

# Vitamin E Succinate (VES) Induces Fas Sensitivity in Human Breast Cancer Cells: Role for $M_r$ 43,000 Fas in VES-triggered Apoptosis<sup>1</sup>

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## ABSTRACT

Fas (CD95/APO-1) is an important mediator of apoptosis. We show that Fas-resistant MCF-7, MDA-MB-231, and MDA-MB-435 human breast cancer cells become responsive to anti-Fas (CD95) agonistic antibody-triggered apoptosis after pretreatment or cotreatment with vitamin E succinate (VES; RRR- $\alpha$ -tocopheryl succinate). In contrast, no enhancement of anti-Fas agonistic antibody-triggered apoptosis was observed following VES pretreatment or cotreatment with Fas-sensitive primary cultures of human mammary epithelial cells, immortalized MCF-10A cells, or T47D human breast cancer cells. Although VES is itself a potent apoptotic triggering agent, the 6-h pretreatment procedure for Fas sensitization did not initiate VES-mediated apoptosis. The combination of VES plus anti-Fas in pretreatment protocols was synergistic, inducing 2.8-, 3.0-, and 6.3-fold enhanced apoptosis in Fas-resistant MCF-7, MDA-MB-231, and MDA-MB-435 cells, respectively. Likewise, cotreatment of Fas-resistant MCF-7, MDA-MB-231, and MDA-MB-435 cells with VES plus anti-Fas enhanced apoptosis 1.9-, 2.0-, and 2.6-fold, respectively. Functional knockout of Fas-mediated signaling with either Fas-neutralizing antibody (MCF-7-, MDA-MB-231-, and MDA-MB-435-treated cells) or Fas antisense oligomers (MDA-MB-435-treated cells only), reduced VES-triggered apoptosis by ~50%. Analyses of whole cell extracts from Fas-sensitive cells revealed high constitutive expression of  $M_r$  43,000 Fas, whereas Fas-resistant cells expressed low levels that were confined to the cytosolic fraction. VES treatment of the Fas-resistant cells caused a depletion of cytosolic  $M_r$  43,000 Fas with a concomitant increase in  $M_r$  43,000 membrane Fas. These data show that VES can convert Fas-resistant human breast cancer cells to a Fas-sensitive phenotype, perhaps by translocation of cytosolic  $M_r$  43,000 Fas to the membrane and show that VES-mediated apoptosis involves  $M_r$  43,000 Fas signaling.

## INTRODUCTION

VES<sup>3</sup> (RRR- $\alpha$ -tocopheryl succinate), a derivative of vitamin E, is currently being characterized for its chemopreventive and chemotherapeutic potential (1). VES has been shown to inhibit the proliferation of several transformed cell types, including human breast cancer cells (2-6). Previous studies have shown VES to inhibit tumor cell growth by a variety of mechanisms, including: induction of apoptosis,  $G_1$  cell cycle blockage, induced cellular differentiation, DNA synthesis arrest, induced secretion and activation of TGF- $\beta$ s, and enhanced expression of TGF- $\beta$  type II receptors (3-15).

VES is a potent inducer of apoptosis in human breast cancer cells, and it appears that a minimum of two apoptotic triggering pathways may be involved. For example, previous studies by our laboratory

have demonstrated a partial role for TGF- $\beta$  signaling and involvement of JNK and c-Jun (15-17). Turley *et al.* (18) have proposed a role for  $M_r$  48,000 Fas signaling in VES-induced apoptosis in human breast cancer cells.

Fas, also called Apo-1 or CD95, is a member of the tumor necrosis factor receptor and nerve growth factor receptor superfamily, which can trigger programmed cell death upon trimerization with Fas ligand (CD95L) or upon cross-linking with Fas-specific antibodies (also called agonistic anti-Fas antibodies). Fas is expressed on the cell surface of a variety of cell types, including T cells, B cells, and macrophages as well as cells of the liver, spleen, lung, testis, heart, brain, intestine, and breast (19-22). Although Fas-induced apoptosis has been studied most in T lymphocytes, recent studies show Fas expression in a wide range of tissues, including breast (18, 19, 22, 23). Furthermore, studies show that Fas signaling is altered in some breast, prostate, colon, and central nervous system cancer cells (18, 19, 23-29).

Apoptosis or programmed cell death is a well-established phenomenon in tissue development and homeostasis (30). Apoptotic pathways can generally be classified into three distinct phases: the initiation phase, which may differ, depending on the apoptosis triggering stimulus; the common effector phase, in which the decision to die is made; and the degradation phase, in which the cell dies and exhibits the morphological and biochemical characteristics of apoptosis (31-34). It appears that the degradation phase uses the same cascading molecular events and produces similar morphological characteristics; however, apoptotic initiating or triggering events use several pathways, and the utilization of a specific pathway(s) may vary, depending on environmental context of cells and apoptotic triggering agent (30, 32, 35). Defects in key components of apoptotic pathways provide a survival advantage to cells and have been implicated as important factors in tumorigenesis (26, 32). Because therapeutic drug-induced apoptosis is a critical component in the treatment of most cancers, alterations in key apoptotic pathways may be critical to drug resistance (36). For example, Fas signaling has been demonstrated to be defective in certain cancer cell types, and studies show that sensitivity to Fas-mediated apoptosis can be restored (23-25, 35). Furthermore, studies show enhanced apoptosis when immunochemotherapy is given in combination with CD95 Fas ligand (37).

Here, we examine the apoptotic inducing abilities of VES and Fas agonistic antibody, administered separately and in combination, on non-Fas-responsive MCF-7, MDA-MB-435, and MDA-MB-231 breast cancer cells and on Fas-sensitive T47D breast cancer cells, HMECs, and immortalized but nontumorigenic MCF-10A human mammary cells. With regard to Fas signaling, we show that VES has no major effects on Fas-sensitive cells but is capable of converting Fas-resistant human breast cancer cells to Fas-sensitive cells. Whole-cell extracts from Fas-sensitive cells constitutively express high levels of  $M_r$  43,000 Fas, whereas whole-cell extracts from Fas-resistant cells expressed lower levels of  $M_r$  43,000 Fas, which were confined to the cytosolic fraction. VES sensitization of the three Fas-resistant cell lines is associated with a decrease in cytosolic  $M_r$  43,000 Fas, with a concomitant increase in  $M_r$  43,000 cell surface membrane Fas. Func-

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<sup>3</sup> The abbreviations used are: VES, vitamin E succinate; TGF- $\beta$ , transforming growth factor- $\beta$ ; JNK, c-Jun NH<sub>2</sub>-terminal kinase; HMEC, human mammary epithelial cell; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorting; VEH, vehicle; DAPI, 4',6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tional knockout of Fas signaling via Fas-neutralizing antibodies or Fas antisense oligomers partially inhibits VES-triggered apoptosis.

## MATERIALS AND METHODS

**Chemicals and Reagents.** RRR- $\alpha$ -tocopheryl succinate, succinic acid, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents for morphological analyses of apoptosis were purchased from Boehringer Mannheim (Indianapolis, IN).

