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Cholesteryl-hemisuccinate-induced apoptosis of promyelocytic leukemia HL-60 cells through a cyclosporin A-insensitive mechanism

Katsuhiko Yamada[a,b], Kayo Arita[b,c], Hirotsugu Kobuchi[d], Shinji Yamamoto[a], Tamotsu Yoshioka[b], Hiroshi Tamai[a], Kozo Utsumi[b,*]

[a]*Department of Pediatrics, Osaka Medical College, 2-7 Daigakucho, Takatsuki 569-8686, Osaka, Japan*
[b]*Institute of Medical Science, Kurashiki Medical Center, Kurashiki 710-8522, Japan*
[c]*Doonan Institute of Medical Science, Ishikawa-cho, Hakodate 041-8502, Japan*
[d]*Department of Cell Chemistry, Institute of Molecular and Cell Biology, Okayama University Medical School, Okayama 700-8558, Japan*

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Abstract

We reported previously that α-tocopheryl-succinate (VES) induced apoptosis of cultured human promyelocytic leukemia cells (HL-60) (Free Radic Res 2000;33:407–18). We have now studied the effect of cholesteryl-hemisuccinate (CS) on the fate of HL-60 cells to clarify whether CS has an effect similar to that of VES. CS inhibited the growth of HL-60 cells without differentiation to granulocytes and induced DNA fragmentation and ladder formation. CS inhibited the phosphorylation of pleckstrin homology domain-containing protein kinase B (Akt) and initiated the activation of a caspase cascade. CS triggered the reaction leading to the cleavage of Bid and also released cytochrome *c* (Cyt. *c*) from mitochondria. In addition, CS induced mitochondrial membrane depolarization and translocation of Bax to mitochondria in HL-60 cells. However, CS did not induce an increase in the concentration of intracellular calcium ions in HL-60 cells. The membrane depolarization, Cyt. *c* release, and DNA fragmentation were inhibited by z-VAD-fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor, but not by cyclosporin A, an inhibitor of membrane permeability transition. These results suggested that CS-induced apoptosis of HL-60 cells might be caused by inhibiting Akt phosphorylation following cleavage of Bid through caspase-8 activation and subsequently via an Apaf complex–caspase cascade pathway.

Keywords: Akt; Apoptosis; Bid; Caspase; Cholesteryl-hemisuccinate; Cytochrome *c*; HL-60 cells; Membrane permeability transition

1. Introduction

Recently, the possible role of apoptosis in the pathogenesis of a wide array of diseases has been highlighted. The modulation of this pivotal process has become an emerging new concept in the treatment of malignant disorders. Thus, the selective induction of apoptosis of target cells is important for treating patients with cancer.

Succinate esters of cholesterol and α-tocopherol interact with intracellular and membrane lipids and affect enzyme activities, such as ATPase [1]. These effects were observed with intact VES and CS, but not with α-tocopherol, cholesterol, or succinate. It has been reported that succinate esters of α-tocopherol selectively inhibit cancer cell proliferation, but no adverse effect on the growth of murine normal bone marrow cells was observed [2–7]. In a previous study, we found that VES induced apoptosis of promyelocytic leukemia cells (HL-60) in a way that involved the mitochondrial MPT pathway [4]. These results suggested that CS might also induce a selective apoptosis of HL-60 cells.

Accumulated evidence shows that apoptosis is a cellular death process involving the sequential activation of a series of caspases and an endonuclease. Mitochondria have been shown to play a key role in apoptosis induction. However, the sequence of changes that occur in mitochondria in the initial steps of apoptosis has not been elucidated clearly.

* Corresponding author. Tel.: +81-86-426-8618; fax: +81-86-426-8618.
E-mail address: utsumiko@mx3.kct.ne.jp (K. Utsumi).

Abbreviations: Akt, pleckstrin homology domain-containing protein kinase B; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; CS, cholesteryl-hemisuccinate; CsA, cyclosporin A; Cyt. *c*, ferricytochrome *c*; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzinidazol carbocyanine iodide; KRP, Krebs-Ringer-phosphate buffer; MPT, membrane permeability transition; pCPT-cAMP, 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate; PKC, protein kinase C; ROS, reactive oxygen species; VES, α-tocopheryl-succinate; z-VAD-fmk, z-Val-Ala-Asp(OMe) fluoromethylketone.

