator-1) inhibited VES-induced apoptosis of human breast MDA-MB-435 cells (12). As another example of cell type-specific effects, Bang *et al.* (52) have demonstrated that VES-initiated apoptosis of human leukemia promyelocytic HL-60 cells occurs through the activation of protein kinase Cα; whereas, Neuzil *et al.* (6) have reported that VES-induced apoptosis of human Jurkat T lymphoma cells involves the inhibition of protein kinase Cα. Other investigators have reported that the activation of caspase-8 (as well as of capases-3, -6, and -9) and the modulation of mitochondrial membrane function play a role in VES-induced apoptosis of HL-60 cells (53), whereas we see no indication of caspase-8 activity playing a role in VES-mediated mitochondrial-dependent apoptosis of MDA-MB-435 cells (Figs. 3 and 4).

One interesting outcome of these studies is the first identification of a role for Bax and Bak in VES-induced apoptosis. Recently, Bax has been shown to be a critical requirement for apoptosis induced by multiple stimuli in human epithelial cancer cells (54). Data reported here suggest that VES-induced apoptosis involves the translocation of Bax and Bak from the cytosol to the mitochondria. Furthermore, data reported here suggest that JNK is involved upstream of Bax translocation. Other studies have shown that JNK is required for Bax and Bak activation caused by exposure of mouse fibroblast cells to stress (32). A goal for future studies will be to determine the mechanism by which VES-activated JNK regulates Bax and Bak relocation. Bax is a pro-apoptotic member of the Bcl-2 protein family that is predominately localized in the cytosol, despite the presence of a typical membrane-anchoring sequence near its COOH terminus, and translocates to the outer mitochondrial membrane after the initiation of apoptosis by a variety of death stimuli (29, 30). On translocation and activation, Bax plays an important role in mitochondrial disruption and the release of cytochrome *c* (31, 55). Cellular processes involved in Bax translocation to the mitochondria have not been fully characterized; however, conformational changes of both NH_2 and COOH termini appear to be involved (30, 35). Studies of immortalized nontumorigenic human breast epithelial cells that are responsive to agonistic anti-Fas antibody-triggered cell death, showed a role for conformational changes in the NH2 terminus of the Bax protein that preceded Bax translocation from the cytosol to the mitochondria (33).

Investigations of a novel antimicrotubule agent epothilone B analogue BMS-247550-mediated apoptosis in human breast cancer cells (MDA-MB-468) demonstrated that the drug triggered a conformational change in the NH_2 terminus of the Bax protein and the Bax translocation from the cytosol to the mitochondria that was accompanied by cytochrome *c* release, and that this pathway was suppressible by overexpression of Bcl-2 (36).

Several lines of evidence taken together suggest that VES may trigger a Fas-, DAXX-, JNK-, and mitochondrial-dependent death-signaling pathway in human breast cancer cells that is neither the typical type I nor the type II form of Fas signaling (56); namely: (*a*) VES conversion of Fas-resistant human breast cancer cells to a Fas-sensitive phenotype involves translocation of cytosolic Fas to the membrane (19); (*b*) functional knockout of Fas with either Fas-neutralizing antibody or Fas antisense oligomers blocks VES-induced apoptosis by 50% or more (16, 19); (*c*) VES does not activates caspase-8 nor does a caspase-8 inhibitor block VES-induced apoptosis (Figs. 3 and 4); (*d*) VES does not induce cleavage of Bid[4]; (*e*) DAXX c, a dominant-negative inhibitor of the Fas-DAXX pathway blocks VES-induced apoptosis, JNK activation, and cytochrome *c* release[4]; (*f*) ectopic expression of wild-type Bcl-2 blocks VES-induced apoptosis (Fig. 6); and (*g*) a chemical inhibitor of JNK blocks VES-induced Bax conformational change (Fig. 7*B*), cytochrome *c* release (Fig. 7*C*), and PARP cleavage (Fig. 7*D*). Thus, it appears VES may trigger one of the multiple pathways of cell-death signaling mediated by Fas (multiple Fas signaling pathways reviewed by Mundle and Raza; Ref. 57). Additional experiments are required to confirm VES signaling via this unique sequence of events downstream of Fas and to investigate cellular events involved in VES-triggered TGF-β-mediated death signaling.

In summary, VES-induced apoptosis involves Fas and TGF-β death receptor pathways that converge to produce a strong and prolonged activation of JNK, which is a critical event. Studies reported here identified JNK involvement, upstream of Bax conformational change and translocation of Bax and Bak from the cytosol to the mitochondria, that results in an apoptotic execution phase involving cytochrome *c* release from mitochondria, activation of caspases -9 and -3, and cleavage of PARP. Additional studies will be required to understand more about the biochemical events involved in apoptosis induced by this derivative of vitamin E.

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