**Cell Culture and VES Treatment.** MDA-MB-435 (provided by Dr. Janet E. Price, Department of Cell Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX; Ref. 38) and MDA-MB-231 (American Type Culture Collection, Manassas, VA) are non-estrogen-responsive breast cancer cell lines. T47D (American Type Culture Collection) and MCF-7 (provided by Dr. Suzanne Fuqua, University of Texas Health Science Center, San Antonio, TX; Ref. 39) are estrogen-responsive breast cancer cell lines. MCF-10A (American Type Culture Collection) is an immortalized nontumorigenic breast cell line. HMECs (provided by Dr. C. Marcelo Aldaz, University of Texas M. D. Anderson-Science Park Research Division, Smithville, TX) are primary cultures of mammary cells derived from normal mammary specimens. MDA-MB-435 cells were cultured in MEM with Earle's balanced salts (Life Technologies, Inc., Grand Island, NY) supplemented with 5% FBS (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). MDA-MB-231 and MCF-7 cells were cultured in MEM with Earle's balanced salts (Life Technologies, Inc.) supplemented with 10% FBS (Hyclone Laboratories) plus 2 mM glutamine, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin, 1 $\times$  (v/v) nonessential amino acids, and 10 mM HEPES (Sigma). T47D cells were cultured in DMEM containing 10% FBS plus 2 mM glutamine, 100  $\mu$ g/ml streptomycin, and 100 IU/ml penicillin. MCF-10A cells were cultured in DMEM/F12 medium (Life Technologies, Inc.) supplemented with 5% horse serum plus 2 mM glutamine, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin, 0.25  $\mu$ g/ml ampicillin B, 100 ng/ml cholera toxin, 20 ng/ml epidermal growth factor, 0.5  $\mu$ g/ml hydrocortisone, and 10  $\mu$ g/ml insulin. HMECs were cultured with basal serum free mammary epidermal cell growth medium supplemented with 0.4% bovine pituitary extract, 10 ng/ml epidermal growth factor, 5  $\mu$ g/ml insulin, 0.5 ng/ml hydrocortisone, 50  $\mu$ g/ml gentamicin, and 50 ng/ml amphotericin B (Clonetics, San Diego, CA). All experiments with primary cultures of normal mammary epithelial cells were conducted with second-passage HMECs.

For experiments, the percentage of serum was reduced to 2% for MDA-MB-435 cells and 5% for the other cell lines (HMEC experiments were conducted in serum-free medium). In these media, growing cells were plated at  $5 \times 10^6$  cells per T-75 flask for Western blotting and FACS analyses and at  $1.5 \times 10^5$  cells/well in 12-well plates for apoptosis analyses. Treatments were conducted at 5, 10, or 20  $\mu$ g/ml VES in 0.2% ethanol (F.C. v/v) or VEH, which consisted of an equivalent or highest amount of sodium succinate in 0.2% ethanol used in the experiments.

**Determination of Apoptosis.** Apoptosis was assessed based on nuclear morphology using the fluorescent DNA dye DAPI, as described previously (13, 15). Briefly, cells were cultured at  $1.5 \times 10^5$  cells/well in 12 well plates overnight to permit attachment. Next, the cells were treated with VEH or VES. After treatment, the cells were washed twice with PBS, and floating cells plus trypsin-released adherent cells were pelleted, washed, and stained with 2  $\mu$ g/ml DAPI (Boehringer Mannheim, Indianapolis, IN) in 100% methanol for 20 min at 37°C. Cells were viewed at  $\times 400$  magnification with a Zeiss ICM 405 fluorescent microscope using a 487701 filter. 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 *versus* nonapoptotic cells, as visualized by DAPI staining, within a cell population by counting a minimum of five different locations/slide and counting a minimum of 100 cells per location for a minimum of 500 cells counted per slide. Apoptotic data are presented as mean  $\pm$  SD for three independent experiments.

**Cell Fractionation.** Membrane and cytosol fractions were isolated as described by Koli and Arteaga (40). Cells were washed with cold PBS and resuspended in fractionation buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 25 mM sodium fluoride, 1 mM DTT, 2 mM sodium orthovanadate, 1  $\mu$ g/ml of

aprotinin, and 1  $\mu$ g/ml leupeptin (Sigma)]. The cells were sheared by passing through a 26-gauge needle five times and then incubated on ice for 5 min. After centrifugation at  $100,000 \times g$  for 1 h at 4°C, the supernatants (cytosol fraction) were transferred to a clean tube, and Triton X-100 was added to a final concentration of 0.8%. The pellets (membrane fraction) were resuspended in fractionation buffer and 0.8% Triton X-100, incubated for 20 min on ice, and centrifuged at  $15,000 \times g$  for 15 min at 4°C. Whole-cell protein extracts were prepared as described previously (3). After washing twice with PBS, the cells were lysed with lysis buffer (1 $\times$  PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) plus 1  $\mu$ g/ml of aprotinin and leupeptin, 1 mM DTT, and 2 mM sodium orthovanadate, and incubated on ice for 30 min. Lysates were then centrifuged at  $15,000 \times g$  for 15 min at 4°C. Protein concentrations were determined using the Bio-Rad Dye Binding protein assay (Bio-Rad Laboratories). Membrane and cytosolic preparations were analyzed by Western/ECL analyses for the presence of the cytosolic enzyme of glycolysis, GAPDH. Cytosolic preparations exhibited readily detectable high levels of GAPDH protein, whereas GAPDH protein in the membrane fractions was detectable only after prolonged exposure times (data not shown).

**Western Blot Analyses.** Fas protein was detected in membrane, cytosol, and whole-cell lysates by Western blot analyses. Fifty to 100  $\mu$ g of protein were loaded per well, separated using SDS-PAGE on a 10% gel, and electroblotted onto a nitrocellulose membrane (Optitrans BA-S supported nitrocellulose for 0.2  $\mu$ m pore; Schleicher and Schuell, Keene, NH). Equal loading was verified using GAPDH antibody (a gift from Dr. D. Hwang, Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, LA; Ref. 41) and by staining the membrane with Ponceau red stain. Immunoblotting was performed using primary rabbit antihuman Fas polyclonal antibody (Santa Cruz Biotechnology) and peroxidase-conjugated goat antirabbit as the secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) followed by detection with ECL (Pierce, Rockford, IL).

**Cell Surface Fas Detection by Flow Cytometry.** Cell surface Fas expression was measured by flow cytometry using a modification of methods of Keane *et al.* (23). Following VEH and VES treatment, MDA-MB-435 cells ( $1 \times 10^6$ ) were washed twice with PBS and incubated at 4°C for 60 min with 20  $\mu$ g/ml antihuman Fas mouse monoclonal IgM (clone CH-11; Upstate Biotechnology, Inc., Lake Placid, NY) or 20  $\mu$ g/ml irrelevant mouse IgM (Sigma) in PBS containing 1% FCS and 0.5 mM EDTA. The specificity of the primary antibody was verified by Western immunoblotting and was determined to detect a  $M_r$  43,000 Fas molecule in whole-cell extracts of the human breast cancer cell lines. After two washings with PBS, the cells were incubated for 30 min at 4°C with constant rotation with 10  $\mu$ g/ml of affinity-purified FITC-conjugated goat antimouse IgG plus IgM (Kirkegaard & Perry, Gaithersburg, MD) in PBS containing 1% FCS and 0.5 mM EDTA. After two more washings, the cells were resuspended in PBS. Immediately before analyzing, cells were filtered through a 60- $\mu$ m mesh to prevent clumping and then analyzed by flow cytometry, using a 488-nm argon laser. FACSscan and CELLquest software were used to analyze data.

**Blockage of Fas Signaling with Anti-Fas Neutralizing Antibody and Fas Antisense Oligonucleotides.** Cells were treated for 48 h with VEH and VES (10 or 20  $\mu$ g/ml) plus 0.5  $\mu$ g/ml of an apoptosis-neutralizing monoclonal Fas antibody (clone ZB4; Immunotech, Westbrook, ME) or 0.5  $\mu$ g/ml control antibody (mouse IgG; Sigma). Apoptotic cells were counted after staining with DAPI.

For antisense experiments,  $1.5 \times 10^5$  cells/well were transiently transfected with sense or antisense Fas oligonucleotides. Briefly, cells were allowed to adhere overnight. The next day they were washed twice with MEM culture media without serum or antibiotics, followed by addition of 0.8 ml of this media plus a mixture of 2  $\mu$ g of sense or antisense oligonucleotide and 8  $\mu$ g of lipofection reagent Lipofectamine (Life Technologies, Inc.) in 200  $\mu$ l of serum free Opti-MEM (Life Technologies, Inc.). After 4 h, the cells were treated with VES or VEH in 2% serum-containing medium and cultured for an additional 48 h for apoptosis analyses and 18 h for Western immunoblot analyses. Apoptosis was monitored using DAPI staining; whole-cell extract Fas expression was analyzed by Western immunoblotting, and cell surface Fas was analyzed by FACS as described above. Oligomer sequences for Fas sense (ATG CTG GGC ATC TGG ACC CTC) and antisense (GAG GGT CCA GAT GCC CAG CAT) oligonucleotides were directed against the ATG translation start site in Fas mRNA and were modified with phosphorothioate at every third nucleotide (Operon Technologies, Alameda, CA).

