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Mechanism of α-Tocopheryl Succinate-Induced Apoptosis of Promyelocytic Leukemia Cells

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Selective induction of apoptosis in tumor cells is important for treating patients with cancer. Because oxidative stress plays an important role in the process of apoptosis, we studied the effect of α-tocopheryl succinate (VES) on the fate of cultured human promyelocytic leukemia cells (HL-60). The presence of fairly low concentrations of VES inhibited the growth and DNA synthesis of HL-60 cells, and also induced their apoptosis via a mechanism that was inhibited by z-VAD-fluoromethylketone (z-VAD-fmk), an inhibitor of pan-caspases. VES activated various types of caspases, including caspase-3, 6, 8, and 9, but not caspase-1. VES triggered the reaction leading to the cleavage of Bid, a member of the death agonist Bcl-2 family, and released cytochrome c (Cyt.c) from the mitochondria into the cytosol by a z-VAD-fmk-inhibitable mechanism. VES transiently increased the intracellular calcium level $[Ca^{2+}]i$ and stimulated the release of Cyt.c in the presence of inorganic phosphate (Pi). However, high concentrations of VES (~100 μM) hardly induced swelling of isolated mitochondria but depolarized the mitochondrial membrane potential by a cyclosporin A (CsA)-insensitive mechanism. These results indicate that VES-induced apoptosis of HL-60 cells might be caused by activation of the caspase cascade coupled with modulation of mitochondrial membrane function.

Keywords: Apoptosis, HL-60 cells, α-tocopheryl succinate, mitochondria, membrane permeability transition, cytochrome c, Bid, caspase

Abbreviations: $[Ca^{2+}]i$, intracellular concentration of Ca^{2+}; CsA, cyclosporin A; Cyt.c, ferricytochrome c; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; HE, hydroethidine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzinidazol carbocyanine iodide; KRP, Krebs–Ringer-phosphate buffer; PKC, protein kinase C; ROS, reactive oxygen species; VE, α-tocopherol; VEA, α-tocopheryl acetate; VES, α-tocopheryl succinate; z-VAD-fmk, z-Val-Ala-Asp (OMe) fluoromethylketone

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INTRODUCTION

Selective killing of tumor cells is important for achieving effective chemotherapy in patients with cancer. For example, induction of apoptosis in transformed cells by some agents, such as all-trans retinoic acid, has attracted much attention as an effective chemotherapy for patients with leukemia. Although apoptosis plays an important role in determining the fate of cancer cells, the mechanism and factors leading to cell death differ from one cell type to another. However, activation of certain caspases plays a central role in the mechanism of apoptosis.[1,2] The activated caspases cleave various proteins, thereby triggering apoptosis in a wide variety of cells.

Reactive oxygen species (ROS) have been postulated to underlie the mechanism of apoptosis,[3–5] so endogenous as well as exogenous antioxidants are expected to influence the apoptotic process. For example, α-tocopherol (VE) and its derivatives are reported to influence apoptosis induced by oxidative stress.[6–14] In addition to their antioxidant activity, VE and related compounds inhibit protein kinase C (PKC) and activation of NF-κB, thereby affecting signal transduction and gene expression.[8,9] However, only limited information is available regarding the effects of VE and its derivatives, such as α-tocopheryl succinate (VES) and trolox, on the fate of transformed cells.[10–15] Among various derivatives of VE, VES has been shown to induce apoptosis of various types of cells.[10–15] Thus, VE and related compounds may have therapeutic potential to eliminate malignant cells by inducing their apoptosis.[16]

Changes in the expression of transcription factors and apoptotic factors and/or opening of the mitochondrial membrane permeability transition (MPT) pore[17] have been postulated to play a role in cellular events leading to apoptosis of certain types of cells.[15] Because VES administered to animals becomes localized in various organs and subcellular fractions,[18] this compound may interact with various components in cellular organelles, thereby inducing apoptosis. HL-60 cells have been shown to undergo differentiation and/or apoptotic death particularly when they are exposed to agents causing oxidative stress such as hydrogen peroxide.[3] The present study showed that VES could induce apoptosis of HL-60 cells by modulating mitochondrial function and activating the caspase cascade.

MATERIALS AND METHODS

Chemicals

Ferricytochrome c (Cyt.c), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), hydroethidine (HE), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzinidazol carbocyanine iodide (JC-1), ribonuclease (RNase) A and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies against Cyt.c and Bid were purchased from PharMingen (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Fluorogenic tetrapeptide substrates for caspases, including acetyl-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA) for caspase-1, acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA) for caspase-3, acetyl-Val-Glu-Ile-Asp-MCA (Ac-VEID-MCA) for caspase-6, acetyl-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA) for caspase 8 and acetyl-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA) for caspase-9, and the pan-caspase inhibitor z-VAD-fmk were obtained from the Peptide Institute (Osaka, Japan). VE, VES and α-tocopheryl acetate (VEA) were kindly supplied by Eisai Co. (Tokyo, Japan). All other chemicals used were of analytical grade and were obtained from Nacalai Tesque (Kyoto, Japan).