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One of the important events is the opening of the MPT pore, a CsA-sensitive mitochondrial channel [8–10]. In this context, it has been suggested that regulation of the MPT pore may play a role in intracellular calcium homeostasis in a variety of forms of cell death [11,12].

Based on these experimental findings, we have investigated the effect of CS on HL-60 cells and analyzed the mechanism of CS-induced apoptosis of the cells. Here we show that CS induced the apoptosis of HL-60 cells through suppression of Akt phosphorylation [13,14] following cleavage of Bid and subsequently by initiating apoptosis through the activation of a caspase cascade in a CsA-insensitive mechanism.

2. Materials and methods

2.1. Chemicals

CS, VES, Cyt. *c*, JC-1, ribonuclease (RNase) A, proteinase K, and pCPT-cAMP were obtained from the Sigma Chemical Co. Monoclonal antibody against Cyt. *c* was purchased from PharMingen, antibodies against Bcl-2, Bax, and Bid from Santa Cruz Biotechnology, and antibody against phospho-Akt from Cell Signaling Technology (New England Biolabs, Ltd.). Fluorogenic tetrapeptide substrates, such as 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, a pan-caspase inhibitor, z-VAD-fmk, and an inhibitor of caspase-8, Ac-ITED-CHO, were obtained from the Peptide Institute. MitoSensor (ApoAlert^{TM}) was purchased from Clontech. All other chemicals were of analytical grade and were obtained from Nacalai Tesque.

2.2. Cell line

HL-60 cells were maintained at 0.2 to 1×10^6/mL in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies Inc.), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were grown in a humidified incubator at 37° under 5% CO_2/95% air and used for assays during the exponential growth phase. Cells were routinely counted to maintain a low population density, and their viability was assayed by the trypan blue exclusion method [15].

2.3. Treatment of cells with cholesterol and CS

HL-60 cells (8×10^5) were generally suspended in 2 mL of RPMI-1640 medium containing 10% fetal bovine serum and various reagents and then incubated in a CO_2 incubator. Before adding the reagents, preincubation was normally performed for at least 1 hr. Cultures were initiated at a density of 1.5×10^5 cells/mL in the presence or absence of 50 μM cholesterol or its derivatives, which were dissolved in ethanol and added to the medium [15]. Cyclosporin A, z-VAD-fmk, and Ac-ITED-CHO were dissolved in DMSO.

2.4. Evaluation of differentiated cells

After 24 hr of incubation with cholesterol or CS, cells were centrifuged onto a glass slide by a cytospin system (Kubota Inc.). Differentiation of HL-60 cells to granulocytes was evaluated by staining for nonspecific esterase activity [16]. On each slide, at least 200 cells were counted under a light microscope.

2.5. Discrimination of apoptotic cells by flow cytometry and sorting

Flow cytometric analyses of apoptotic cells and the cell cycle were carried out as previously described [17]. Briefly, HL-60 cells (2×10^6) were fixed in 200 μL of 70% ethanol at 4° for 4 hr, and then were incubated in 40 mL of phosphate-citrate buffer (pH 7.5) at room temperature for 30 min. Following treatment with 0.1 μg/mL of RNase A in 100 μL of PBS at 37° for 30 min, the cells were stained with 50 μg/mL of propidium iodide in 1 mL of PBS at 37° for 30 min in the dark. Analytic flow cytometric measurements were performed using a FACScan flow cytometer with argon laser excitation at 488 nm (Becton Dickinson), and fluoresence (FL2;DNA) was detected through a 564–606 nm band pass filter. Twenty thousand cells were analyzed in each sample.

2.6. Subcellular fraction for Cyt. c assay

After harvesting HL-60 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 phenylmethylsulfonyl fluoride] (1×10^7 cells) and homogenized in a Teflon homogenizer. The homogenate was centrifuged at 750 *g* for 3 min at 4°. The supernatant was then centrifuged at 10,000 *g* for 15 min at 4°. The resulting pellet (mitochondrial fraction) was resuspended in buffer A. The supernatant was further centrifuged at 100,000 *g* for 60 min at 4°. The final supernatant represented the cytosolic fraction. Aliquots of 50 μL were used for western blot analysis of Cyt. *c* [18].

