

# Activation of Extracellular Signal-regulated Kinase and c-Jun-NH<sub>2</sub>-terminal Kinase but not p38 Mitogen-activated Protein Kinases Is Required for RRR- $\alpha$ -Tocopheryl Succinate-induced Apoptosis of Human Breast Cancer Cells<sup>1</sup>

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## ABSTRACT

RRR- $\alpha$ -tocopherol succinate (vitamin E succinate, VES) is a potent, selective apoptotic agent for cancer cells but not normal cells. VES has been shown to inhibit the growth of a wide variety of tumor cells in cell culture and animal models. Studies addressing mechanisms of action of VES-induced apoptosis have identified transforming growth factor- $\beta$ , Fas/CD95-APO-1, and mitogen-activated protein kinase (MAPK) signaling pathway involvement. Here we show that MAPKs, the extracellular signal-regulated kinases (ERK), and the stress-activated protein kinases, c-Jun NH<sub>2</sub>-terminal kinases (JNK), but not p38, are critical mediators in VES-induced apoptosis of human breast cancer MDA-MB-435 cells. VES activates ERK1/2 and JNK both in level and duration of kinase activity. Expression of dominant negative mutants of ERK1, MAPK/ERK activator-1, or JNK1 but not p38 blocked phosphorylation of the substrate glutathione S-transferase-c-Jun and inhibited VES-induced apoptosis. Increased phosphorylation and transactivation activity of nuclear transcription factors c-Jun, ATF-2, and Elk-1 are observed after VES treatments; however, only c-Jun and ATF-2 appear to be involved in VES-induced apoptosis based on antisense blockage experiments. Collectively, these results imply a critical role for ERK1 and JNK1 but not p38 in VES-induced apoptosis of human MDA-MB-435 breast cancer cells.

## INTRODUCTION

VES<sup>3</sup> is a potent growth inhibitor of various cancer cell types *in vitro* and *in vivo* (1–5). VES-induced growth inhibition is produced by cell cycle blockage, induced cellular differentiation, increased expression of biologically active TGF- $\beta$ , and TGF- $\beta$  type II receptor expression (4). VES triggered apoptosis via TGF- $\beta$  and Fas signaling pathways and inhibition of protein kinase C (1, 5–9). VES is noteworthy not only for its antiproliferative and proapoptotic effects on tumor cells but also for its low toxicity toward normal cell types (2, 4).

VES is a succinate ester of natural vitamin E (RRR- $\alpha$ -tocopherol) and is used as a vitamin E source in some commercial supplements. Vitamin E in blood and tissues is usually in the unesterified RRR- $\alpha$ -

tocopherol form; however, intact succinate esters of vitamin E can be taken up directly by cells in culture and *in vivo* (10–13). VES is a member of a group of compounds including retinoids and deltanoids (vitamin D metabolites and analogues), tamoxifen (an antiestrogen), and monoterpenes that inhibit the growth of tumor cells and have in common the ability to induce tumor cells to secrete biologically active TGF- $\beta$  and to trigger apoptosis (14–17).

MAPKs represent a family of kinases that transduce diverse extracellular stimuli (mitogenic growth factors, environmental stresses, and proapoptotic agents) to the nucleus via kinase cascades to regulate proliferation, DNA synthesis arrest, differentiation, and apoptosis (18–22). There are three well-defined MAPK pathways in mammalian cells: the ERK1/ERK2 cascade (18); and the stress-activated JNK (19, 20) and p38 (20) MAPK cascades.

MAPKs are activated through phosphorylation of specific threonines and tyrosines by dual specificity kinases via a four-step kinase cascade: MAPK<sup>4</sup> (MAP Kinase Kinase Kinase Kinase, MKKKK/MEKKKs; TAB1); MAPK<sup>3</sup> (MAP Kinase Kinase Kinase, MKKK/MEKKs; Raf; TAK); MAPK<sup>2</sup> (MAP Kinase Kinase; MEKK/MEKs); and MAPK<sup>1</sup> (MAP Kinase; ERK1/ERK2, JNKs, and p38s). Key targets of MAPKs include the nuclear transcription factors c-Myc, Sap1, Elk-1, c-Jun, activating transcription factor-2, nuclear factor  $\kappa$ B, Max, and MEF2 (18–22). Because MAPKs play important roles in permitting cells to perceive and react to numerous extracellular stimuli, it is not surprising that the MAPK cascades have multiple substrates including shared, overlapping substrates, cross-cascade interactions, and interconnections with other signal transduction systems, all of which enable the MAPKs to mediate coordinated responses but also make their study difficult because of the high degree of complexity. Furthermore, MAPKs exhibit cell type-specific and stimulus-specific responses (18).

Previous studies by our lab have documented the involvement of AP-1 in induction of apoptosis by VES in MCF-7 human breast cancer cells (23). These studies demonstrated that VES treatment resulted in sustained, elevated levels of c-jun mRNA and c-Jun protein levels and increased AP-1 consensus element binding activity. Expression of a dominant negative interfering mutant of c-Jun in the MCF-7 human breast cancer cells partially protected the cells against VES-initiated apoptosis (23). Studies in MDA-MB-435 cells supported the notion that c-Jun plays a critical but not totally sufficient role in VES-induced apoptosis, and implicates JNK activation in this process (24).

To better define how VES induces apoptosis, we have additionally addressed the involvement of MAPKs. These data demonstrate: (1) VES treatment of human MDA-MB-435 breast cancer cells produces an early, transient activation of ERK1/2, a slightly later, prolonged activation of JNK1, but no activation of p38; (2) dominant negative mutants of ERK1 and JNK1, but not a dominant negative mutant of p38, can partially block VES-induced apoptosis; (3) VES increases the phosphorylation status and transactivation activity of nuclear transcription factors c-Jun, ATF-2, and Elk-1; and (4) antisense oli-

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<sup>3</sup> The abbreviations used are: VES, vitamin E succinate (RRR- $\alpha$ -tocopheryl succinate); AA, arachidonic acid; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; ATF-2, activating transcription factor-2; DAPI, 4',6-diamidino-2-phenylindole; Dox, doxycycline; DN, dominant negative mutants capable of binding substrate but incapable of activation by phosphorylation; Elk-1, ETS domain protein; ERK, extracellular signal-regulated kinase; Fas, CD95/APO-1; GAL4, yeast transcription factor involved in regulation of galactose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; GST-c-Jun/GST-ATF-2/GST-Elk-1, critical phosphorylation protein domains of transcription factors c-Jun, ATF-2, and Elk-1 fused to GST; JNK, c-Jun NH<sub>2</sub>-terminal kinase also called stress-activated protein kinase; MEK, MAPK kinase; MDA-MB-435, estrogen nonresponsive human breast cancer cells; p38, HOG1 kinases; TGF- $\beta$ , transforming growth factor- $\beta$ ; UT, untreated; VEH, vehicle.

gomers to c-Jun and ATF-2, but not antisense oligomers to Elk-1, can partially block VES-induced apoptosis. Taken together, these data support the hypothesis that ERK1 and JNK1 are critical yet not totally sufficient mediators of VES-induced apoptosis of human breast cancer cells.