Cell Line

HL-60 cells were maintained at $0.2 \sim 1 \times 10^6$ cells/ml in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin.

Cells were cultured in a humidified incubator at 37°C under 5% CO_2/95% air and used for assays during the exponential phase of growth. Cells were routinely counted to maintain a low population density and their viability was assayed by the trypan blue exclusion method.[19]

Treatment of Cells with α-Tocopherol and its Derivatives

HL-60 cells (8×10^5 cells) were generally suspended in 2 ml of RPMI-1640 medium containing 10% fetal bovine serum and various reagents and then cultured in a CO_2 incubator. Prior to experiments, the cells were generally incubated for longer than 30 min.[19]

Evaluation of Cell Differentiation

Cultures were initiated at a density of 1.5×10^5 cells/ml in the presence or absence of 50 μM VES. After 24 h of incubation with VES, the cells were centrifuged onto a glass slide by a cytospin system (Kubota Inc., Tokyo). Differentiation of HL-60 cells to granulocytes was evaluated by staining for nonspecific esterase activity.[20] On each slide, at least 200 cells were counted under a light microscope.

Isolation of Rat Liver Mitochondria

After male Wistar rats weighing 200 g were fasted overnight, their livers were removed and homogenized in 250 mM sucrose containing 10 mM Tris–HCl buffer (pH 7.4) and 1 mM EGTA at 4°C. Mitochondria were isolated by the method of Hogeboom.[21]

Subcellular Fraction for Cyt.c Assay

After harvesting HL-60 cells (1×10^7 cells), they were suspended in 50 μl of ice-cold buffer A (250 mM sucrose, 20 mM HEPES buffer pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and 0.1 mM PMSF) and homogenized in a Teflon homogenizer. The homogenate was centrifuged at 750× *g* for 10 min at 4°C, after which the supernatant was centrifuged at 10,000× *g* for 15 min at 4°C. The resulting pellet (the mitochondrial fraction) was resuspended in buffer A and the supernatant was further centrifuged at 100,000× *g* for 60 min at 4°C. The final supernatant was used as the cytosolic fraction. Aliquots of 50 μl were employed for Western blot analysis of Cyt.c.

Assay of [^{3}H]-Thymidine Incorporation into DNA

Incorporation of [^{3}H]-thymidine into cellular DNA was measured by the method of Heck *et al.*[22] Briefly, HL-60 cells (1×10^5 cells/ml) were incubated with medium alone or medium containing the indicated amounts of VES and were pulsed with 1 mCi [^{3}H]-thymidine during the last 4 h of a 24-h incubation period. After washing the cells with PBS, 1 ml of 10% trichloroacetic acid (TCA) was added. Subsequent steps were conducted at 4°C. After centrifugation, the pellet was washed three times with 5% TCA, lysed with 0.5 N NaOH and neutralized with 1 N HCl. The insoluble fraction was trapped on a Whatman GF/C filter. After washing three times with 100% ethanol, the filter was dried and radioactivity was measured in a liquid scintillation counter.

Assay for Mitochondrial Swelling and Membrane Potential

Mitochondria (100 μg protein/ml) were incubated in 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M KCl (Tris–KCl solution) at 25°C. Large amplitude swelling of mitochondria (representing the MPT[17]) was measured spectrophotometrically at 540 nm using a dual beam spectrophotometer (Shimadzu UV-3000) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer.

For analysis of the mitochondrial membrane potential, mitochondria (100 μg protein/ml) were

incubated in Tris–KCl solution at 25°C in the presence of 0.1 nM cyanine dye diS-C3-(5). Then, the fluorescence intensity was recorded at 672 nm using excitation light at 650 nm in a fluorospectrophotometer (Hitachi 650-10LC) at 25°C.[23]

Analysis of Intracellular Generation of ROS

ROS were measured using oxidation-sensitive fluorescent probes, DCFH-DA and HE.[24] Before and after incubation with various concentrations of VES, cells were incubated for 30 min with 20 μM DCFH-DA or 10 μM HE in PBS containing 5 mM glucose, 0.3 mM $CaCl_2$ and 0.62 mM $MgCl_2$. Then cells were washed with PBS and analyzed with a FACS Calibur flow cytometer (Becton Dickinson).

Measurement of $[Ca^{2+}]i$

Values of $[Ca^{2+}]i$ were calculated from changes in the fluorescence intensity of Fura-2-loaded HL-60 cells. Loading with Fura-2 and the calibration of Fura-2-Ca^{2+} as a function of $[Ca^{2+}]i$ were carried out essentially as described previously.[25] Briefly, HL-60 cells (1×10^7 cells/ml) were incubated in Krebs–Ringer-phosphate buffer (KRP) containing 10 mM glucose and 5 μM Fura-2 AM for 1 h at 37°C. The incubated cells were washed twice with KRP solution, resuspended in glucose containing KRP, and kept on ice until use. The fluorescence intensity of Fura-2-loaded HL-60 cells (5×10^5 cells/ml) in KRP containing glucose was measured with a calcium analyzer (Jasco CAF 110) at 37°C, using excitation at 340 nm and emission at 500 nm, and $[Ca^{2+}]i$ was calculated by the method of Tsien.[25]