2.7. 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 of Fura-2 and the calibration of Fura-2-Ca^{2+} as a

function of $[Ca^{2+}]_i$ were carried out essentially as described previously [4,19]. Briefly, HL-60 cells (1×10^7/mL) were incubated in KRP containing 10 mM glucose and 5 μM Fura-2 AM for 1 hr at 37°. The incubated cells were washed twice with KRP solution, resuspended in glucose containing KRP, and kept on ice until used. Excitation and emission wavelengths used were 340 and 500 nm, respectively. The fluorescence intensity of Fura-2-loaded HL-60 cells (5×10^5/mL) in KRP containing glucose was measured with a calcium analyzer (Jasco CAF 110) at 37° and calculated by the method of Grynkiewicz *et al*. [20].

2.8. Assay of mitochondrial membrane potential in HL-60 cells

HL-60 cells (2×10^5/mL) were incubated with medium alone or with 50 μM CS for various periods, and the mitochondrial membrane potential in the cells was measured by the method of Salvioli *et al*. [21]. The cells were washed with PBS, stained with 2 ng/mL of JC-1 for 15 min at room temperature in PBS, and analyzed using a FACScan flow cytometer (Becton Dickinson).

HL-60 cells were incubated on an 8-well culture slide (0.8×10^5/well) for the analysis of the mitochondrial membrane potential after staining with MitoSensor (ApoAlert™, Clontech) for 15 min at room temperature. After incubation in culture medium containing 50 μM CS for various periods, the cells were washed with culture medium, and stained with MitoSensor for 20 min at 37°. The stained cells were observed by fluorescence microscopy.

2.9. Analysis of DNA fragmentation

The extent of DNA fragmentation was determined spectrophotometrically by the diphenylamine method [15]. After incubation with cholesterol or CS, 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° for 10 min. The lysate was centrifuged at 13,000 *g* at 4° for 20 min to separate intact and fragmented chromatin. Both the pellet and the supernatant were precipitated by 6% perchloric acid at 4° for 30 min. The precipitates were sedimented further at 13,000 *g* for 20 min at 4°. The DNA specimens were heated at 70° 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° in the dark, they were measured spectrophotometrically at 600 nm, and the percentage of DNA fragmentation was calculated. DNA fragmentation was also detected by agarose gel electrophoresis [22]. The lysate (fragmented DNA fraction) was treated with 400 μg/mL of RNase A at 37° for 1 hr and 400 μg/mL of proteinase K at 37° for 1 hr. The fragmented DNA samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The fragmented DNA in the aqueous phase was then electrophoresed at 100 V through a 2% agarose gel containing 0.1 mg/mL of 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 Polaroid 667 film.

2.10. Western blot analysis

Cell lysates were prepared as described elsewhere [22]. Cells (2×10^6) 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° for 5 min. The samples were then subjected to SDS–PAGE. Proteins in the gel were transferred onto an Immobilon filter (Millipore Co.), and then incubated with primary antibody (1:1000 dilution for Cyt. *c* and phospho-Akt, Bcl-2 and Bax; 1:500 dilution for Bid) and finally with horseradish peroxide-linked second antibody (1:2000 dilution for Cyt. *c*; 1:50,000 for Bid, Bcl-2, and Bax) and analyzed using an ECL plus kit (Amersham). Protein concentration was determined by the method of Bradford [23] using bovine serum albumin as a standard.

2.11. Assay for caspase activity

Caspase activity was determined as described previously [22] in 20 mM HEPES buffer (pH 7.5) containing 0.1 M NaCl and 5 mM dithiothreitol at 37° using a 10 μM concentration of either 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 is defined as the amount of enzyme required for the hydrolysis of 1 μmol substrate per 1 hr.