## MATERIALS AND METHODS

**Cell Culture and VES Treatment.** MDA-MB-435 human breast cancer cells (provided by Dr. Janet E. Price, Department of Cell Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX) are an estrogen receptor-negative cell line isolated from the pleural effusions of a human with breast cancer (25). Cells were grown as monolayers on plastic (Corning Plastic Ware, Corning, NY) and maintained in MEM with Earle's balanced salts (Life Technologies, Inc., Grand Island, NY), supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, UT) plus 2 mM glutamine, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin, 1  $\times$  (v/v) nonessential amino acids, 2  $\times$  (v/v) MEM vitamins, and 1 mM sodium pyruvate (Sigma Chemical Co., St. Louis, MO). Cultures were routinely examined to verify absence of *Mycoplasma* contamination. For experiments, the percentage of fetal bovine serum was reduced to 2%, and exponentially growing cells were plated at  $1.6 \times 10^6$  cells/T-25 flask for protein and kinase activity assays and  $1.5 \times 10^5$  cells/well in 12-well plates for apoptosis analyses and allowed to attach overnight. Treatments were conducted at 10 or 20  $\mu$ g/ml VES in 0.2% ethanol (F.C. volume for volume) or VEH control, which consisted of an equivalent or highest amount of sodium succinate in 0.2% ethanol used in the experiment. VES and sodium succinate were purchased from Sigma Chemical Co.

**Western Immunoblot Analyses.** Whole-cell protein extracts were prepared as described previously (8), and 100  $\mu$ g of protein were loaded per lane, separated using SDS-PAGE on a 10–12% gel under reducing conditions, and electroblotted onto a nitrocellulose membrane (0.2  $\mu$ m pore Optitrans BA-S-supported nitrocellulose; Schleicher and Schuell, Keene, NH). Equal loading was verified using GAPDH antibody (produced in house). Immunoblotting was performed using primary rabbit antibodies with specificity for active (dually phosphorylated) ERK, JNK, or p38 (Promega, Madison, WI). Primary rabbit antibodies with specificity for ERK1/2, JNK1, c-Jun, ATF-2, Elk-1, MEK1, HA-tag, and primary goat antibody specific for p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine antibodies to the Flag-tag were purchased from Sigma Chemical Co. Horseradish peroxidase-conjugated goat antirabbit or goat antimouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA). Horseradish peroxidase-conjugated donkey antigoat serum was purchased from Santa Cruz Biotechnology. Immune complexes were visualized using enhanced chemiluminescence detection (Pierce Chemical Co., Rockford, IL). Fold differences in level of chemiluminescence were determined by densitometric analyses.

**DAPI Staining and Apoptotic Evaluation.** Apoptosis was assessed based on nuclear morphology using the fluorescent DNA dye DAPI, as described previously (8). Cells in which the nucleus contained clearly condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Apoptotic data are reported as the percentage of apoptosis, obtained by determining the number of apoptotic cells *versus* the total number of cells. For each sample, a minimum of 3 counts involving  $\geq 100$ –200 cells/count were scored. Apoptotic data are presented as mean  $\pm$  SD for three independent experiments.

**MAPK Activity Assays (Immune Complex Kinase Assays).** MAPK activity assays were conducted as described previously (18). Briefly, whole cell lysates (100–200  $\mu$ g/sample) were first immunoprecipitated with antibodies specific for MAPKs (ERK1/2, JNK1, or p38) before assaying for substrate phosphorylation. Antibodies used included a rabbit antibody to ERK that recognized primarily ERK1 but also detected ERK2, a rabbit antibody that recognized JNK1, and a goat antibody that recognized p38 (Santa Cruz Biotechnology). Immune precipitated MAPKs were reacted directly with 2  $\mu$ g of GST-c-Jun (amino acids 1–79; a gift of Dr. Roger Davis, Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA).

**Assessment of Whole-Cell Kinase Activity Capable of Phosphorylating Nuclear Transcription Factors c-Jun, ATF-2, and Elk-1.** *In vitro* kinase assays were conducted as described previously (24). In addition to the GST-

c-Jun (1–79) substrate described above, GST-ATF-2 (1–109), and GST-Elk-1 (307–428; gifts from Dr. Roger Davis) were used for these studies.

**c-Jun-, ATF-2-, and Elk-1-Transactivation Activity Assay.** c-Jun-, ATF-2-, and Elk-1-transactivation activities were determined as described previously (24). Briefly, MDA-MB-435 cells were cotransfected with a chimeric GAL4 DNA-binding domain construct coupled with the transactivation domains of c-Jun (1–79), ATF-2 (1–109), or Elk-1 plus the pGSE1bLuc reporter construct, which contains five GAL-4 sites cloned upstream of a minimal promoter element and the firefly luciferase gene (kind gifts from Dr. Roger Davis) plus a  $\beta$ -galactosidase plasmid for normalization of transfection efficiencies. Light units were determined for luciferase expression, and absorbance was determined for  $\beta$ -galactosidase activity. Data are presented as relative light units, which is based on the ratio of luciferase light units to  $\beta$ -galactosidase absorbance units.

**Transient Transfection of Cells with DN ERK1, MEK1, JNK1, and p38 Constructs.** Constructs containing dominant negative acting mutants of ERK1 and MEK1 were gifts from Dr. Jacques Pouyssegur (Center de Biochimie, Center National de la Recherche Scientifique, Universite de Nice, Nice, France; 26–28). Constructs containing dominant negative acting mutants of JNK1 and p38 were gifts from Dr. Roger Davis (29).

DN ERK1 [pcDNA expression (CMV promoter) vector encoding HA/p44T192A<sup>mapk</sup>] has threonine 192 replaced with alanine (27, 28) and DN MEK1 (pECE-HA/S222A<sup>MEK1</sup>) has amino acid serine 222 replaced with alanine (26). DN-JNK1 [pcDNA3-Flag-JNK1(APF)] has the tyrosine 185 and threonine 183 amino acids, which require phosphorylation for activity replaced with alanine and phenylalanine, respectively. DN p38 [pCMV-Flag-p38(AGF)] has the tyrosine 182 and threonine 180 amino acids, which require phosphorylation for activity replaced with alanine and phenylalanine, respectively (29). DN JNK1 and DN p38 contain the Flag epitope (-Asp-Try-Lys-Asp-Asp-Asp-Asp-Lys-), and DN ERK1 and DN MEK1 contain a sequence coding for a nine-residue immunodominant peptide from influenza virus hemagglutinin, which permits detection by immunoblotting using reagents specific for the Flag-tag and the HA-tag, thus allowing discrimination of DN proteins from endogenous proteins.