Analysis of DNA Fragmentation

The extent of DNA fragmentation was determined spectrophotometrically by the diphenylamine method.[19] After incubation with VES, cells were lysed in 200 μl of lysis buffer (10 mM Tris–HCl buffer pH 7.4, 10 mM EDTA and 0.5% Triton X-100) at 4°C for 10 min. The lysate was centrifuged at 13,000× *g* at 4°C for 20 min to separate intact and fragmented chromatin. Both the pellet and the supernatant were precipitated with 6% perchloric acid at 4°C for 30 min, and the precipitates were sedimented at 13,000× *g* for 20 min at 4°C. The DNA specimens were heated at 70°C for 20 min in 50 μl of 6% perchloric acid, and were mixed with 100 μl of 1.5% diphenylamine solution containing 1.5% sulfuric acid and 0.01% acetaldehyde in acetic acid. After overnight incubation at 30°C in the dark, measurement was done spectrophotometrically at 600 nm and the percentage of fragmented DNA was calculated.

DNA fragmentation was also detected by agarose gel electrophoresis.[26] The lysate was treated with 400 μg/ml RNase A at 37°C for 1 h and 400 μg/ml proteinase K at 37°C for 1 h. DNA samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol. Total DNA in the aqueous phase was then electrophoresed at 100 V through a 2% agarose gel containing 0.1 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate buffer, pH 8.3, containing 2 mM EDTA). DNA bands were visualized under ultraviolet illumination and photographed on a Polaroid 667 film.

Western Blot Analysis

Cell lysates were prepared as described elsewhere.[26] Cells (2×10^6 cells) were dissolved in SDS-sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and boiled at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel were transferred onto an Immobilon filter (Millipore Co.), and then incubated with the primary antibody (1:1000 dilution for Cyt.c, 1:500 dilution for Bid) and subsequently with the horseradish peroxidase-linked secondary antibody (1:2000 dilution for Cyt.c, 1:50,000 dilution for Bid). Detection was done using an ECL plus kit (Amersham). The protein concentration was determined by the method of

Lowry *et al.*[27] using bovine serum albumin as a standard.

Assay of Caspase Activity

Caspase activity was determined as described previously.[26] Incubation was done in 20 mM HEPES buffer (pH 7.5) containing 0.1 M NaCl and 5 mM dithiothreitol at 37°C, using 10 μM of the appropriate substrate: Ac-YVAD-MCA, Ac-DEVD-MCA, Ac-VEID-MCA, Ac-IETD-MCA or Ac-LEHD-MCA for caspase-1, 3, 6, 8 and 9, respectively. One unit of the enzyme activity was defined as the amount of enzyme required for the hydrolysis of 1 μmol substrate per 1 h.

RESULTS

Effect of VES on Cell Growth, DNA Synthesis and Differentiation

The effect of VES on the growth and DNA synthesis of HL-60 cells was investigated. Under the standard conditions, HL-60 cells showed a normal growth pattern with a doubling time of about 24 h. The presence of VES inhibited the growth of HL-60 cells in a dose-dependent manner (Figure 1A), and cell growth was completely inhibited by VES at concentrations higher than 30 μM. VE had no appreciable effect on the growth of HL-60 cells. In order to determine the extent of apoptosis induced by VES, cells were analyzed by flow cytometry at various times after treatment. Typically arrested cells were not observed after treatment with 20 μM VES for 48 h, although the number of apoptotic cells increased during the incubation (data not shown). Therefore, the effect of VES on DNA synthesis of HL-60 cells was examined. As shown in Figure 1B, incorporation of [^{3}H]-thymidine into DNA was also inhibited by VES in a concentration dependent manner (Figure 1B).

Since some reagents, such as retinoic acid and phorbol ester, induce the differentiation of HL-60 cells,[28] the effect of VES on the properties of HL-60 cells was also investigated. VES was found to have induced morphological changes characteristic of apoptosis, such as cell shrinkage and nuclear fragmentation (Figure 2). Such changes in morphology occurred with a concomitant increase in the number of cells showing fragmentation of DNA and the nucleus. However,