**Fas and VES Sensitivity and Synergism Assay.** For the Fas sensitivity assay, cells were treated with 0.5  $\mu\text{g/ml}$  antihuman Fas mouse monoclonal IgM antibody (clone CH-11; Upstate Biotechnology, Inc., Lake Placid, NY) or 0.5  $\mu\text{g/ml}$  irrelevant mouse IgM antibody (Sigma) for 2 days, and then analyzed for percentage of cells undergoing apoptosis. For VES modulation of Fas sensitivity assays, cells were pretreated with VEH or VES at 5, 10, and 20  $\mu\text{g/ml}$  for 6 h; washed in medium to remove VEH and VES; treated with 0.5  $\mu\text{g/ml}$  anti-Fas or 0.5  $\mu\text{g/ml}$  irrelevant antibody for another 42 h; and then analyzed for percentage of apoptotic cells. For the synergism assays, cells were cotreated with VEH or VES at 5, 10, and 20  $\mu\text{g/ml}$  plus 0.5  $\mu\text{g/ml}$  of anti-Fas or 0.5  $\mu\text{g/ml}$  of irrelevant antibody for 30 h prior to analyzing for apoptosis. Apoptosis was monitored using DAPI staining.

## RESULTS

**Fas and VES-mediated Apoptosis.** HMECs, MCF-10A cells, and T47D cells were sensitive to the apoptotic inducing properties of anti-Fas, exhibiting 36, 52 and 47% apoptosis after 2 days of treatment with 0.5  $\mu\text{g/ml}$  anti-Fas, respectively, whereas MCF-7, MDA-MB-231, and MDA-MB-435 cells were resistant to anti-Fas-induced apoptosis, exhibiting background levels of 3–5% apoptosis (Fig. 1A). In contrast, Fas-resistant MCF-7, MDA-MB-231, and MDA-MB-435 cells were sensitive to the apoptotic inducing properties of VES, exhibiting 32, 48, and 35% apoptosis after 2 days of treatment with 10  $\mu\text{g/ml}$  VES, respectively, whereas the Fas-sensitive HMECs, MCF-10A cells, and T47D cells were resistant to VES-induced apoptosis (Fig. 1B).

**Pretreatment of Fas-resistant Cells with VES Renders Them Fas-sensitive.** Fas-resistant MDA-MB-435 cells pretreated with 5, 10, or 20  $\mu\text{g/ml}$  VES for 6 h were rendered sensitive to subsequent anti-Fas induced apoptosis, exhibiting 4-, 5-, and 5.4-fold increases in

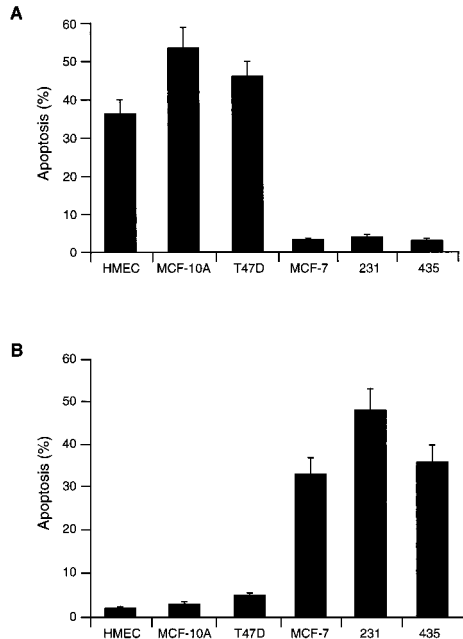


Fig. 1. HMECs (three different mammary specimens were analyzed) and MCF-10A cells (immortalized but nontumorigenic) and transformed human breast cancer cells (T47D, MCF-7, MDA-MB-231, and MDA-MB-435) cultured at  $1.5 \times 10^5$  cells/well in 12-well plates were examined for sensitivity to two separate apoptotic inducing agents: agonistic anti-Fas antibody and VES. All cells were monitored for apoptosis (%) by morphological characterization of nuclei from DAPI-stained cells, as described in "Materials and Methods." A, for detection of Fas sensitivity, all cells were treated for 48 h with either 0.5  $\mu\text{g/ml}$  anti-Fas apoptotic triggering antibody or 0.5  $\mu\text{g/ml}$  irrelevant antibody. B, for detection of VES sensitivity, all cells were treated for 48 h with either 10  $\mu\text{g/ml}$  VES or VEH. Irrelevant antibody-treated (A) and VEH-treated (B) cells did not exhibit apoptosis (data not shown). Columns, mean percentage apoptosis for three independent experiments; bars, SD.

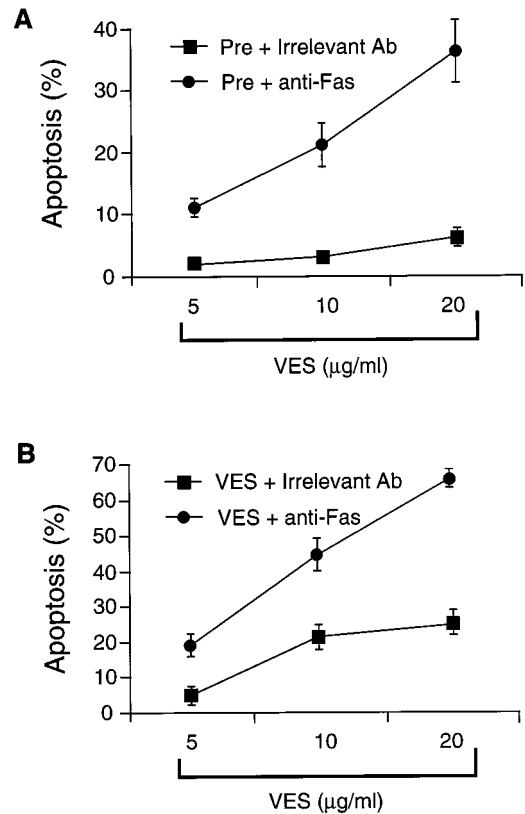


Fig. 2. Fas-resistant MDA-MB-435 cells ( $1.5 \times 10^5$  cells/well in 12-well plates) were pretreated for 6 h with VES or cotreated with VES plus anti-Fas and examined for apoptosis. A, cells were pretreated for 6 h with 5, 10, and 20  $\mu\text{g/ml}$  VES, washed, and cultured for 42 h with 0.5  $\mu\text{g/ml}$  anti-Fas or 0.5  $\mu\text{g/ml}$  irrelevant antibody. Following treatment, nuclei were DAPI-stained and examined for apoptosis. B, cells were cocultured with 5, 10, and 20  $\mu\text{g/ml}$  VES plus 0.5  $\mu\text{g/ml}$  anti-Fas or VES plus 0.5  $\mu\text{g/ml}$  irrelevant antibody for 30 h. Cells were DAPI-stained and examined for apoptosis. Data points, means for three independent experiments; bars, SD.

apoptosis compared to VES-pretreated cells cultured with irrelevant antibody for the same time period, namely, 42 h (Fig. 2A). MDA-MB-435 cells that were cotreated with VES plus anti-Fas for 30 h exhibited 3.6-, 2.1-, and 2.6-fold increases in apoptosis when compared to cells treated with VES plus irrelevant antibody (Fig. 2B). In the absence of pretreatment or cotreatment with VES, MDA-MB-435 cells do not exhibit apoptosis to anti-Fas agonistic antibody (Fig. 1A).

Further evidence that pretreatment of Fas-insensitive MCF-7, MDA-MB-231, and MDA-MB-435 cells with VES for 6 h rendered the cells sensitive to anti-Fas agonistic antibody triggered apoptosis (42 h culture with anti-Fas) can be seen in data presented in Table 1. MCF-7, MDA-MB-231, and MDA-MB-435 cells pretreated with VES and triggered to undergo apoptosis with anti-Fas exhibited 2.8-, 3.0-, and 6.3-fold increases in apoptosis compared to the lower levels of apoptosis induced by pretreatment of the cells with VES for 6 h followed by treatment with irrelevant antibody (Table 1). Levels of apoptosis induced by anti-Fas alone (no pretreatment) were less than those obtained in cultures in which cells were pretreated with VES for 6 h and then incubated with irrelevant antibody (Table 1). Pretreatment of the Fas-sensitive MCF-10A cells for 6 h with VES had no enhancing effect on anti-Fas triggered apoptosis compared to the levels of apoptosis induced by anti-Fas treatment alone. Pretreatment of the Fas-sensitive T47D cells with VES resulted in a modest 1.2-fold increase in anti-Fas-triggered apoptosis. As expected, the Fas-sensitive cells treated with anti-Fas resulted in Fas-triggered apoptosis (Table 1).