Transient transfection of cells with expression plasmids coding for DN-interfering proteins was conducted using LipofectAMINE and Plus Reagent (Life Technologies, Inc.) following the instructions of the manufacturer. Briefly, MDA-MB-435 cells at  $1.5 \times 10^5$ /well in 12-well plates were used for apoptosis assessment, and cells at  $1.6 \times 10^6$ /T-25 flask were used for Western immunoblot analyses. The cells were allowed to adhere overnight, then washed twice with serum-free medium (MEM) and incubated for 3–4 h with either 0.5 ml of MEM-Option serum-free medium (Life Technologies, Inc.) containing 100  $\mu$ l of plasmid/Plus reagent/LipofectAMINE complex for 12-well plates or 3 ml of MEM-Option serum-free medium containing 700  $\mu$ l of plasmid/Plus reagent/LipofectAMINE complex for T-25 flasks. The plasmid/Plus reagent/LipofectAMINE complex was made by mixing 0.7  $\mu$ g of plasmid/50  $\mu$ l of serum-free medium with 5  $\mu$ l of Plus reagent followed by 15 min incubation, then mixing the reaction with 4  $\mu$ g of LipofectAMINE Reagent/50  $\mu$ l of serum-free medium followed by another 15 min incubation. The cells were cultured in normal culture medium for 20 h followed by VES- or VEH-treatments for different time periods.

Transient transfection efficiency in MDA-MB-435 cells with Flag-tagged DN JNK was  $\sim 50\%$ , as determined by immunohistochemical staining using mouse anti-Flag primary antibody, antimouse horseradish peroxidase-labeled secondary antibody, and substrate (data not shown). Briefly, 20 h after transfection with either Flag-tagged DN JNK or vector control, cells ( $1.5 \times 10^5$ /well in 12-well plates) were washed with PBS twice then fixed with 4% paraformaldehyde for 20 min at 4°C. After fixation, cells were washed with PBS twice, pretreated with PBS containing 1% FCS for 10 min at room temperature, then incubated with 2  $\mu$ g/ml anti-Flag antibody (Sigma Chemical Co.) in PBS containing 1% FCS for 1 h at 4°C. The cells were washed with PBS twice then reacted with secondary antibody and peroxidase substrate contained in the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions.

**Stable Transfection of Cells with DN JNK1-inducible (TET-on) Construct.** MDA-MB-435 cells were stably transfected with a TET-on-inducible expression plasmid (Clontech) and TRE-Flag-JNK1(APF) plasmid encoding DN JNK1(APF) described above [produced in-house using the Clontech system (K1620–1)]. To generate inducible clones, MDA-MB-435 cells were

first transfected with the pTet-On vector, and stable clones were selected by growing the cells in the presence of 0.5 mg/ml of G418 (Sigma Chemical Co.) as a selective antibiotic, followed by transfection with pTRE DN JNK1(APF) and selection of stably transfected clones using 0.5 mg/ml of G418 and 0.2 mg/ml of hygromycin B (Clontech) as selective antibiotics. Transfections were performed using LipofectAMINE Plus Reagent described as above. Inducible clones were screened by Western immunoblot analyses to determine levels of endogenous JNK1 and Flag-tagged DN JNK1 expression after 2  $\mu$ g/ml of doxycycline treatment for 2 days.

**Antisense Knockout of c-Jun, ATF-2, and Elk-1.** Phosphothiorate-modified antisense and sense DNA oligonucleotides to c-Jun (antisense, CGT TTC CAT CTT CGT AGT CAT and sense, ATG ACT GCA AAG ATG GAA ACG), ATF-2 (antisense, CAC ATG TAA CTT GAA TTT CAT and sense, ATG AAA TTC AAG TTA CAT GTG), and Elk-1 (antisense, CAG CGT CAC AGA TGG GTC CAT and sense, ATG GAC CCA TCT GTG ACG CTG) were purchased from Operon Technologies (Alameda, CA) and transfected into MDA-MB-435 cells using LipofectAMINE (Life Technologies, Inc.) as described previously (24) and as described above without the Plus reagent. Briefly, the cells were transfected with 0.5 ml of MEM-Option serum-free medium (Life Technologies, Inc.) plus 100  $\mu$ l of oligomer/LipofectAMINE complex for 12-well plates and 3 ml of MEM-Option serum-free medium plus 700  $\mu$ l of oligomer/LipofectAMINE complex for T-25 flasks. The oligomer/LipofectAMINE complex was made by mixing 2  $\mu$ g oligomers/50  $\mu$ l MEM-Option serum-free medium with 8  $\mu$ g of LipofectAMINE/50  $\mu$ l MEM-Option serum-free medium for 45 min.

## RESULTS

**VES Treatment of MDA-MB-435 Cells Induces Activation of MAPKs ERK1/2 and JNK1 but not p38.** We showed previously that VES treatments increase c-Jun transactivation activity and phosphorylation of GST-c-Jun substrate via JNK in MDA-MB-435 cells (24). In this study we characterize the activated status of MAPKs ERK, JNK, and p38 after various VES-treatment times using antiactive MAPK antibodies that recognize the dually phosphorylated peptide sequence representing the catalytic core of active MAPK enzymes. VES induces early and sustained activation of ERK1/2 starting at 1 h, peaking at 2 h, and returning to barely detectable levels by 6 h after treatment (Fig. 1A, top panel). Levels of ERK1/2 proteins were not increased by VES treatment (Fig. 1A, bottom panel). Densitometric analyses showed peak ERK1 (p44) activation at 2 h to be 5-fold higher than at 1 h after VES treatment, whereas peak ERK2 (p42) activation at 2 h was 17-fold higher than the VEH control. VES induced a prolonged JNK1 activation starting at 1.5 h and continuing through 24 h after treatment initiation (Fig. 1B, top panel). Levels of JNK1 were not increased with VES treatment (Fig. 1B, bottom panel). Densitometric analyses showed JNK activation at 12 h after VES treatment to be 10-fold higher than JNK activity after treatment with VES for 1.5 h. VES treatment did not activate p38 (Fig. 1C, top panel). MDA-MB-435 cells treated with 20  $\mu$ M AA for 15 min were used as positive controls for activation of p38. p38 protein levels were not modulated by VES treatment (Fig. 1C, bottom panel).