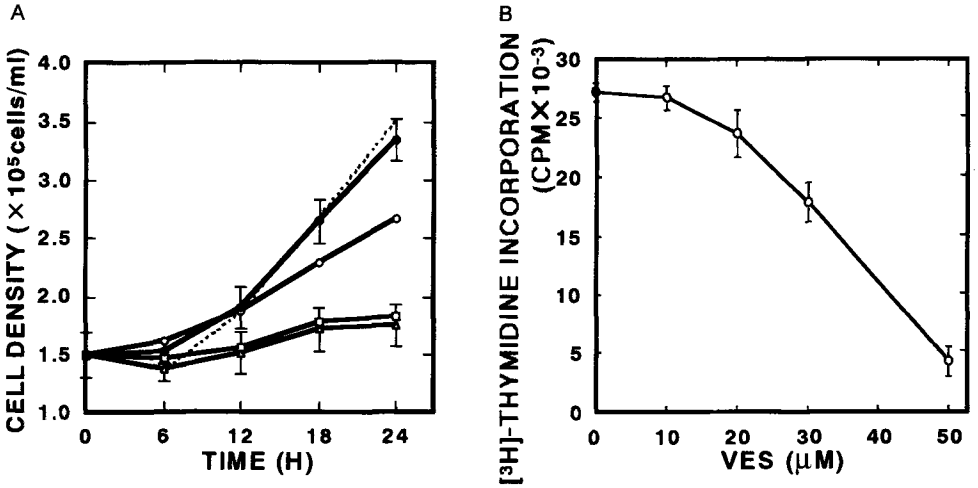


FIGURE 1 *Effect of VES on the growth and DNA synthesis of HL-60 cells.* HL-60 cells were exposed to 0 (●), 10 (○), 30 (□), and 50 μM of VES (△) or 50 μM of VE (---) as described in Materials and Methods. At the indicated times, the number of viable cells was determined by the trypan blue exclusion test (A). The effect of VES on the incorporation of [^{3}H]-thymidine into DNA was measured as described in Materials and Methods (B). Experiments were performed at least three times with similar results.

VES did not increase the number of granulocytes, as measured by staining for nonspecific esterase activity.[20]

Effect of VES on Cellular DNA

Incubation of HL-60 cells with VES induced the fragmentation of cellular DNA in a dose- and time-dependent manner (Figure 3). Kinetic analysis revealed that DNA fragmentation became apparent at 12–24 h after incubation with 50 μM VES. When incubation was done with VES at concentrations higher than 30 μM for 24 h, DNA ladder was observed on agarose gel electrophoresis. In contrast, VE and VEA (data not shown) had no appreciable effect on cellular DNA even at a concentration of 50 μM.

Effect of z-VAD-fmk on VES-Induced DNA Fragmentation

As caspases play crucial roles in the process of apoptosis, we investigated the effect of z-VAD-fmk, a pan-caspase inhibitor,[29,30] on VES-induced

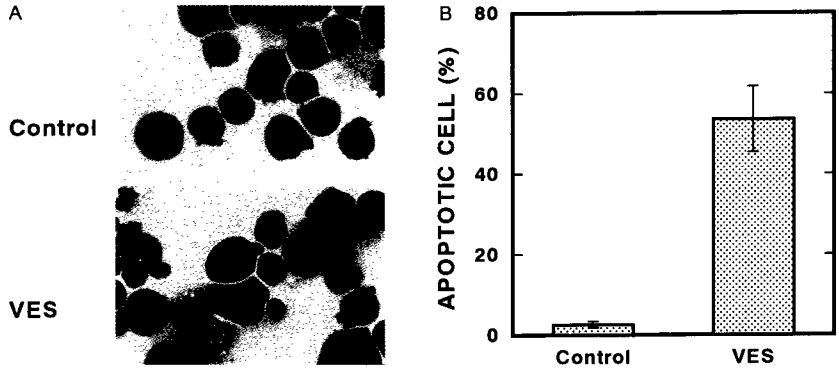


FIGURE 2 *Effect of VES on the morphology of HL-60 cell.* HL-60 cells were incubated with 50 μM VES for 48 h (A) and the percentage of cells showing fragmented nuclei (B) was calculated. Other conditions were as in Figure 1. Results show mean ± SD derived from three separate experiments. (See Color plate VII at the end of this issue.)

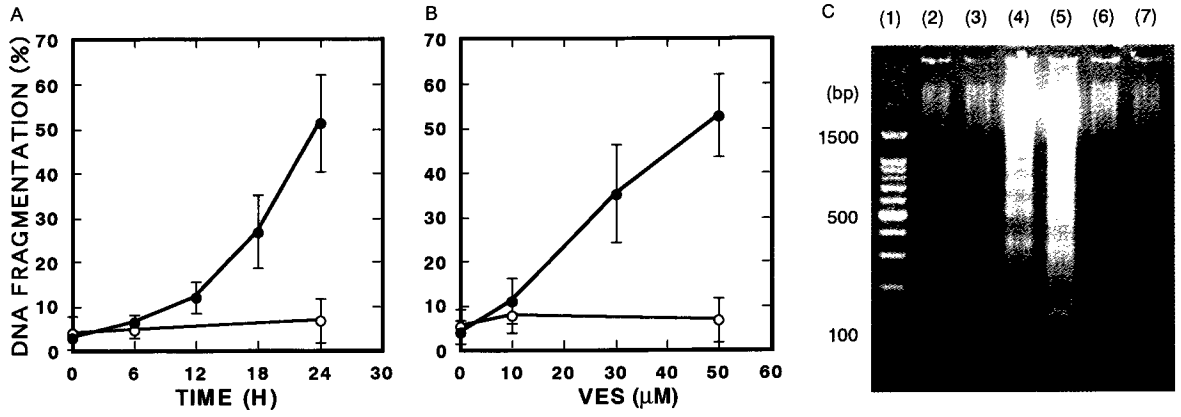
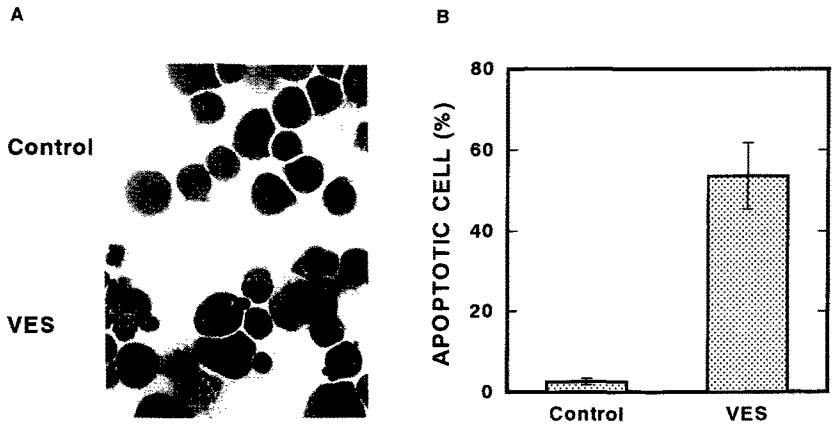