Table 1 Fas-insensitive human breast cancer cells pretreated with VES for 6 h become sensitive to the induction of apoptosis by anti-Fas agonistic antibody<sup>a</sup>

Cell lines	Pretreatment	Treatment	Apoptosis (%) <sup>b</sup>	Enhanced apoptosis
Fas-insensitive				
MCF-7	VES	+ irrelevant Ab	5 ± 1	
	None	Anti-Fas	3 ± 0.6	
MDA-MB-231	VES	+ Anti-Fas	14 ± 3	2.8 <sup>c</sup>
	None	Anti-Fas	4 ± 1	
MDA-MB-435	VES	+ Anti-Fas	33 ± 4	3.0 <sup>c</sup>
	None	Anti-Fas	11 ± 2	
Fas-sensitive				
MCF-10A	VES	+ irrelevant Ab	5 ± 1	
	None	Anti-Fas	52 ± 5.7	
T47D	VES	+ Anti-Fas	49 ± 6	0 <sup>d</sup>
	None	Anti-Fas	2 ± 1	
T47D	VES	+ irrelevant Ab	47 ± 5	
	None	Anti-Fas	55 ± 6	1.2 <sup>d</sup>

<sup>a</sup> Fas-insensitive MCF-7, MDA-MB-231, and MDA-MB-435 cells and Fas-sensitive MCF-10A and T47D cells at  $1.5 \times 10^5$  cells/well were pretreated with 20  $\mu\text{g/ml}$  VES for 6 h, cells were washed in medium to remove VES and then incubated for an additional 42 h with 0.5  $\mu\text{g/ml}$  anti-Fas or 0.5  $\mu\text{g/ml}$  irrelevant antibody. As a control for Fas sensitivity/insensitivity, cells pretreated with medium were treated with 0.5  $\mu\text{g/ml}$  anti-Fas for the 42-h treatment time.

<sup>b</sup> Cells were DAPI-stained and morphologically examined for apoptotic cells. Apoptotic cells (%) were determined by counting the number of apoptotic cells versus non-apoptotic cells. Data are depicted as mean  $\pm$  SD of three independent experiments.

<sup>c</sup> Enhanced apoptosis, presented as fold increase, for Fas-insensitive cells was calculated by dividing the number of apoptotic cells obtained with VES pretreated plus anti-Fas with the number of apoptotic cells obtained with VES pretreated plus irrelevant antibody.

<sup>d</sup> Fold increase for Fas-sensitive cells was calculated by dividing the number of apoptotic cells obtained with VES pretreated plus anti-Fas with the number of apoptotic cells obtained with cells treated with anti-Fas alone.

**Cotreatment of Fas-insensitive Cells with VES and Anti-Fas Renders Them Fas-sensitive.** Similar results were obtained when the Fas-insensitive cells (MCF-7, MDA-MB-231 and MDA-MB-435 cells) were cotreated with 20  $\mu\text{g/ml}$  VES plus anti-Fas for 30 h (Table 2). MCF-7, MDA-MB-231, and MDA-MB-435 cells exhibited 1.9-, 2.0-, and 2.6-fold increases in apoptosis compared to apoptosis induced by cotreatments of VES plus irrelevant antibody. Fas-insensitive cells treated with anti-Fas only did not exhibit apoptotic characteristics. VES and anti-Fas cotreatments had little effect on levels of apoptosis induced in Fas sensitive MCF-10A and T47D cells. Although the level of apoptosis expressed by T47D cells cotreated with VES plus anti-Fas was higher than cells treated with anti-Fas alone, the increased levels were not markedly different between the two treatment groups. In an effort to see if VES might synergize with anti-Fas at lower concentrations of anti-Fas, Fas-sensitive MCF-10A cells were cotreated with a 5-fold lower concentration of agonistic anti-Fas (namely, 0.1  $\mu\text{g/ml}$ ) plus VES (20  $\mu\text{g/ml}$ ); however, no synergistic effects were observed (data not shown).

**VES-triggered Apoptosis Involves Fas Signaling.** Because Fas-resistant cells are sensitive to VES-triggered apoptosis (Fig. 1B) and because Turley *et al.* (18) reported that VES-induced apoptosis involves Fas signaling, it was of interest to see whether VES-triggered apoptosis of these cell lines also involved Fas signaling. VES- (20  $\mu\text{g/ml}$ ) and VEH-treated MCF-7, MDA-MB-231, and MDA-MB-435 cells were cultured in the presence of 0.5  $\mu\text{g/ml}$  neutralizing antibody to Fas for 48 h. Identically treated cells cocultured with 0.5  $\mu\text{g/ml}$  of irrelevant antibody served as controls (Fig. 3). Fas neutralizing antibody reduced the ability of VES to induce apoptosis by 57, 54, and 53% for the MCF-7, MDA-MB-231, and MDA-MB-435 cells, respectively (Fig. 3).

An alternate approach to knocking out Fas signaling used transient transfections with Fas antisense oligomers. MDA-MB-435 cells transiently transfected with antisense oligomers to Fas exhibited a 54% reduction in VES-induced apoptosis (48 h culture with 20  $\mu\text{g/ml}$  VES

and VEH) compared to VES-treated cells transiently transfected with Fas sense oligomers (Fig. 4A). VEH-treated cells exhibited background levels of apoptosis (Fig. 4A). Western/immunoblot analyses of whole-cell extracts from MDA-MB-435 cells (after 18 h of 20  $\mu\text{g/ml}$  VES treatment) transiently transfected with antisense or sense oligomers to Fas showed reduced levels of  $M_r$  43,000 Fas in the anti-sense-treated cells when compared to the levels of  $M_r$  43,000 Fas in

Table 2 Cotreatment of Fas-insensitive human breast cancer cells with VES plus anti-Fas agonistic antibody is synergistic<sup>a</sup>

Cell lines	Treatment	Apoptosis (%) <sup>b</sup>	Fold synergism
Fas-insensitive			
MCF-7	VES + irrelevant Ab	24 ± 3	
	Anti-Fas	3 ± 1	
	VES + Anti-Fas	46 ± 5	1.9 <sup>c</sup>
MDA-MB-231	VES + irrelevant Ab	40 ± 5	
	Anti-Fas	4 ± 1	
	VES + Anti-Fas	79 ± 8	2.0 <sup>c</sup>
MDA-MB-435	VES + irrelevant Ab	25 ± 3	
	Anti-Fas	3 ± 1	
	VES + Anti-Fas	66 ± 8	2.6 <sup>c</sup>
Fas-sensitive			
MCF-10A	VES + irrelevant Ab	3 ± 1	
	Anti-Fas	52 ± 6	
	VES + Anti-Fas	50 ± 5	0 <sup>d</sup>
T47D	VES + irrelevant Ab	5 ± 1	
	Anti-Fas	47 ± 5	
	VES + Anti-Fas	57 ± 6	1.2 <sup>d</sup>

<sup>a</sup> Fas-insensitive MCF-7, MDA-MB-231, and MDA-MB-435 cells and Fas-sensitive MCF-10A and T47D cells at  $1.5 \times 10^5$  cells/well were cotreated with 20  $\mu\text{g/ml}$  VES plus 0.5  $\mu\text{g/ml}$  anti-Fas or 20  $\mu\text{g/ml}$  VES plus 0.5  $\mu\text{g/ml}$  of irrelevant antibody for 30 h. As a control for Fas sensitivity/insensitivity, cells were treated with 0.5  $\mu\text{g/ml}$  anti-Fas for 30 h.