Additional evidence that VES treatment of MDA-MB-435 cells induces activation of MAPKs comes from studies in which the individual MAPKs were immunoprecipitated and then tested for ability to phosphorylate a target substrate, GST-c-Jun (1-79). This substrate encompasses the NH<sub>2</sub>-terminal region of c-Jun that contains the activation domain of this transcription factor, which on appropriate phosphorylation leads to increased transcriptional activity of c-Jun (19). ERK1/2, JNK1, or p38 proteins were immunoprecipitated from whole cell extracts obtained from cells treated with VEH or VES (20  $\mu$ g/ml) for various time periods. The ability of ERK1/2 to phosphorylate GST-c-Jun was detected by 1 h, peaked by 2 h, and returned to VEH treatment levels by 4 h after VES treatment (Fig. 2A, top panel). Densitometric analyses showed peak ERK-mediated phosphorylation

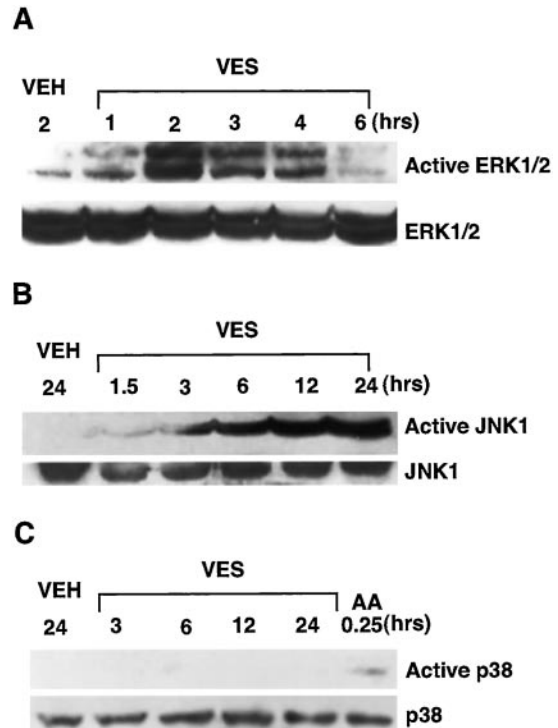


Fig. 1. Time course of MAPK activation by VES. MDA-MB-435 cells were treated with 20  $\mu$ g/ml of VES or VEH and harvested at the indicated times. A, ERK1/2; B, JNK1; and C, p38 activation was determined by Western immunoblotting analyses using antibodies that recognize the phosphorylated form of the respective active MAPKs (top panels). Protein levels of the MAPKs were also determined by Western immunoblot analyses using antibodies to the respective enzymes (bottom panels). A positive control for activated p38 involved treating the MDA-MB-435 cells with 20  $\mu$ M AA for 15 min (C). A-C, data are representative of two independent experiments.

of GST-c-Jun at 2 h after VES treatment to be 4.4-fold higher than phosphorylation of substrate after VEH treatment. JNK1 immunoprecipitated from VES-treated cells was capable of phosphorylating GST-c-Jun as early as 3 h after treatment, and JNK kinase activity remained active for  $\geq$ 12 h after VES treatment (Fig. 2B, top panel). Densitometric analyses showed the peak JNK activity for the GST-c-Jun substrate at 12 h after VES treatment to be 2.3-fold higher than kinase activity of the VEH control. Phosphorylation of GST-c-Jun by p38 immunoprecipitated from VES-treated cells did not increase with treatment time and did not differ from the VEH control (data not shown). The increased kinase activity of ERK1/2 or JNK1 is not attributable to enhanced protein levels, because the levels of ERK1/2 and JNK proteins did not change after VES treatment (Fig. 2, A and B, bottom panels, respectively).

**ERK1, MEK1, and JNK1 but not p38 Are Involved in VES-induced Apoptosis.** To additionally address the role of MAPK signaling in VES-induced apoptosis, studies were conducted to determine the effects of specific blockage of MAPKs (ERK1, JNK1, or p38) or an activator of ERK (MEK1) using dominant-negative acting mutants.

To evaluate the relevance of ERK activation in VES-induced apoptosis, MDA-MB-435 cells were transiently transfected with an expression construct containing dominant negative ERK1 (T192A) or DN MEK1 (S222A), a direct upstream activator of ERK. DN ERK1 blocked VES-induced apoptosis produced by treating the cells with either 10 or 20  $\mu$ g/ml VES for 2 days by 45 and 49%, respectively (Fig. 3A). Likewise, DN MEK1 blocked VES-induced apoptosis by 42 and 43% (Fig. 3B). Furthermore, both DN ERK1 and DN MEK1 blocked: (a) VES-induction of active ERK1/2 (Fig. 3C, top panel); and (b) VES-induction of ERK kinase activity (as measured by the



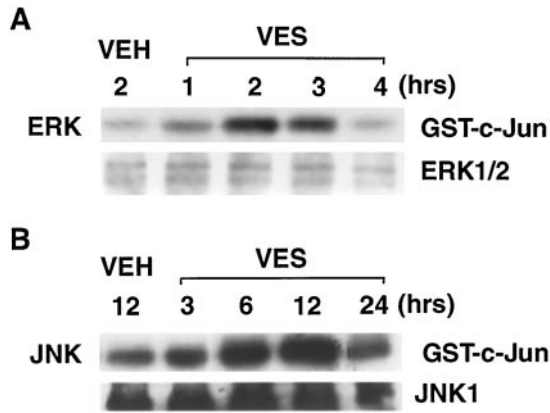


Fig. 2. Time course of VES effects on MAPK phosphorylation of GST-c-Jun. MDA-MB-435 cells were treated with 20  $\mu\text{g/ml}$  of VES or VEH and harvested at the indicated times. ERK1/2 (A) and JNK1 (B) activity was assayed by immunocomplex kinase assays using GST-c-Jun as the substrate (top panels). Protein levels of ERK1/2 and JNK1 were measured by Western immunoblot analyses (bottom panels). A and B, data are representative of two independent experiments.

ability of immunoprecipitated ERK1/2 to phosphorylate GST-c-Jun; Fig. 3C, second panel). Densitometric analyses showed that DN ERK1 in comparison to vector control reduced levels of active ERK1 by  $\sim 48\%$  and decreased phosphorylation of GST-c-Jun by  $\sim 43\%$ . Likewise, densitometric analyses showed that DN MEK1 in comparison to vector control reduced levels of active ERK1 by  $\sim 68\%$  and active ERK2 by  $\sim 48\%$  and decreased phosphorylation of GST-c-Jun by  $\sim 59\%$ . Verification that the MDA-MB-435 cells were expressing DN ERK1 and DN MEK1 comes from Western immunoblot analyses showing three bands (endogenous ERK1/2 and HA-tagged DN ERK1) in cells transfected with DN ERK1 versus two bands (endogenous ERK1/2) in empty vector control cells (Fig. 3C, third panel) and two bands (endogenous MEK1 and HA-tagged DN MEK1) in cells transfected with HA-tagged DN MEK1, versus 1 band (endogenous MEK1) in empty vector control cells (Fig. 3C, fourth panel). Additional evidence of successful transfection was documented by analyses of whole cell extracts using antibodies to the HA epitope of the DN ERK1 and MEK1 proteins (Fig. 3C, fifth panel). Levels of GAPDH were used to verify lane loads (Fig. 3C, sixth panel).