3. Results

3.1. Effect of CS on the proliferation and differentiation of HL-60 cells

Under standard conditions, HL-60 cells showed a normal growth pattern with a doubling time of about 24 hr. The presence of CS inhibited the growth of HL-60 cells in a concentration- and time-dependent manner (Fig. 1A). Cell growth was inhibited completely by CS at concentrations higher than 50 μM at 24 hr. However, no differentiation to granulocytes of HL-60 cells following treatment with CS was observed even at concentrations of more than 50 μM (Fig. 1B).

3.2. Relation between cell cycle and DNA fragmentation after CS

Cells were quantitated by flow cytometry at various times after treatment with 30 μM CS to confirm the state

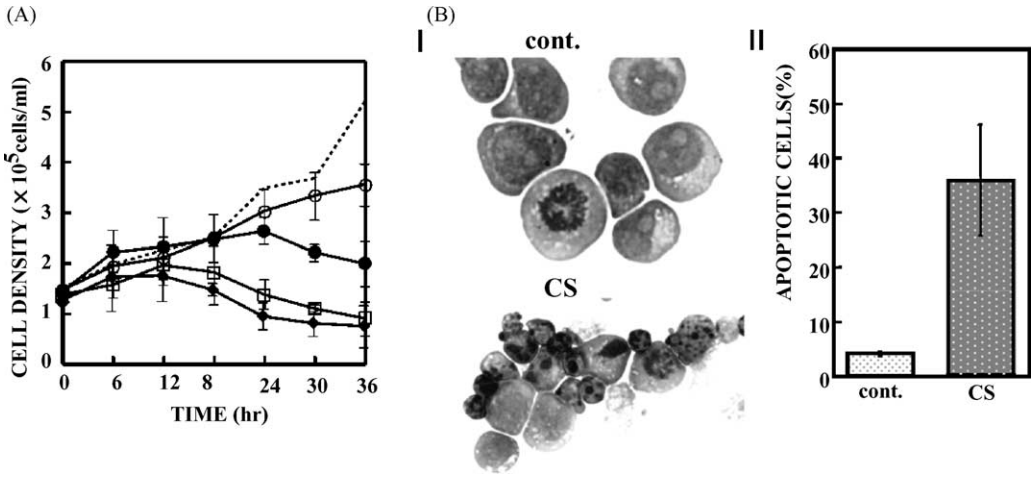

Fig. 1. Effect of CS on growth and morphology of HL-60 cells. (A) Cells were exposed to 0 (···), 25 (○), 50 (●), 75 (□), and 100 (◆) μM CS under the experimental conditions described in Section 2. At the indicated times, the number of viable cells was determined by the trypan blue exclusion test. Experiments were performed at least three times with similar results. (B) HL-60 cells were incubated with 50 μM CS for 24 hr (I), and the percentage of cells showing fragmented nuclei (II) was calculated. Values are means ± SD derived from three separate experiments.

of apoptosis and cell arrest of the treated cells. The number of apoptotic cells increased 24 and 48 hr after treatment with 30 μM CS without arrest in any phase of the cell cycle (Fig. 2).

3.3. Effect of CS on DNA fragmentation and ladder formation and its sensitivity to CsA and z-VAD-fmk

Incubation of HL-60 cells with CS induced fragmentation of cellular DNA in a time- and concentration-dependent manner (Fig. 3A and B). Kinetic analysis revealed that DNA fragmentation became apparent at 6–36 hr after incubation with 50 μM CS (Fig. 3A). DNA ladder formation induced by treatment with CS at a concentration of 50 μM was also observed on agarose gel electrophoresis (Fig. 3C). Ladder formation was started at 6 hr by 50 μM CS and increased in parallel with DNA fragmentation for 24 hr. In contrast, neither cholesterol nor succinate at a concentration of 50 μM showed appreciable effects on DNA ladder formation in these cells (data not shown).

Since MPT and caspases play crucial roles in the process of apoptosis, we investigated the effects of z-VAD-fmk, a pan-caspase inhibitor [24], and CsA, an inhibitor of MPT, on CS-induced apoptosis of HL-60 cells. DNA fragmentation induced by CS was strongly suppressed by pretreating cells with 75 μM z-VAD-fmk for 1 hr (Fig. 3D). However, the CS-induced DNA fragmentation was increased further by pretreating cells with 1 μM CsA (Fig. 3D). The results indicate that CS-induced DNA fragmentation was insensitive to classic MPT but dependent upon the activation of a caspase cascade [10,25].