<sup>b</sup> Cells were DAPI-stained and morphologically examined for apoptotic cells. Apoptotic cells (%) were determined by counting the number of apoptotic cells versus non-apoptotic cells. Data are depicted as mean  $\pm$  SD of three independent experiments.

<sup>c</sup> Fold synergism, presented as fold increase, for Fas-resistant cells was calculated by dividing the number of apoptotic cells obtained when cells were triggered with VES + anti-Fas with the number of apoptotic cells obtained when cells were cotreated with VES + irrelevant antibody.

<sup>d</sup> Fold synergism for Fas sensitive cells was calculated by dividing the number of apoptotic cells obtained when cells were triggered with VES plus anti-Fas with the number of apoptotic cells obtained when cells were triggered with anti-Fas alone.

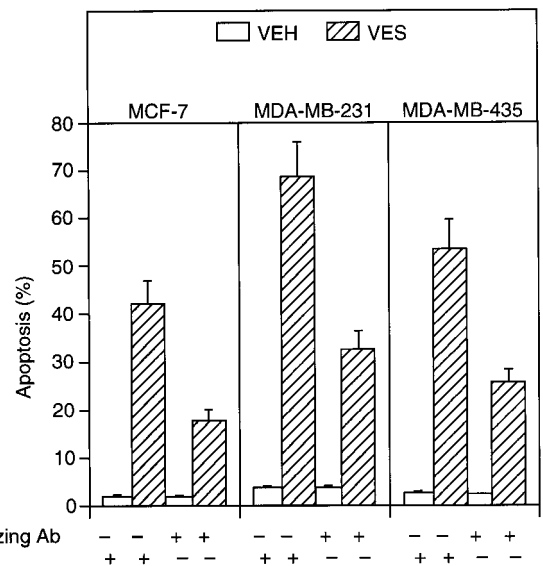


Fig. 3. Inhibitory effects of Fas-neutralizing antibody on VES-induced apoptosis in Fas resistant MCF-7, MDA-MB-231, and MDA-MB-435 cells. Cells ( $1.5 \times 10^5$  cells/well in 12-well plates) were cultured with 0.5  $\mu\text{g/ml}$  mouse monoclonal Fas-neutralizing antibody or 0.5  $\mu\text{g/ml}$  control mouse IgG plus 20  $\mu\text{g/ml}$  of VES (▨) or VEH (□) for 48 h. Apoptosis was monitored by DAPI staining. Columns, mean percentage apoptosis of three independent experiments; bars, SD.

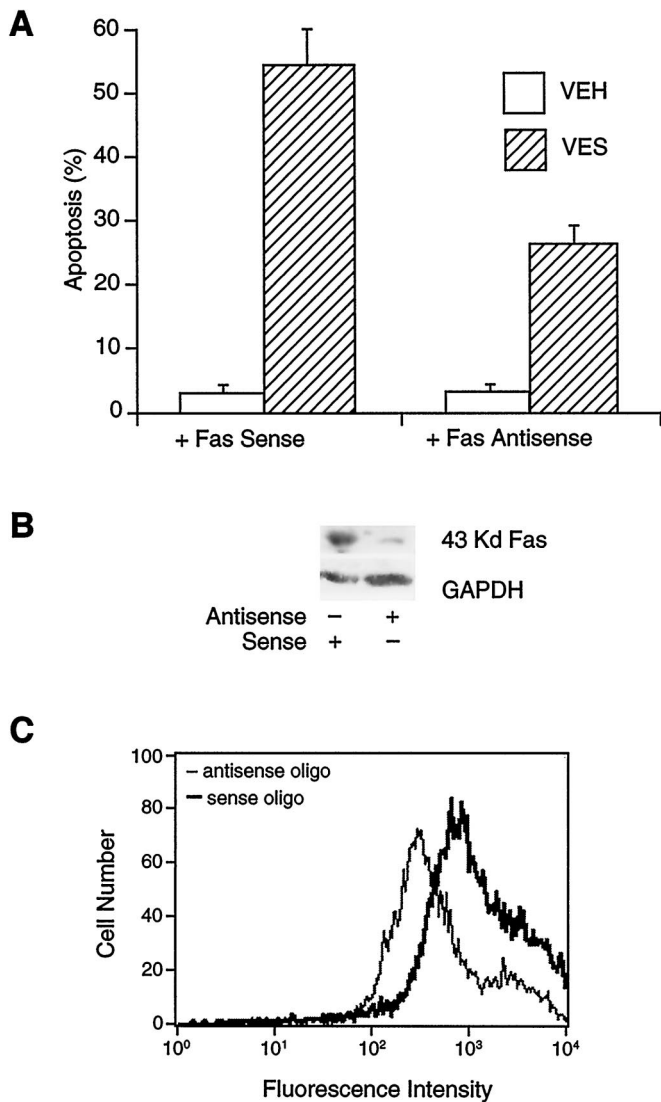


Fig. 4. Effects of Fas antisense oligonucleotides on VES-induced apoptosis and  $M_r$  43,000 Fas expression in MDA-MB-435 cells. MDA-MB-435 cells ( $1.5 \times 10^5$  cells/well) were transiently transfected with Fas sense or antisense oligomers, as described in "Materials and Methods." A, Fas sense- and antisense-transfected MDA-MB-435 cells were cultured with VEH ( $\square$ ) or 20  $\mu\text{g/ml}$  VES ( $\text{hatched}$ ) for 48 h. After treatment, the cells were DAPI-stained and assayed for apoptosis. Columns, means for three independent experiments; bars, SD. B, replica experiments were conducted to verify transfection efficiency. Following 18 h treatment with VES, levels of  $M_r$  43,000 Fas in 100  $\mu\text{g}$  of whole-cell extracts were determined as described in "Materials and Methods." Analyses of GAPDH levels served as a control for lane loads. C, to further verify Fas antisense effects, we assayed MDA-MB-435 cells for membrane Fas levels by flow cytometry as described in "Materials and Methods." MDA-MB-435 cells transiently transfected with either Fas sense or antisense oligomers were cultured with VES (20  $\mu\text{g/ml}$ ) for 18 h. Cell surface Fas levels, as detected with mouse antibody to Fas (clone CH-11) plus FITC-conjugated goat antimouse immunoglobulin were determined by flow cytometry, using a 488-nm argon laser. Data were analyzed by FACScan and CELLQuest software.

the sense oligomer-treated control cells (Fig. 4B). GAPDH protein levels served to establish protein lane loads (Fig. 4B). A comparison of the fluorescent profile of sense-transfected (thick line) and antisense-transfected (thin line) MDA-MB-435 cells cultured with 20  $\mu\text{g/ml}$  VES and analyzed for cell surface Fas expression by flow cytometry show a reduction in fluorescence by cells transfected with Fas antisense, suggesting that the antisense treatment reduced the cell surface expression of Fas (Fig. 4C).

**Expression of  $M_r$  43,000 Fas.** In an effort to understand how VES was rendering Fas-resistant cells Fas-sensitive, levels of  $M_r$  43,000 Fas protein were determined. Whole-cell extracts from the Fas-sen-

sitive cell lines (HMECs, MCF-10A, and T47D) exhibited high levels of constitutively expressed  $M_r$  43,000 Fas (Fig. 5A). In contrast, whole-cell extracts from Fas-resistant cell lines (MCF-7, MDA-MB-231, and MDA-MB-435) did not express detectable levels of  $M_r$  43,000 Fas (Fig. 5A). However, prolonged exposure of immunoblots of whole-cell extracts from VEH-treated and VES-treated (20  $\mu\text{g/ml}$ ) MCF-7, MDA-MB-231, and MDA-MB-435 cells revealed detectable levels of  $M_r$  43,000 Fas (Fig. 5B). [Please note that differences between levels of Fas in Fig. 5, A and B are due to differences in amounts of whole-cell extract proteins analyzed (50 versus 100  $\mu\text{g/lane}$ , respectively) and differences in autoradiogram exposure times (10 versus 60 s, respectively)]. Fas levels in whole cell extracts were not affected by VES treatment (Fig. 5B). Levels of  $M_r$  43,000 Fas expressed by T47D cells treated with VEH and VES (20  $\mu\text{g/ml}$ ), after prolonged immunoblot exposure, are presented for comparative purposes (Fig. 5B). VES treatment had little to no effect on levels of  $M_r$  43,000 Fas expressed in T47D whole-cell extracts (Fig. 5B).