The biological relevance of increased JNK activity to VES-induced apoptosis and whether or not p38 was involved in VES-induced apoptosis was assessed using MDA-MB-435 cells transiently transfected with expression vectors carrying dominant negative constructs. DN JNK reduced VES-induced apoptosis by 47 and 44% in MDA-MB-435 cells transiently transfected with Flag-tagged DN JNK(APF) and treated with 10 and 20  $\mu\text{g/ml}$  of VES when compared with levels of apoptosis in VES-treated empty vector control cells (Fig. 4A). Levels of endogenous JNK + DN Flag-tagged JNK expressed by the transfected cells are depicted in Fig. 4B, top panel. Levels of Flag-tagged DN JNK expressed are depicted in Fig. 4B, middle panel. GAPDH levels served to help verify lane loads (Fig. 4B, bottom panel). The ability of whole cell extracts from DN JNK-transfected cells to inhibit VES-mediated phosphorylation of GST-c-Jun, in comparison to VES-treated empty vector control cells, is depicted in Fig. 4B, third panel. Densitometric analyses showed that DN JNK, in comparison to vector control, decreased phosphorylation of GST-c-Jun by  $\sim 53\%$ . Transient transfection efficiency in MDA-MB-435 cells with Flag-tagged DN JNK was  $\sim 50\%$  as determined by color reaction using mouse anti-Flag primary antibody, antimouse horseradish peroxidase-labeled secondary antibody, and substrate (Vectastain ABC Kit; Vector Laboratories; data not shown).

VES-induced apoptosis was not modulated in MDA-MB-435 cells

transiently transfected with DN p38(AGF), because levels of apoptosis in VES-treated cells transiently transfected with DN p38 were essentially the same levels as VES-treated empty vector control cells (Fig. 4A). Although high levels of Flag-tagged protein were expressed in cells transiently transfected with DN p38 (Fig. 4B, second panel) there was no reduction in phosphorylation of GST-c-Jun by whole cell extracts from the DN p38-transfected cells (Fig. 4C, third panel).

To additionally confirm the role of JNK in VES-induced apoptosis, yet another approach, namely the establishment of stable clones of

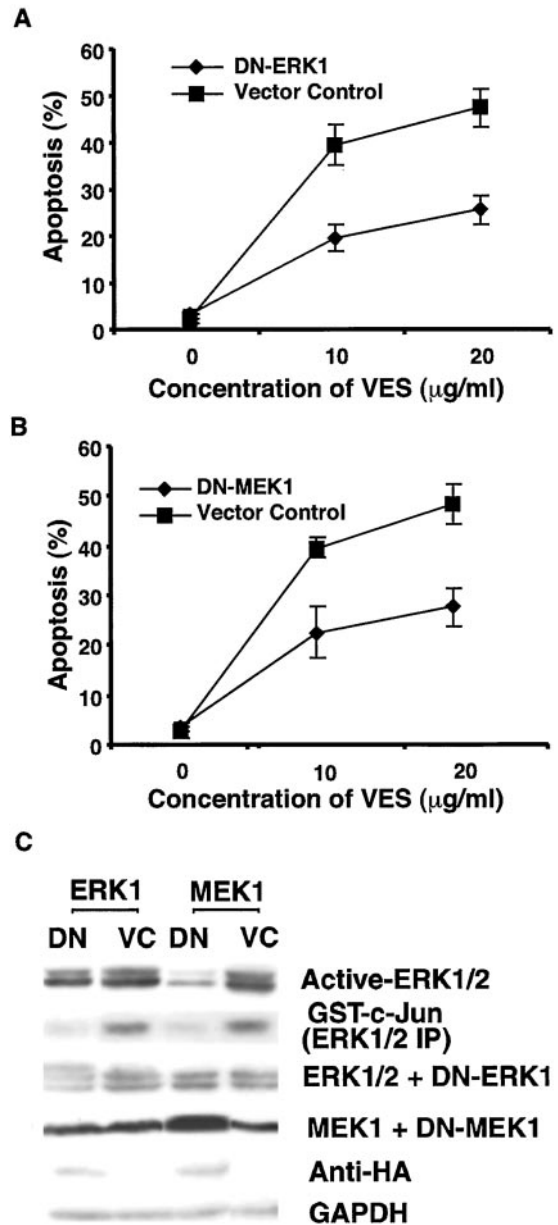


Fig. 3. Blockage of VES-induced apoptosis by DN ERK1 and MEK1 mutants. MDA-MB-435 cells transiently transfected with DN ERK1, DN MEK1, or empty vector control were treated with 0 (VEH), 10, or 20  $\mu\text{g/ml}$  of VES for 2 days. Apoptosis was measured by analyses of morphology of nuclei of DAPI stained cells (A and B). C, various parameters of DN or empty vector control (VC) transfected cells were measured. ERK1/2 activation was measured by Western immunoblot analyses using antiactive ERK1/2 antibody after VES treatment for 2 h (top panel). ERK1/2 activity was assayed by immunocomplex kinase assay using GST-c-Jun as the substrate after VES treatment for 2 h (panel 2). Protein levels of ERK (endogenous ERK1/2 + HA-tagged DN ERK1; panel 3), protein levels of MEK1 (endogenous + HA-tagged DN MEK1; panel 4), levels of HA-tagged DN ERK1 and HA-tagged DN MEK1 (panel 5), and GAPDH protein levels (bottom panel) for lane loads were measured by Western immunoblotting analyses. A and B, data are depicted as mean of three experiments; bars,  $\pm$  SD. C, data are representative of two independent experiments.