3.4. Caspase activation of HL-60 cells by CS

Since CS-induced apoptosis was inhibited by z-VAD-fmk, we also examined the effect of CS on the activation of caspase-1, -3, -6, -8, and -9 in HL-60 cells using specific synthetic substrates for each caspase. CS activated various types of caspase-like proteases, such as caspase-3, -6, -8, and -9, but not caspase-1. Cellular activities of caspases markedly increased after incubation for 12 hr with 50 μM

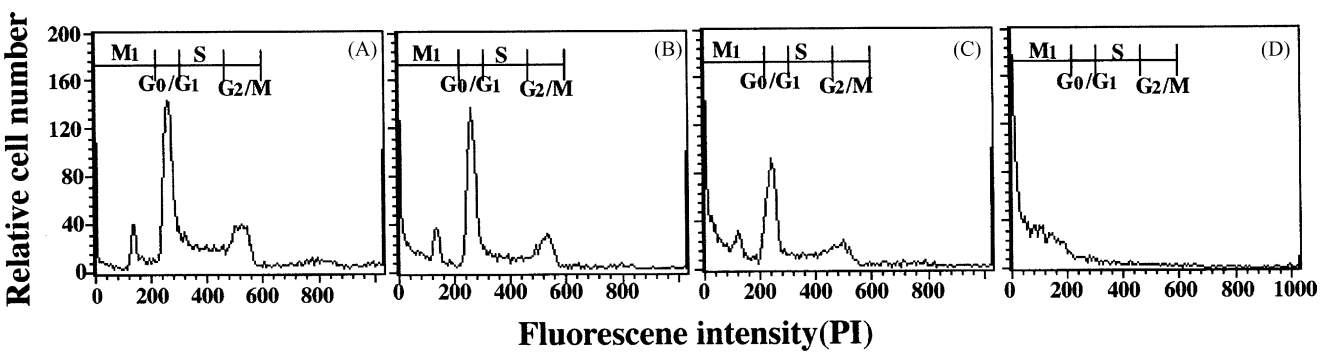

Fig. 2. Changes in DNA content of HL-60 cells treated with CS. Untreated HL-60 cells (A) and following treatment with 30 μM CS for 12 (B), 24 (C), and 48 (D) hr. CS-treated cells were fixed in 200 μL of 70% ethanol at 4° for longer than 4 hr, and suspended in phosphate-citrate buffer and maintained at room temperature for 30 min prior to propidium iodide staining.