FIGURE 3 *DNA fragmentation in VES-treated HL-60 cells.* Cells were incubated in the presence (●) or absence (○) of 50 μM VES at 37°C (A). At the indicated time, fragmented DNA in the cells was determined by the diphenylamine method. The effect of various concentrations of VES on the fragmentation of DNA was also observed (B). (●), VES; (○), without VES. The cells (8×10^5 cells) were incubated with various concentrations of VES and VE for 24 h and then subjected to 2% agarose gel electrophoresis. Lines 1, 2, 3, 4, 5, 6 and 7 are molecular size markers, control cells, cells treated with 10, 30 and 50 μM VES, and cells treated with 10 and 50 μM VE, respectively (C).



Color Plate VII (see page 412, figure 2) *Effect of VES on the morphology of HL-60 cell.* HL-60 cells were incubated with 50 μM VES for 48 h (A) and the percentage of cells showing fragmented nuclei (B) was calculated. Other conditions were as in Figure 1. Results show mean ± SD derived from three separate experiments.

apoptosis of HL-60 cells. DNA fragmentation induced by VES was effectively suppressed by pretreatment of cells with 30 μM z-VAD-fmk for 1 h (Figure 4). This result indicated that activation of caspases was also crucial for VES-induced apoptosis of HL-60 cells.

Effect of VES on the Activity of Caspases

Since VES-induced apoptosis was inhibited by z-VAD-fmk, we also examined the effect of VES on the activation of caspase-1, 3, 6, 8 and 9 in HL-60 cells using specific substrates. Cellular activities of caspases significantly increased after incubation with 50 μM VES for 24 h. VES activated caspase-3 in a time- and concentration-dependent manner that was concomitant with DNA fragmentation (Figure 5). VES also activated caspase-6 strongly and caspase-8 and 9 slightly. However, VES did not activate caspase-1.

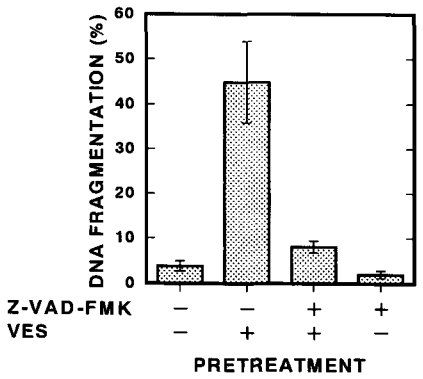


FIGURE 4 *Effect of z-VAD-fmk on the VES-induced fragmentation of cellular DNA.* Cells were incubated in the presence or absence of 30 μM z-VAD-fmk for 1 h and concurrently with or without 100 μM VES for 6 h. DNA fragmentation was determined by the diphenylamine method. Experiments were carried out at least three times with similar results.

Release of Cyt.c from Mitochondria by VES

Since VES activated caspase-9, Cyt.c might possibly be released from the mitochondria into the cytosol.[31] As expected, a significant fraction of the Cyt.c was released from the mitochondria of VES-treated cells (Figure 6). In this context, a preliminary experiment showed depolarization of the mitochondria in HL-60 cells by VES using JC-1.[32] These results indicated that VES induced the mitochondrial MPT and that the release of Cyt.c from the mitochondria might play an important role in VES-induced apoptosis of HL-60 cells.

Effect of VES on Cellular Bid

Bid cleaved by caspase-8 directly triggers the release of Cyt.c from the mitochondria[33,34] via