**Modulation of Membrane Fas Levels by VES.** Analyses of  $M_r$  43,000 Fas expressed in membrane and whole cell preparations obtained from Fas-sensitive (MCF-10A and T47D) cells showed high levels of  $M_r$  43,000 Fas and showed that VES treatment did not alter the levels of membrane Fas. Likewise, based on data presented in Fig. 5B, the overall cellular content (whole-cell extracts) of  $M_r$  43,000 Fas did not markedly change following VES treatments of Fas-insensitive MCF-7, MDA-MB-435, and MDA-MB-435 cells. In contrast, analyses of membrane (Fig. 6B) versus cytosolic (Fig. 6C) preparations of Fas-insensitive cells showed a reduction in cytosolic  $M_r$  43,000 Fas following VES treatment (Fig. 6C) and an increase in levels of  $M_r$  43,000 Fas in membrane preparations (Fig. 6B) following VES treatments. Furthermore, membrane preparations from MDA-MB-435 cells treated with 20  $\mu\text{g/ml}$  VES for 3, 6, 12, and 24 h showed a time-dependent increase in membrane levels of Fas expressed when compared to the 24 h VEH-treated cells (Fig. 6D). Taken together, these data suggest that VES treatment causes translocation of  $M_r$  43,000 Fas from the cytosol to the membrane in Fas-resistant cell lines.

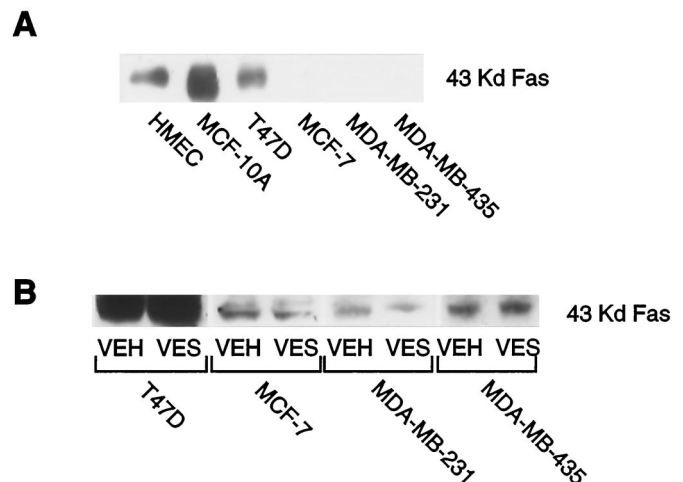


Fig. 5.  $M_r$  43,000 Fas expression in whole cell extracts obtained from ( $5 \times 10^6$  cells) Fas-sensitive (HMEC, MCF-10A, and T47D) and Fas-insensitive (MCF-7, MDA-MB-231, and MDA-MB-435) cells. A, to determine constitutive levels of  $M_r$  43,000 Fas, 50  $\mu\text{g}$  of whole cell extracts (prepared from each of the untreated cell lines) were electrophoresed, and proteins were immunoblotted and analyzed with Fas antibody (cell fractionation and Western immunoblot/ECL procedures are described in "Materials and Methods"). B,  $M_r$  43,000 Fas levels in 100  $\mu\text{g}$  of whole cell extracts from Fas-sensitive T47D and Fas-resistant MCF-7, MDA-MB-231, and MDA-MB-435 cells cultured for 24 h with VEH or 20  $\mu\text{g/ml}$  VES were determined by Western immunoblot/ECL procedures described in "Materials and Methods." Immunoblots were over exposed (as seen in the extracts from Fas-sensitive T47D) to detect  $M_r$  43,000 Fas in the Fas-resistant cell lines.

Validation of the Western immunoblot data were achieved using FACS analyses of membrane Fas expressed by MDA-MB-435 cells treated with VEH and VES (20  $\mu\text{g}/\text{ml}$ ), using a mouse monoclonal antihuman Fas reagent that specifically recognizes a  $M_r$  43,000 Fas molecule as the primary antibody and FITC-labeled goat antimouse immunoglobulin as the secondary antibody. These analyses showed that MDA-MB-435 cells treated with VES exhibited increased membrane Fas compared to VES- and VEH-treated control cells (MDA-MB-435 cells treated with VES and VEH and analyzed using an irrelevant primary antibody; Fig. 7, *left*). For these studies, VES-treated cells incubated with irrelevant primary antibody served as an important control for VES-treated cells incubated with anti-Fas. As

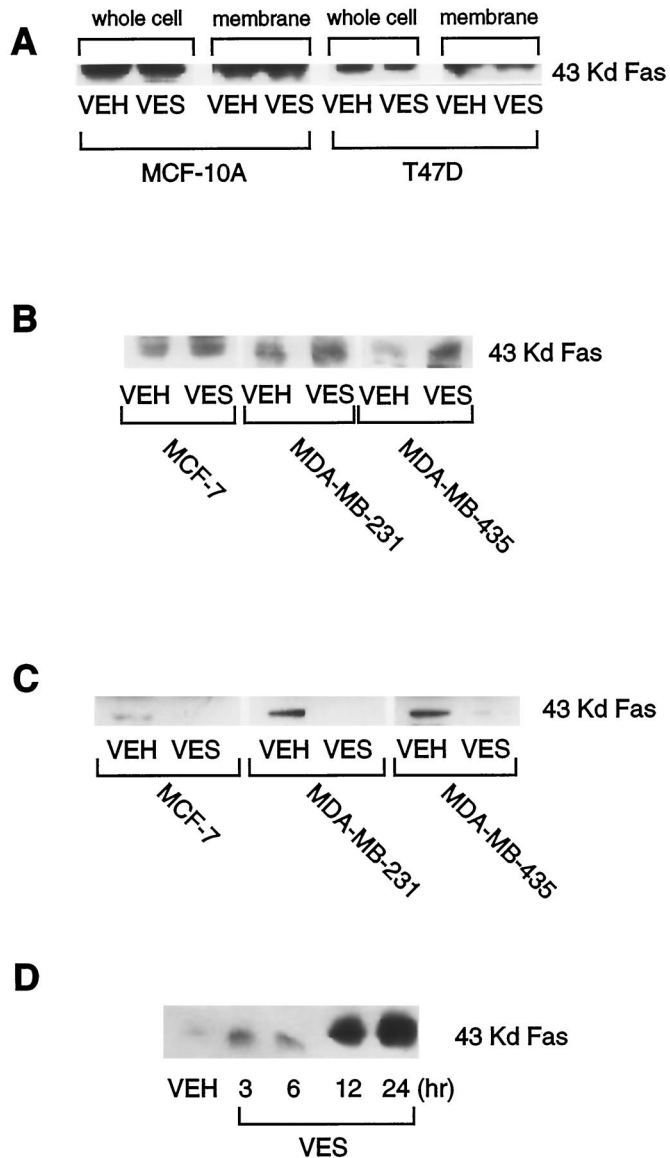


Fig. 6. Expression of  $M_r$  43,000 Fas in whole cell, cytosolic, and membrane preparations. Levels of  $M_r$  43,000 Fas expressed by Fas-sensitive (MCF-10A and T47D) and Fas-resistant (MCF-7, MDA-MB-231, and MDA-MB-435) cells were determined by Western immunoblot/ECL analyses as described in "Materials and Methods." A, levels of  $M_r$  43,000 Fas in 50  $\mu\text{g}$  of whole cell and membrane preparations obtained from Fas-sensitive MCF-10A and T47D cells treated with VEH or 20  $\mu\text{g}/\text{ml}$  VES for 24 h. Levels of  $M_r$  43,000 Fas in 100  $\mu\text{g}$  of membrane preparations (B) or 100  $\mu\text{g}$  of cytosolic preparations (C) obtained from Fas-resistant MCF-7, MDA-MB-231, and MDA-MB-435 cells treated with VEH or 20  $\mu\text{g}/\text{ml}$  VES for 24 h. D, levels of  $M_r$  43,000 Fas in 100  $\mu\text{g}/\text{ml}$  membrane preparations obtained from Fas resistant MDA-MB-435 cells cultured with 20  $\mu\text{g}/\text{ml}$  VES for 3, 6, 12, and 24 h (24-h VEH control).