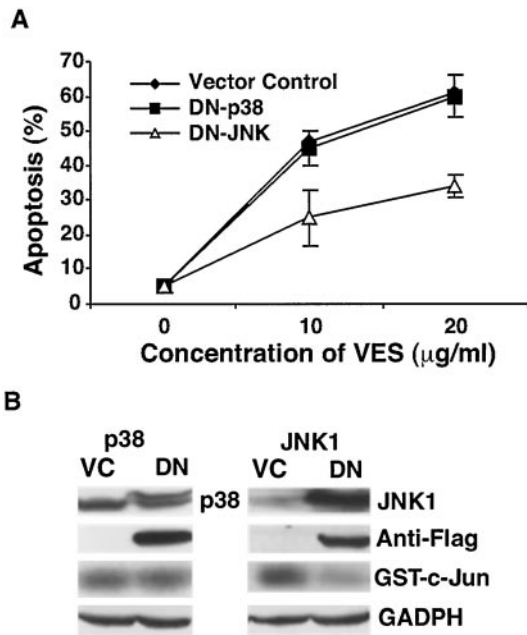


Fig. 4. Involvement of JNK1 but not p38 in VES-induced apoptosis and phosphorylation of GST-c-Jun. **A**, MDA-MB-435 cells transiently transfected with DN p38, DN JNK1, or vector control were treated with 0 (VEH), 10, or 20 µg/ml of VES for 2 days, and apoptosis was determined by morphological analyses of nuclei of DAPI stained cells. **B**, MDA-MB-435 cells transiently transfected with DN p38, DN JNK1, or empty vector control (VC) were treated with 20 µg/ml of VES for 12 h and various parameter measured. Protein levels of p38 and JNK1 (endogenous + flag-tagged DN; *top panel*), Flag-tagged DN p38 and Flag-tagged DN JNK1 (*panel 2*), and GAPDH (*bottom panel*) were determined by Western immunoblot analyses. Ability of whole cell extracts to phosphorylate GST-c-Jun was measured using *in vitro* kinase assay (*panel 3*). **A**, data are depicted as the mean of three experiments; *bars*, ± SD. **B**, data are representative of two independent experiments.

MDA-MB-435 cells with inducible DN JNK(APF), was used. MDA-MB-435 cells were stably transfected with an inducible (Tet-on) Flag-tagged DN JNK construct, and clones expressing high levels of DN JNK after 2 days of treatment with 2 µg/ml of Dox were chosen (three such clones designated 19, 35, and 53 are reported here). For experiments, Dox-induced and noninduced cells were treated with 20 µg/ml of VES for 2 days. Induction of DN JNK in clones 19, 35, and 53 by Dox reduced the ability of VES to increase the phosphorylation status of GST-c-Jun (Fig. 5A, *top panel*). Densitometric analyses of these data showed 2.4-, 1.4-, and 1.8-fold reductions in comparison to levels of GST-c-Jun substrate phosphorylation after VES-treatment of noninduced cells for clones 19, 3, and 53, respectively. JNK1 (endogenous + DN JNK1) protein levels expressed in induced (+) clones 19, 35, and 53 were 2.0-, 3.2-, and 6.4-fold higher than JNK (endogenous only) levels expressed by noninduced (-) clones (Fig. 5A, *lower panel*). In comparison to VES-treated noninduced cells from clones 19, 35, and 53, VES-induced apoptosis of Dox treated clones 19, 35, and 53 was reduced by 52, 42, and 49%, respectively (Fig. 5B).

**VES Treatments Increase Phosphorylation Status and Transactivation Potential of c-Jun, ATF-2, and Elk-1.** Analyses of cellular extracts from MDA-MB-435 cells treated with 20 µg/ml of VES for varying times show that VES induces kinase activity capable of increasing the phosphorylation status of all three of the transcription factor substrates tested, namely GST-c-Jun, GST-ATF-2, and GST-Elk-1 at 3, 6, 12, and 24 h after VES treatment. Peak phosphorylation of the substrates was seen at 12 h after VES treatment (Fig. 6A). Densitometric analyses of the 12 h data showed peak phosphorylation for c-Jun, ATF-2, and Elk-1 to be 3.5-, 10.2-, and 5.3-fold higher in the VES *versus* VEH control samples, respectively.

Assessments of transactivation potential of the transcription factors after VES treatment were performed using cells transiently cotransfected with an expression vector encoding a fusion protein consisting of a GAL4 DNA-binding domain fused with the activation domain of one of the transcription factors (c-Jun, ATF-2, or Elk-1) plus a vector encoding a reporter construct (the firefly luciferase gene) under the transcriptional regulation of the GAL4 fusion protein. Expectations are that activation of the transcription factor-specific activation domain by phosphorylation by activated MAPKs would result in increased reporter gene transcription and translation, measurable as relative light units of luciferase activity. Treatment of the cotransfected cells with VES (20 µg/ml for 12 or 24 h) resulted in evidence of increased transactivation activities reported as fold increases in luciferase activity in comparison with VEH control-treated cell samples (Fig. 6B). VES treatments for 12 or 24 h produced increases in luciferase activities of 1.9- and 3.6-fold for c-Jun-, 2.0- and 5.3-fold for ATF-2, and 4.0- and 6.3-fold for Elk-1-mediated transactivation in comparison with the levels of transactivation measured in VEH control-treated cells (Fig. 6B). Cells cotransfected with a control GAL4 DNA-binding domain construct containing no transactivation domain plus the luciferase reporter construct exhibited low levels of luciferase activity after either VES or VEH treatment (data not shown).

**Antisense Oligomers to c-Jun and ATF-2 but not to Elk-1 Reduce VES-induced Apoptosis.** Because previous studies by our lab had shown that c-Jun is critical to VES-induced apoptosis (24), it was of interest to determine whether or not either ATF-2 or Elk-1 might be playing a necessary role in VES-induced apoptosis, so we conducted functional knockout experiments using antisense oligonu-

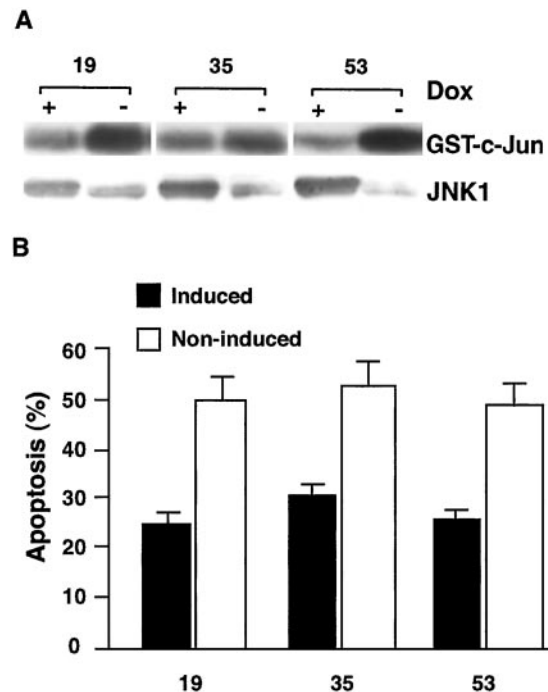


Fig. 5. Cells stably expressing inducible DN JNK1 are resistant to VES-induced apoptosis. MDA-MB-435 cell clones designated 19, 35, and 53, which are stably transfected with a tet-on inducible DN JNK1 construct, were treated with 2 µg/ml of Dox for 2 days to induce DN JNK1 expression before VES treatment. **A**, Dox-induced (+) cells exhibit a marked reduction in the ability of VES (20 µg/ml for 12 h) to induce kinase activity capable of phosphorylating GST-c-Jun as measured by *in vitro* kinase assay in comparison to uninduced (-) cells (*top panel*). JNK1 protein levels (endogenous + flag-tagged DN JNK1) were measured by immunoblotting. **B**, Dox-induced DN JNK1 expression in cells resulted in resistance to VES (20 µg/ml for 2 days)-induced apoptosis. Apoptosis was detected by analyses of morphology of nuclei of DAPI stained cells. **A**, data are representative of two independent experiments. **B**, data are depicted as the mean of three separate experiments; *bars*, ± SD.