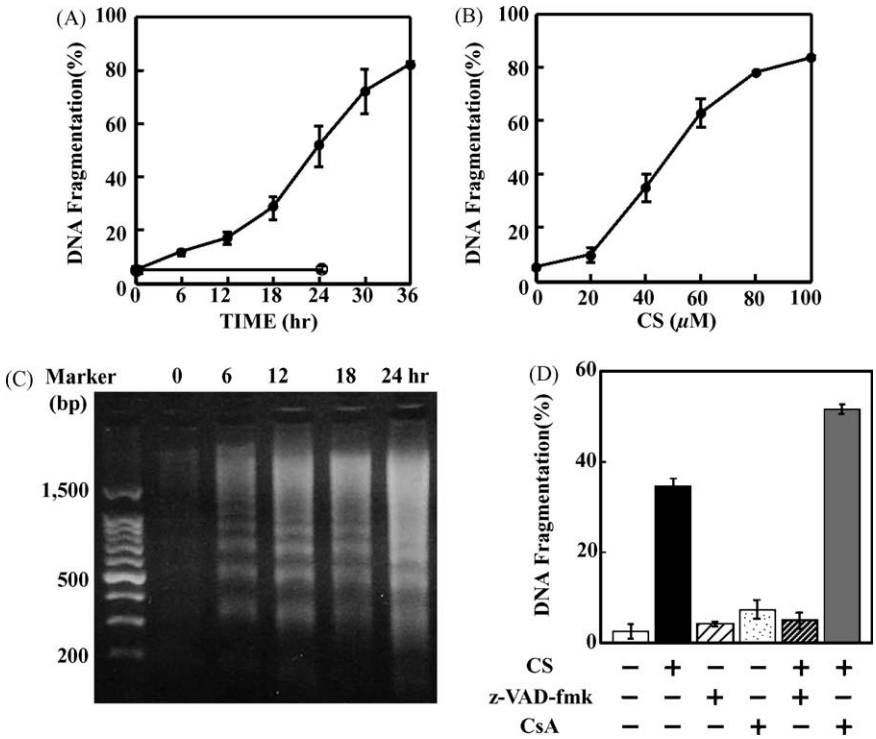

Fig. 3. DNA fragmentation of CS-treated HL-60 cells and its sensitivity to z-VAD-fmk and CsA. (A) Cells were incubated in the presence of 50 μM CS (●) or 50 μM cholesterol (○) at 37°. At the indicated times, fragmented DNA in the cultured cells was determined by the diphenylamine method. (B) The effect of various concentrations of CS (●) on fragmentation of DNA was also observed. The cells (1.5×10^5) were incubated with various concentrations of CS for 24 hr. Values are means ± SD derived from three separate experiments. (C) DNA ladder formation after treatment with 50 μM CS was detected by gel electrophoresis (2% agarose). Lanes 1, 2, 3, 4, 5, and 6 are molecular size markers, control cells at 24 hr, and cells treated with 50 μM CS for 6, 12, 18, and 24 hr, respectively. Experiments were carried out at least three times with similar results. (D) Cells were preincubated in the presence or absence of 75 μM z-VAD-fmk or 1 μM CsA for 1 hr and subsequently with or without 100 μM CS for 9 hr. DNA fragmentation was determined by the diphenylamine method. Values are means ± SD derived from three separate experiments.

CS and reached a maximum at 24 hr (Fig. 4A). Activation by CS, while pronounced for caspase-3 and -6, was much less for caspase-8 and -9 (Fig. 4B). The results obtained in these experiments suggest that a cascade of caspase proteases may be involved in CS-induced apoptosis of HL-60 cells.

3.5. Cyt. c release from mitochondria of HL-60 cells by CS and its sensitivity to z-VAD-fmk

Since CS activated caspase-9, Cyt. *c* might possibly be released from the mitochondria into the cytosol [9]. As

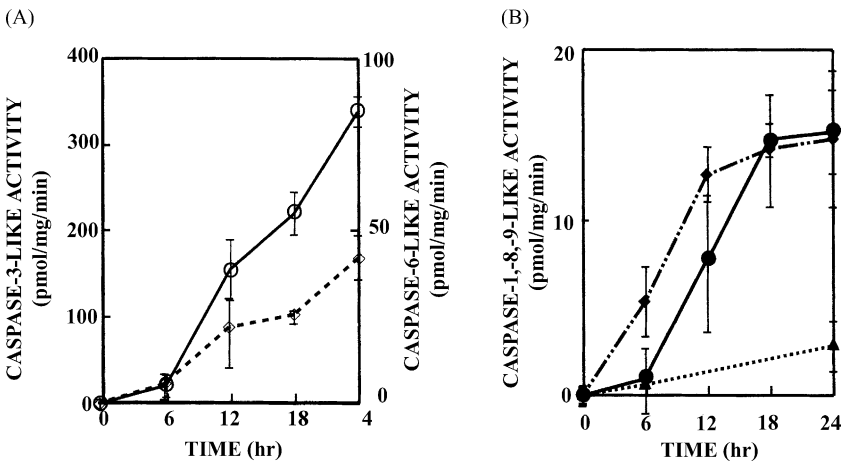

Fig. 4. Effect of CS on the activities of various caspases. (A) Cells were incubated with 50 μM CS, and time-dependent changes in the activity of caspase-3 (○) and -6 (◇) were determined as described in the text. (B) The effect of 50 μM CS on the activity of caspase-1 (▲), -8 (●), and -9 (◆) was also determined. Cell extracts (50 μL) were incubated with 10 μM fluorogenic peptide substrates at 37° for 1 hr. Values are means ± SD derived from three separate experiments.