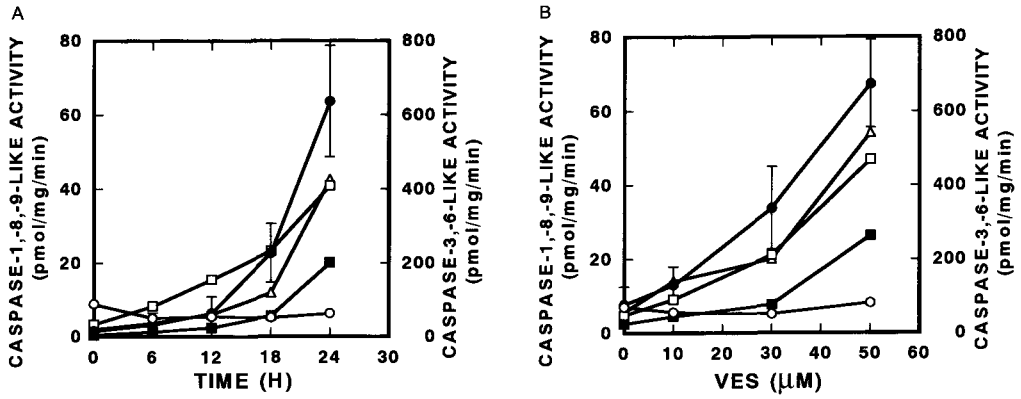


FIGURE 5 *Effect of VES on the caspase activity in HL-60 cells.* Cells were incubated with 50 μM VES and time-dependent changes in the activity of caspase-1 (○), 3 (●), 6 (■), 8 (□) and 9 (△) were determined (A). The effect of varying doses of VES on the activity of caspase-1 (○), 3 (●), 6 (■), 8 (□) and 9 (△) was also determined (B). Cell extracts (50 μl) were incubated with the specific fluorogenic peptide substrates (10 μM) at 37°C for 1 h.

a Bcl-2-inhibitable mechanism,[31] thus relaying an apoptotic signal from the cell surface to the mitochondria.[34] Therefore, we studied the effect of VES on cellular levels of cleaved Bid. Western blot analysis revealed that Bid was present as a 22 kDa protein in intact HL-60 cells. Incubation of cells with VES resulted in the formation of 16 and 15 kDa fragments of Bid (Figure 7). The cleavage of Bid was inhibited by the presence of z-VAD-fmk.

Effect of VES on the Generation of ROS by HL-60 Cells

It has been reported that VE and its derivatives affect the process of apoptosis induced by oxidative stress.[6–14] Thus, we also studied the effect of VES on the generation of ROS by HL-60 cells. Figure 8 shows the change in fluorescence intensity of DCFH which is an oxidation-sensitive fluorescent probe.[24] When exposed to 30 μM VES for 24 h, the fluorescence intensity increased significantly, suggesting the generation of ROS (Figure 8A). In contrast, the generation of ROS was not enhanced by 30 μM VE (data not shown). The generation of ROS was dependent on both the VES concentration and the incubation time (Figure 8B). However, ROS generation only became apparent after the onset of the cellular events leading to apoptosis. Moreover, no detectable change in the generation of the superoxide radicals was found to occur as measured by HE,

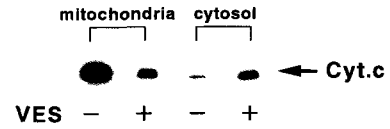


FIGURE 6 *Effect of VES on the distribution of Cyt.c in HL-60 cells.* Cells were treated with 50 μM VES for 24 h. Then, the Cyt.c in mitochondrial and cytosolic fractions was determined by Western blotting. Experiments were carried out at least three times with similar results.

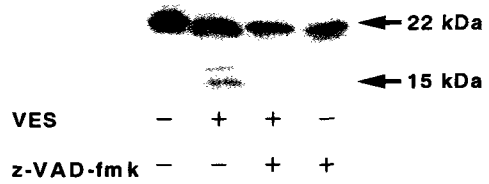


FIGURE 7 *Effect of VES on the processing of Bid.* Cells were incubated in the presence or absence of 100 μM z-VAD-fmk and/or 50 μM VES for 24 h and then cell lysates (50 μl) were analyzed by immunoblotting. Experiments were carried out at least three times with similar results. 22 kDa, pro-Bid; 15 kDa, processed Bid.

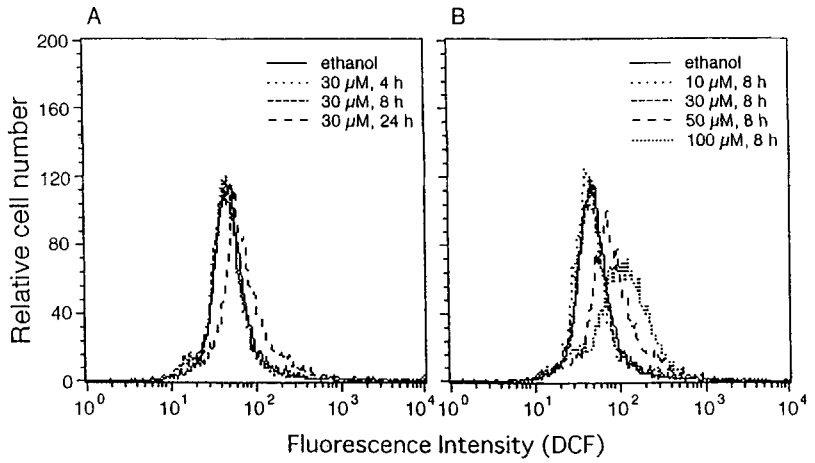


FIGURE 8 *Generation of ROS by VES-treated cells.* ROS were measured using an oxidation-sensitive fluorescent probe (DCFH-DA).[24] Cells were treated with various concentration of VES and incubated for 30 min with 20 μM DCFH-DA at 37°C. After washing with PBS, the fluorescence intensity of the cells was analyzed with a FACS Calibur flow cytometer. (A) Time-dependence of the effect of 30 μM VES; (B), VES concentration-dependence after 8 h of treatment.

a specific probe for superoxide[24] (data not shown). These results suggested that ROS might not be involved in the mechanism of VES-induced apoptosis.