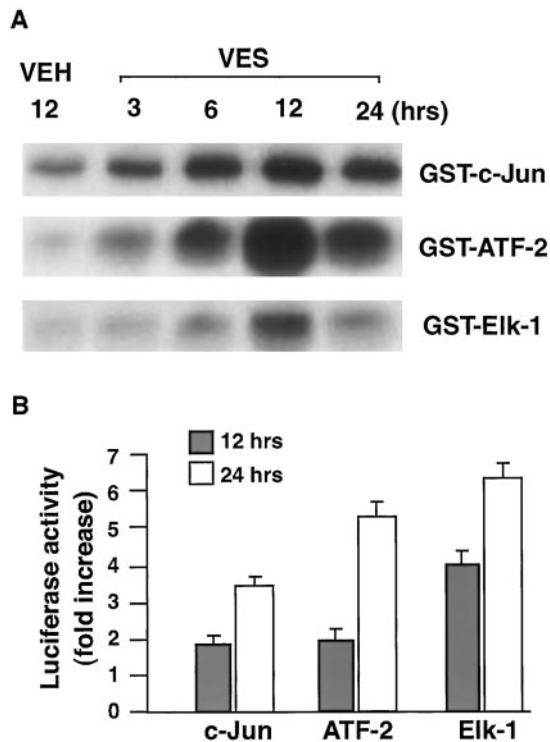


Fig. 6. VES induces c-Jun, ATF-2, and Elk-1 phosphorylation and transactivation activity. *A*, *in vitro* phosphorylation of GST-c-Jun, ATF-2, and Elk-1 was measured using whole cell extracts from MDA-MB-435 cells treated with VES (20 µg/ml) for 3, 6, 12, and 24 h or VEH for 12 h. *B*, transactivation activity of c-Jun, ATF-2, and Elk-1 was measured in MDA-MB-435 cells transiently transfected with GAL-4/transactivation luciferase reporter systems after treatment of the cells with 20 µg/ml of VES or VEH for 12 or 24 h. The fold increase in luciferase activity (mean) was determined by comparison to VEH control data. Data in *A*, data are representative of two independent experiments. *B*, data are depicted as the mean of three experiments; bars, ± SD.

cleotides. Repeat analyses of antisense oligonucleotide functional knockout of *c-jun* were performed for comparative purposes. MDA-MB-435 cells transiently transfected with antisense or sense oligomers to *c-Jun*, *ATF-2*, and *Elk-1* were cultured with 20 µg/ml of VES for 2 days. Antisense oligomers to *c-Jun* and *ATF-2*, in comparison to cells transfected with corresponding sense oligomers, blocked VES-induced apoptosis by 49 and 39%, respectively (Fig. 7A). VEH-treated cells gave basal levels (<5%) of apoptosis (data not shown). In contrast, antisense oligomers to *Elk-1* had little to no effect on VES-induced apoptosis (Fig. 7A). Evidence for transfection efficiency and functionality of the antisense oligomers to *c-jun*, *ATF-2*, and *Elk-1* is provided by Western immunoblot analyses, demonstrating that cells transiently transfected with antisense oligomers, in comparison to sense oligomers, exhibited reduced expression of *c-Jun*, *ATF-2*, and *Elk-1* (Fig. 7B, top panel). Densitometric analyses showed a 3.9-, 2.3-, and 2.7-fold reduction, respectively. GAPDH levels were used as lane load controls (Fig. 7B, bottom panel).

**DISCUSSION**

VES is a potent inhibitor of human breast cancer cell growth *in vitro*, inducing human breast cancer cells to undergo DNA synthesis arrest and apoptosis (6–8). VES does not induce apoptosis of either normal human mammary epithelial cells or immortalized but nontumorigenic human mammary MCF-10A cells (4, 8). However, VES does induce human mammary epithelial cells and MCF-10A cells to undergo DNA synthesis arrest (4). Human breast cancer cells, including MDA-MB-435 cells, are nonresponsive to apoptosis induced by exogenous TGF-β or agonistic anti-Fas-triggering antibodies (1, 8). A

unique and important feature of VES-induced apoptosis in human breast cancer cells is the ability of VES to restore both TGF-β and Fas signaling pathways and to use both of these signaling pathways in the induction of apoptosis (1, 8). The objective of the current studies was to analyze the contributions of MAPK signaling to VES-induced apoptosis.

Studies presented here document that VES-triggered apoptosis involves ERK1, MEK1, and JNK1 but not p38. Both ERK1/2 and JNK1 were activated after VES treatment as documented by detection of the active (phosphorylated) forms of these kinases using antibodies specific for the active enzyme as well as kinase activity assays using immunoprecipitated kinases and using GST-c-Jun as substrate. Furthermore, transient expression of dominant-negative mutants of ERK1 and JNK1 (also stably transfected inducible DN JNK) blocked VES-induced phosphorylation of GST-c-Jun substrate as well as blocking VES-induced apoptosis by ~50%, indicating that activated ERK1 and JNK1 are involved in VES-induced apoptosis. Transient transfections with DN p38 had no effect on either phosphorylation of GST-c-Jun substrate or apoptosis induced by VES. Thus, collectively, our results indicate a critical role for ERK1 and JNK1 but not p38 in VES-induced apoptosis of human MDA-MB-435 cells.