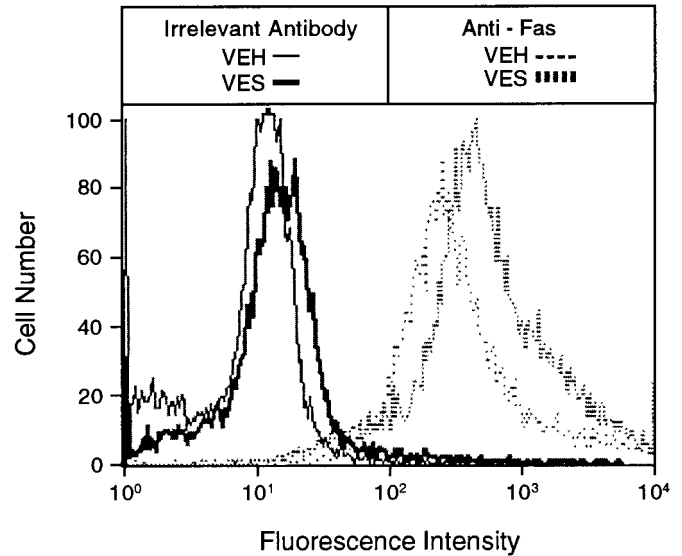


Fig. 7. FACS analyses of MDA-MB-435 cells for membrane Fas levels. MDA-MB-435 cells were cultured with VEH or VES (20  $\mu\text{g}/\text{ml}$ ) for 24 h. Cells were then processed for flow cytometry analyses as described in "Materials and Methods." Cell surface Fas levels were determined by FACS analyses with a 488-nm argon laser. Data were analyzed by FACScan and CELLQuest software. To determine Fas-related fluorescence, compare the nonspecific fluorescent shift obtained with VES treated cells analyzed with irrelevant antibody with the fluorescent shift obtained with VES-treated cells analyzed with anti-Fas.

can be seen in Fig. 7, a subpopulation of cells treated with VES plus irrelevant antibody exhibited a shift in fluorescence compared to the VEH- plus irrelevant antibody-treated control. Furthermore, VES-treated cells (no primary or secondary antibodies) showed a similar fluorescence shift (data not shown). These shifts most likely represent apoptotic cells induced by VES because dead or dying cells exhibit more natural fluorescence than nonapoptotic cells. However, the magnitude of the fluorescence shift in the VES-treated plus irrelevant antibody-assayed cells is less than the shift obtained for the VES-treated cells assayed with anti-Fas antibody (Fig. 7).

## DISCUSSION

Studies reported here demonstrate that: (a) VES converts Fas-resistant human breast cancer cells to Fas-responsive; (b) VES and agonistic anti-Fas antibody act synergistically to induce apoptosis of Fas-resistant cells; (c) functional knockout of Fas in Fas-resistant cells reduced VES-triggered apoptosis (Fas knockout verified by reduced  $M_r$  43,000 Fas expression); (d) VES treatment of Fas-resistant cells results in the translocation of  $M_r$  43,000 Fas from the cytosol to the cell surface; (e) VES treatments did not modify or only modestly modified the response of Fas-sensitive human breast cancer cells to anti-Fas agonistic antibody; and (f) VES-mediated apoptosis involves Fas signaling, confirming an earlier report by Turley *et al.* (18).

Human Fas is a transmembrane glycoprotein found on multiple cell types (20). Also known as APO-1 (for apoptosis-1) and CD95, Fas can mediate apoptosis when it is triggered by its natural ligand, Fas ligand, or when cross-linked with specific anti-Fas agonistic antibodies (reviewed in Ref. 42). Fas signaling is complex, and although Fas may be expressed, it may be inactive or may even signal accelerated cell growth (43).

Recent evidence suggests that tumor cells can exploit the Fas system by down-regulating the expression of functional Fas to resist Fas ligand-mediated killing by cytotoxic T cells, whereas some tumor cells, which exhibit a Fas-resistant phenotype themselves, may actually use the Fas system in mounting a "counterattack" for deletion of

tumor-infiltrating T-cells via up-regulation of tumor cell-derived Fas ligand (42–45). Recent studies indicated that anticancer drugs such as doxorubicin lead to the induction of the Fas system (46), and as tumors develop mechanisms of drug resistance, they also develop mechanisms of resistance to Fas-mediated apoptosis (36). Due to the importance of Fas signaling of apoptotic cell death of tumor cells, characterization of the molecular events involved in Fas resistance and identification of agents that can stimulate the expression of responsive Fas are needed.

In the *in vitro* studies reported here, VES is demonstrated to be an effective agent for switching Fas-resistant human breast cancer cells to a Fas-sensitive phenotype. Studies by Keane *et al.* (23) showed both primary normal HMECs and the immortalized nontumorigenic HMEC line (MCF10A) as well as T47D tumor cells to be Fas sensitive, whereas six breast cancer cell lines (MCF-7, ZR 75–1, MDA-MB-231, MDA-MB-435, MDA-MB-468, and SKBr3) were shown to be resistant to anti-Fas antibody-triggered apoptosis. Furthermore, Keane *et al.* (23) demonstrated the expression of a  $M_r$  43,000 Fas molecule by Fas-sensitive cells (HMECs, MCF10A, and T47D cells), which was not observed in Fas-resistant breast cancer cell lines. Our data are in complete agreement with the data of Keane *et al.* (23), regarding the responses to anti-Fas antibody-triggered apoptosis by HMECs, MCF10A, and T47D cells; the lack of anti-Fas antibody triggered apoptosis by MCF-7, MDA-MB-231, and MDA-MB-435 breast cancer cell lines that we studied; and the high constitutive expression of a  $M_r$  43,000 Fas molecule by the Fas-sensitive cells and the comparatively low level of expression of the  $M_r$  43,000 Fas molecule (namely, detectable only after long autorad exposure) by the Fas-resistant cells.

Although Keane *et al.* (23) showed that treatment of the Fas-resistant breast cancer cells with IFN- $\gamma$  for 4 days sensitized them to Fas-mediated apoptosis, they reported little or no increase in the cell surface expression of Fas following IFN- $\gamma$  treatment. In contrast to IFN- $\gamma$  sensitization, VES sensitization of Fas-resistant cells is correlated with an increase in cell surface Fas expression. In our studies, pretreatment of Fas-resistant cells with VES for 6 h rendered the cells sensitive to the apoptotic inducing actions of the anti-Fas agonistic antibody, and although analyses of Fas levels in whole cell extracts showed that the overall levels of Fas did not change, analyses of cytosolic and membrane enriched extracts showed cytosolic levels of Fas to decrease while membrane levels of Fas increased, suggesting that VES was inducing Fas-resistant cells to become Fas-sensitive via translocation of the  $M_r$  43,000 Fas molecule from the cytosol to the membrane. Thus, the studies by Keane *et al.* (23) and data reported here show that Fas-resistant human breast cancer cells can be rendered Fas-sensitive by two agents *in vitro*; namely, IFN- $\gamma$  treatment for 4 days which appears to sensitize the cells to anti-Fas antibody-triggered apoptosis via increased interleukin-1 $\beta$ -converting enzyme (caspase-1) expression, and VES treatment for 6 h which appears to sensitize the human breast cancer cells via increasing  $M_r$  43,000 Fas cell surface expression.

Additional support for VES-induced increased levels of cell surface Fas expression comes from FACS analyses, showing that VES-treated MDA-MB-435 cells, analyzed using a monoclonal reagent to Fas, express higher levels of Fas than VES-treated cells analyzed using an irrelevant control antibody. It is important to note that cells induced to undergo apoptosis exhibit an increase in “natural” cellular fluorescence that is detectable in the 488-nm range used to detect fluorescein-tagged antibodies, thus making it critical to distinguish between an increase in fluorescence due to increased apoptotic cell fluorescence from an increase in fluorescence due to an increase in cell surface Fas detected using an anti-Fas reagent in combination with a fluorescein-tagged secondary antibody.