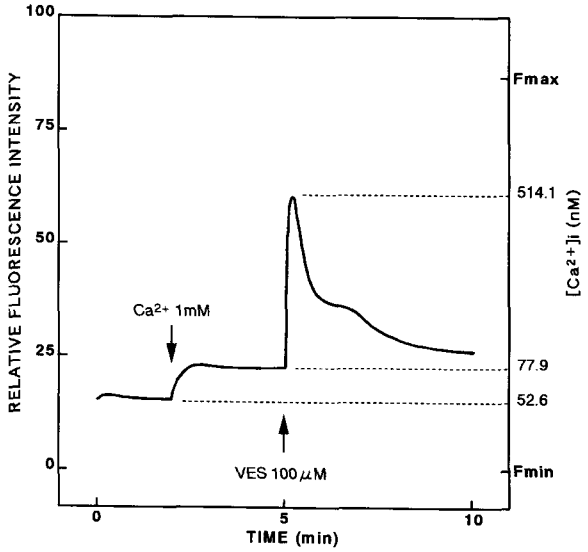


FIGURE 9 *Effect of VES on the Ca^{2+} status in HL-60 cells.* HL-60 cells (1×10^7 cells/ml) were loaded with 5 μM Fura-2 in KRP solution containing 10 mM glucose at 37°C and the fluorescence intensity of Fura-2-loaded cells was monitored by a calcium analyzer (Jasco CAF 110) at 37°C. Then, at the indicated times, 1 mM $CaCl_2$ and 100 μM VES were added to the medium, and the change in fluorescence intensity was monitored. Experiments were carried out at least three times with similar results.

Effect of VES on $[Ca^{2+}]i$ Status of HL-60 Cells

The presence of Ca^{2+} and inorganic phosphate (Pi) has been known to induce MPT of mitochondria, thereby releasing Cyt.c by a CsA-sensitive mechanism.[17] To gain further insights into the mechanism of Cyt.c release, the effect of VES on the $[Ca^{2+}]i$ of HL-60 cells was examined by measuring changes of Fura-2 fluorescence.[5,25] VES was found to increase the $[Ca^{2+}]i$ transiently in a concentration-dependent manner (Figure 9).

Effect of VES on Mitochondrial Functions

Because VES depolarized the mitochondrial membrane potential in HL-60 cells and released Cyt.c, the effect of VES on the function of isolated mitochondria was examined. VES was found to decrease the membrane potential of mitochondria in a CsA-insensitive manner (Figure 10). However, Cyt.c was not released from mitochondria by treating cells with 100 μM VES for 10 min. Large amplitude swelling of mitochondria and Cyt.c release were induced in the presence of Ca^{2+} and Pi by some mechanism that was inhibited by CsA.[10,16,34] These results indicated that VES may

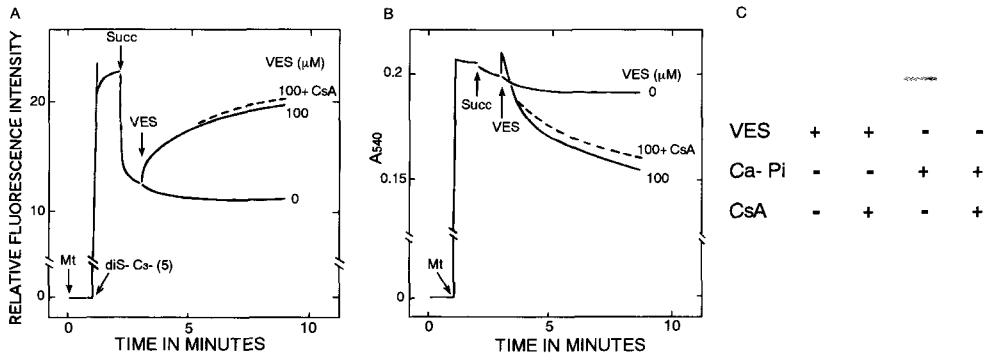


FIGURE 10 *Effect of VES on mitochondrial membranes and Cyt.c.* Rat liver mitochondria were loaded with 0.1 nM dis-C3-(5) in the presence or absence of 1 μM CsA (A). Then 5 mM succinate and 100 μM VES were added to the mixture. The mitochondrial membrane potential was monitored by measuring the change in fluorescence intensity. Large amplitude swelling of mitochondria was induced by adding 100 μM VES and the change in optical density at 540 nm was monitored in the presence or absence of 1 μM CsA (B). Cyt.c released from mitochondria into the medium was also determined by Western blot analysis after incubation in 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M KCl and 5 mM succinate at 25°C for 10 min. Mitochondria were treated with 100 μM VES or 200 μM Ca^{2+} plus 2 mM Pi in the presence or absence of 1 μM CsA.

not directly trigger the release of Cyt.c from the mitochondria.