The duration of JNK activation is important in determining cell fates (30). Mitogenic stimuli produce transient JNK induction, whereas environmental stresses and apoptotic triggering agents produced sustained JNK activation (30). Studies reported here and previous studies by our lab document that JNK levels are elevated for a prolonged time period after VES treatment (24). VES treatments induce activation of JNK1 beginning 1.5 h after VES treatment and produce a sustain elevation for ~24 h with peak levels at 12 h after treatment involving a 10-fold increase over control values. The kinetics of JNK1 but not ERK1/2 activation correlate with the kinetics of increased phosphorylation of c-Jun and ATF-2, which appear to play a role in VES-induced apoptosis and correlate with the onset of early VES-induced apoptotic alterations such as activation of caspase 3 and

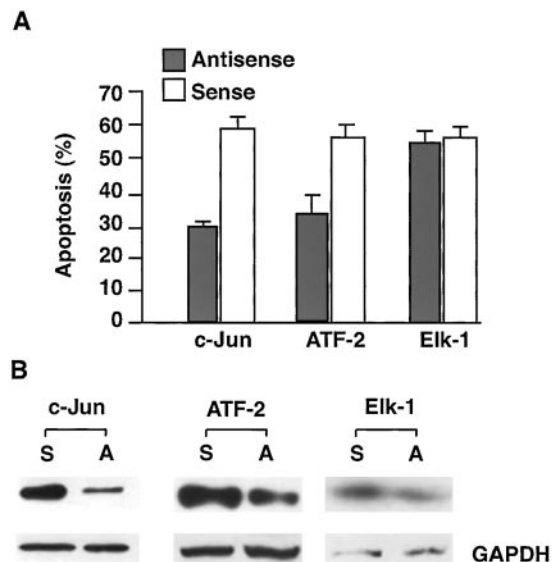


Fig. 7. Antisense oligomers to c-Jun and ATF-2 but not Elk-1 block VES-induced apoptosis. *A*, MDA-MB-435 cells transiently transfected with antisense or sense oligomers to c-Jun, ATF-2, or Elk-1 were treated with 20 µg/ml of VES for 2 days. Apoptosis was detected by morphological analyses of nuclei of DAPI stained cells. *B*, MDA-MB-435 cells transiently transfected with sense (S) or antisense (A) oligomers to c-Jun, ATF-2, or Elk-1 were treated with 20 µg/ml of VES for 12 h. c-Jun, ATF-2, and Elk-1 protein levels were detected by Western immunoblot analyses (top panel). GAPDH protein (bottom panel) was used to establish lane loads. *A*, data are depicted as the mean of three experiments; bars, ± SD. *B*, data are representative of two independent experiments.



cleavage of poly(ADP-ribose) polymerase, which occur ~12 h after VES treatment.<sup>4</sup>

Because either JNK or p38 or both JNK and p38 can be activated by various stressors, it is of interest that VES activates JNK but not p38. To rule out the possibility that this differential activation might be attributable to loss of functionality of the p38 stress-activated protein kinase in this neoplastic cell line, we treated MDA-MB-435 cells with an agent (AA), which is reported in the literature to be capable of activating p38 in human breast cancer cells (31) and showed that indeed the MDA-MB-435 cells used in these studies have an activatable p38 (Fig. 1C).

The functional relevance of ERK1 activation to VES-induced apoptosis was a surprise, because typically, activation of the ERK pathway is associated with responses to mitogenic stimulation (18), and the kinetics of ERK1 activation do not appear to correlate with other apoptotic-related events in VES-treated cells. Although the majority of ERK stimuli induce cell proliferation or differentiation, it appears that some stimuli, for example, ligation of the cell surface receptor Fas (CD95/APO-1) by its specific ligand or by anti-Fas antibodies in human neuroblastoma cells, may use the ERK pathway to trigger apoptosis (32). Furthermore, Mulder *et al.* (33, 34) have reported the involvement of ERK2 (and JNK) in TGF- $\beta$ -mediated negative-growth control of human breast cancer cells that retain responsiveness to TGF- $\beta$ . It should be noted that the increase in ERK2 induced by TGF- $\beta$  was detected 10 min after treatment initiation, was sustained for at least 30 min, and achieved a level 1.7-fold above control values. This is a very different profile from what we observe with VES treatments, which induce activation of ERK1/2 beginning 1 h after VES treatment and produce a sustained elevation for  $\geq 3$ –4 h with peak levels 2 h after treatment involving a 17-fold increase over control values. Thus, the duration and intensity of ERK activation appears to be important in determining cell fate, namely, growth arrest *versus* apoptosis. Both the upstream activators of MEK1 and the downstream substrates of ERK1/2 that are playing a role in VES-induced apoptosis remain to be identified.

Previous studies by our lab have shown that the transcription factor c-Jun is involved in VES-induced apoptosis of human breast cancer cells (23, 24). Data reported here show that in addition to c-Jun, ATF-2 but not Elk-1 is involved in VES-induced apoptosis. Studies by Dam *et al.* (35, 36) have shown that c-Jun/ATF-2 dimers are major regulators of the c-Jun promoter. Previous studies by our lab have shown that VES induces prolonged and enhanced expression of c-Jun mRNA, protein, and AP-1 activity (23, 24). VES-mediated increases in c-jun mRNA occur at the transcriptional level (24), suggesting that perhaps dual activation of c-Jun and ATF-2 may contribute to this event. Unlike c-Jun, the protein levels of ATF-2 are not modulated by VES. Because antisense oligomers to c-Jun can block VES-induced apoptosis, one possible mechanism whereby antisense oligomers to ATF-2 may block VES-induced apoptosis is via reduction of c-Jun protein levels.

We have shown previously that VES treatment of human breast cancer cells converts the cells from a nonresponsive to a responsive phenotype in regard to TGF- $\beta$  signaling and agonistic anti-Fas antibody stimuli (1, 8). VES-treated MDA-MB-435 cells secrete biologically active TGF- $\beta$  ligand and exhibit enhanced cell surface membrane levels of TGF- $\beta$  type II receptors (6). Furthermore, VES treatment of MDA-MB-435 cells induces the translocation of cytosolic Fas to the cell surface membrane (8). Blockage of either the TGF- $\beta$  or Fas signaling pathways with neutralizing antibodies decreases VES-induced activation of c-Jun kinases and decreases VES-

induced apoptosis, suggesting the involvement of both pathways in these VES-mediated events (data not shown). Fas/FADD/caspase 8 signaling (37) does not appear to be occurring in VES-restored Fas signaling, rather, preliminary data suggest that Fas is activating JNK via Daxx. Likewise, preliminary data suggest that VES-restored TGF- $\beta$  signaling does not involve Smads but rather that TGF- $\beta$  activation of JNK may be via Rho/Cdc 42 and TAB/TAK-1 signaling (38–40). Clarification of the contributions of these signaling pathways to VES-induced apoptosis will require additional study.

In summary, the overall goal of our studies is to characterize the signaling pathways producing VES-induced apoptosis, cellular differentiation, and cell growth inhibition. Expectations are that these studies will increase basic knowledge about fundamentally important signaling pathways in cancer cells, which will lead to the development of specifically targeted drugs to achieve improved cancer cell elimination. Studies reported here implicate MAPKs ERK1 and JNK1 but not p38, as well as transcription factors c-Jun and ATF-2 but not Elk-1 as being involved in VES-induced apoptosis.

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## Activation of Extracellular Signal-regulated Kinase and c-Jun-NH<sub>2</sub>-terminal Kinase but not p38 Mitogen-activated Protein Kinases Is Required for RRR- $\alpha$ -Tocopheryl Succinate-induced Apoptosis of Human Breast Cancer Cells

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