In addition to sensitizing Fas-resistant breast cancer cells to anti-Fas triggered apoptosis, the combined treatment of Fas-resistant cells with VES and anti-Fas agonistic antibody produced synergisms. The molecular basis of this synergism is unknown, but it is interesting to note that VES activates latent TGF- $\beta$  and up-regulates TGF- $\beta$  receptor type II expression in human breast cancer cells (2, 3, 15). Furthermore, antisense functional knockouts of either TGF- $\beta$ 1 or TGF- $\beta$  receptor type II or antibody neutralization of TGF- $\beta$ s inhibits VES-induced apoptosis by ~50% (15). Pretreatment of glioma cells *in vitro* with TGF- $\beta$  has been shown to enhance Fas/APO-1 antibody-induced apoptosis (24). More recent studies show that endogenous expression of TGF- $\beta$ 1 by murine glioma cells inhibits growth and tumorigenicity and enhances Fas-mediated apoptosis and that combinations of anti-Fas plus TGF $\beta$ 1 result in a synergistic apoptotic effect (47).

A role for the Fas system in VES-triggered apoptosis was originally reported by Turley *et al.* (18). Although Turley *et al.* (18) did not address VES sensitization of Fas-resistant breast cancer cells and VES plus anti-Fas synergisms, they did show that VES up-regulated both Fas and Fas ligand protein levels in two human breast cancer cell lines (MDA-MB-231 and SKBR-3 cells) and that functional knockouts of the Fas system blocked VES-mediated apoptosis. In contrast to the studies reported here and by Keane *et al.* (23), Turley *et al.* (18) reported on a  $M_r$  48,000 Fas molecule. Turley *et al.* (18) analyzed 300  $\mu$ g of cell lysates per lane using rabbit polyclonal antihuman Fas (Santa Cruz Biotechnology). In our hands, this antibody (albeit a different lot number) detected a prominent  $M_r$  48,000 band the levels of which did not change following VES treatments and a faint  $M_r$  43,000 band. The data reported here used a different antibody to human Fas (rabbit polyclonal antibody reagent; Santa Cruz Biotechnology). This antibody reagent that was used for all of the Western immunoblotting analyses reported here detected a very prominent  $M_r$  48,000 molecule as well as a less prominent  $M_r$  43,000 molecule. The  $M_r$  48,000 molecule was judged to not represent functional Fas for the following reasons. The  $M_r$  48,000 molecule was constitutively expressed by all cells tested (with the notable exception of HMECs) at very high levels in both whole cell and membrane preparations (levels much higher than those observed for the  $M_r$  43,000 molecule); however, expression of the  $M_r$  48,000 protein did not correlate with Fas functionality. HMECs, which are Fas sensitive, expressed only the  $M_r$  43,000 molecule, and functional knockout experiments using antisense oligonucleotides to Fas reduced levels of the  $M_r$  43,000 molecule but not levels of the  $M_r$  48,000 molecule (Note:  $M_r$  48,000 data were not presented in the immunoblots to focus attention to the relevant  $M_r$  43,000 Fas molecule.) Additionally, VES treatments that sensitized Fas-resistant cells to anti-Fas agonistic antibody triggered apoptosis was correlated with marked changes in the expression of cytosolic and membrane  $M_r$  43,000 Fas, whereas no noticeable changes were observed in the expression of the  $M_r$  48,000 molecule following VES treatments in our experiments. Furthermore, although Fas was originally reported to be a  $M_r$  48,000 molecule in SKW6.4 human B cells using SDS-PAGE under nonreducing conditions (48), it has since been reported to be a  $M_r$  43,000 molecule in human fibroblasts (20) and human breast cancer cells (23). Thus, we judged the  $M_r$  48,000 molecule to not be relevant and chose to present data on the  $M_r$  43,000 molecule only (*i.e.*,  $M_r$  48,000 molecules were cutoff).

Studies reported here indicating a role for Fas in VES-triggered apoptosis agree, in principle, with those previously reported by Turley *et al.* (18). Functional knockout (antisense oligonucleotides or neutralizing antibody) of Fas reduced VES-induced apoptosis in MDA-MB-435 cells by ~50%. Experiments reported by Turley *et al.* (18) showed that neutralizing antibody to Fas reversed VES-induced apoptosis by 91% in MDA-MB-231 cells and 97% in SKBR-3. Although the same monoclonal antibody reagent was used in both

studies, differences in cell lines analyzed and methods of determining apoptosis may account for the differences. Although it is unclear at this time whether Fas-signaling is both necessary and totally sufficient for VES-triggered apoptosis in human breast cancer cells, our previous studies suggest that this is unlikely. We have shown that VES-triggered apoptosis involves the TGF- $\beta$  signaling pathway, because blockage of this signaling pathway inhibits VES-induced apoptosis to a similar degree as that we observed in our studies of blockage of the Fas signaling pathway; namely,  $\sim$ 50% (6, 15). Furthermore, our previous studies have indicated an important role for JNK in VES-mediated apoptosis (17). Although oligomerization of Fas results in the activation of JNK, the role of JNK in Fas-induced apoptosis is controversial (43, 49–51). Some studies support a role for JNK in the induction of cell death by Fas because blockage of JNK signaling prevents Fas-induced apoptosis (49, 50), whereas other studies appear to rule out a role for JNK in Fas-mediated apoptosis because Fas still kills cells under conditions in which JNK is blocked (43, 51). A recent report may reconcile the seemingly contradictory data in that Fas-triggered apoptosis can involve two pathways, a JNK-dependent pathway that is triggered by a novel Fas binding protein called Daxx, and a JNK-independent pathway that is triggered by the FADD-mediated pathway (52). The potential interactions among TGF- $\beta$ , Fas, and JNK, all of which appear to be involved in VES-triggered apoptosis of human breast cancer cells, remain to be sorted out.

We do not know why the Fas-sensitive cell lines used in these studies are resistant to VES-induced apoptosis. Because our previous studies have indicated that VES-induced apoptosis requires, at least in part, TGF- $\beta$  signaling (15), it is possible that VES-insensitive cells may have defective or inhibited TGF- $\beta$ -mediated apoptotic signaling pathways. For example, T47D cells are TGF- $\beta$  resistant and do not express TGF- $\beta$  receptor type II mRNA (40), and HMECs undergo DNA synthesis arrest but not apoptosis when treated with 10 ng of exogenous TGF- $\beta$ .<sup>4</sup> Furthermore, VES-mediated apoptosis requires, at least in part, prolonged activation of JNK, and prolonged activation of JNK does not occur in VES insensitive HMECs and T47D cells.<sup>4</sup>

After the submission of this manuscript, a paper was published on cell surface trafficking of Fas in *Science* (53). Fas trafficking in human breast cancer epithelial cells following VES treatment appears to differ from Fas trafficking reported by Bennett *et al.* (53) in at least two aspects. (a) Wild-type p53 is not required in VES-mediated increases of membrane Fas, because two of the cell lines (MDA-MB-231 and MDA-MB-435) studied have been reported to have p53 mutant proteins (54). (b) In contrast to p53-mediated Fas trafficking, which was observed to be rapid and transient (cell surface Fas levels increased within 1 h of treatment and returned to baseline levels 2 h after treatment), VES induced Fas membrane expression occurs within 3 h following treatment and remains for at least 24 h.

In summary, RRR- $\alpha$ -tocopheryl succinate (VES), a derivative of vitamin E, is demonstrated for the first time to be capable of reversing the Fas-resistant phenotype exhibited by Fas-resistant human breast cancer cells. Human breast cancer cells treated with VES for as briefly as 6 h become sensitized to anti-Fas agonistic antibody triggered apoptosis, and this VES-mediated sensitization is correlated with a translocation of cytosolic  $M_r$  43,000 Fas to the cell surface. Our hope is that further characterization of death receptor systems in human breast cancer cells and further characterization of VES-mediated proapoptotic effects will someday aid in the rationale selection of single, combined, or sequential chemotherapeutic regimes for improved treatment of human breast cancer.

<sup>4</sup> W. Yu, B. G. Sanders, and K. Kline, unpublished data.

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