DISCUSSION

The present study clearly demonstrated that VES inhibits DNA synthesis and modulates mitochondrial functions, thereby releasing Cyt.c into the cytosol, activating caspase-3, and inducing apoptosis of HL-60 cells without triggering their differentiation to granulocytes. Kinetic analysis revealed that the MPT coupled with an increase of $[Ca^{2+}]i$ and generation of caspase-8-cleaved Bid may have enhanced the release of Cyt.c from the mitochondria.

During the preparation of this manuscript, we came across a report which stated that VES also induced apoptosis of Jurkat T cells by activating caspase-3.[21] Because VES-induced depolarization of the mitochondria in Jurkat T cells occurred after the rupture of lysosomes, mitochondrial depolarization has been postulated to be the result of lysosomal damage.[12] To test this possibility, we analyzed the effects of VES on cellular events occurring in HL-60 cells, such as decreased accumulation of acridine orange in the lysosomes,[35] depolarization of the mitochondrial membrane potential of cells using JC-1,[32] and the exposure of phosphatidylserine on the cell surface membranes.[36] When cells were exposed to 30 μM VES, the accumulation of lysomotrophic acridine orange in the lysosomes decreased as analyzed by flow cytometry. Moreover, a significant fraction of phosphatidylserine became exposed on the outer surface of the cell membranes after treatment with 30 μM VES and depolarization of the mitochondrial membranes was also induced by treating HL-60 cells with VES. However, VES-induced apoptosis of HL-60 cells was not affected by the presence of 10 μM CA-074Me, a specific inhibitor of cathepsin B, or by 10 μM E-64d, a membrane permeable inhibitor of lysosomal cysteine protease. The activation of caspase-3 by lysosomal enzymes was reported to be suppressed by 1 μM E-64.[37] These observations indicate that the mitochondrial MPT and the subsequent release of Cyt.c might be the major cause of VES-induced apoptosis in HL-60 cells.

Carbon tetrachloride has been known to exhibit hepatotoxicity by generating its carbon centered radicals. VES also revealed a unique property to inhibit liver injury caused by carbon tetrachloride in the rat.[38] Because an equal dose of VE had no appreciable effect on the liver injury induced by carbon tetrachloride, the antioxidant activity of the VE moiety of VES and/or metabolite of VE, a hydrolyzed metabolite of VES, might not be responsible for the protective effect of this ester. In this context, cholesteryl hemisuccinate, but not cholesterol, also exhibited a similar protective effect against carbon tetrachloride-induced liver injury.[38] Preliminary experiments in this laboratory have shown that 75 μM cholesteryl hemisuccinate also induces apoptosis of HL-60 cells. These observations suggest that succinate esters of some hydrophobic compounds, such as VE and cholesterol, may interact with cellular proteins involved in signaling pathway(s) that influence the cytotoxicity of carbon tetrachloride-derived radicals. Thus, it is not surprising that VES affected some signaling pathway(s) leading to cell death.

Intravenously administered VES has been shown to accumulate in microsomes and mitochondria in its intact form.[39] It should be noted that, when hydrolyzed by certain esterases, equimolar amounts of VE and succinate would be generated in cells. Because succinate is a potential substrate for mitochondrial respiration, cells incubated with VES would utilize succinate to maintain their ATP levels, which favors the survival of cells. Preliminary experiments revealed that the presence of fairly high concentrations (~50 μM) of both VE and succinate had no appreciable effect on the viability of HL-60 cells. Thus, hydrolyzed products of VES might not be responsible for the induction of apoptosis. Another possible explanation for the cytotoxicity

of VES would be the unique structure of the VES molecule. Because VES interacts with cellular membranes including lysosomes and mitochondria, the amphipathic nature of this ester might directly affect the stability and function of these membranes and modulate membrane-bound enzymes and/or phospholipids,[40] thereby initiating a sequence of events leading to apoptosis. This possibility should be studied further.

The present study also demonstrated that both activation of caspase-8 and the induction of DNA fragmentation were inhibited by z-VAD-fmk. Recent studies have revealed that caspase-8 is activated by a Fas-receptor-independent mechanism.[41] Furthermore, caspase-6 and 7 have been shown to trigger the release of Cyt.c without processing Bid.[42] Thus, the initial reaction of the caspase activation and the mechanism through which caspase-8 and -6 are activated by VES also remain to be studied.

We also showed that the cellular generation of ROS remained unchanged during 8 h of incubation with 30 μM VES, but increased slightly after 24 h when the cellular events leading to apoptosis had already occurred. Furthermore, antioxidants, such as VE, VEA, and N-acetylcysteine, had no appreciable effect on VES-induced apoptosis (data not shown). These results suggest that the inhibition of cell growth and induction of apoptosis by VES might not be a consequence of its antioxidant activity. In conclusion, the mechanism for the induction of apoptosis in HL-60 cells and Jurkat T cells by VES suggests that this agent may have a therapeutic potential for treating patients with leukemia. This possibility is currently being investigated.

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