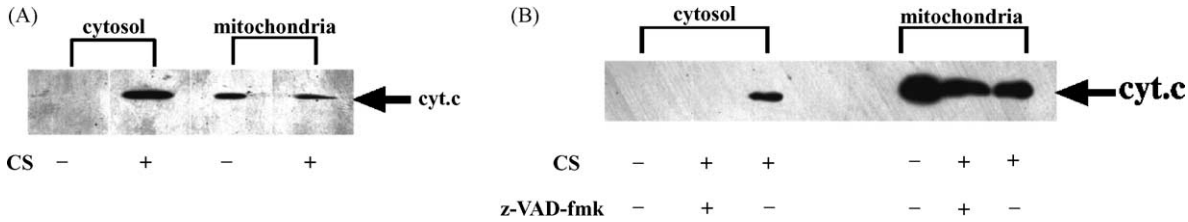

Fig. 5. Effect of CS on the distribution of Cyt. *c* in cells and its sensitivity to z-VAD-fmk. (A) Cells were treated with 50 μM CS for 24 hr. (B) Cells were preincubated in the presence or absence of 100 μM z-VAD-fmk for 1 hr and subsequently with or without 100 μM CS for 18 hr. Cyt. *c* in mitochondrial and cytosolic fractions was detected by western blotting. Experiments were carried out at least three times with similar results.

suspected, a significant fraction of the mitochondrial Cyt. *c* was released into the cytosol by treatment with CS (Fig. 5A). The amounts of Cyt. *c* released by CS increased with the time of CS treatment (data not shown). Furthermore, Cyt. *c* release was suppressed by z-VAD-fmk (Fig. 5B) but increased by CsA (data not shown). These results suggested that the Cyt. *c* release from mitochondria might occur through a caspase-dependent pathway but not through the classic MPT pathway [10] and that the released Cyt. *c* might play an important role in CS-induced apoptosis of HL-60 cells.

3.6. Cleavage of Bid in HL-60 cells by CS and its sensitivity to caspase inhibitors

Bid cleaved by caspase-8 has been shown to directly trigger the release of Cyt. *c* from mitochondria [26–28], which is suppressed by Bcl-2. Thus, an apoptotic signal initiated by CS might be relayed from the cell surface to the mitochondria in a manner that is similar to that described for receptor-mediated apoptosis [27]. Due to this observation, we studied the effect of CS 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 CS resulted in the formation of 16 and 15 kDa fragments from Bid (Fig. 6A). The generation of truncated Bid (tBid) was suppressed by z-VAD-fmk and a caspase-8 inhibitor, Ac-ITED-CHO (Fig. 6B). These results showed that cleaved products of Bid in CS-treated HL-60 cells might also be involved in the mechanism of Cyt. *c* release in cells.

3.7. Changes in mitochondrial membrane potential in HL-60 cells induced by CS

It has been shown that Cyt. *c* is released from mitochondria into the cytosol by opening of the MPT pore, and it has been postulated that pore opening plays a role in cellular events leading to apoptosis of certain types of cells [9]. For these reasons, the effect of CS on mitochondrial membrane potential in intact HL-60 cells was examined by staining with MitoSensor and following up with FACScan analysis of JC-1 fluorescence. CS induced depolarization of the mitochondrial membrane potential in a time- and concentration-dependent manner (data not shown). The reddish fluorescence of the cell mitochondria, stained with MitoSensor, was changed to the green fluorescence of the cytosol during the incubation with 50 μM CS, indicating depolarization of the membrane (Fig. 7A). The depolarization of HL-60 cells by CS was confirmed by a FACScan technique; after treatment of HL-60 cells with 50 μM CS for 6, 12, and 24 hr, the intensity of JC-1 fluorescence was also decreased in a time-dependent manner (Fig. 7A). The depolarization was inhibited by z-VAD-fmk but was stimulated further by CsA (Fig. 7B). Furthermore, the beginning of DNA fragmentation induced by CS was faster than depolarization.

3.8. Effect of CS on the expression and distribution of Bcl-2 and Bax

The MPT of mitochondria has an important role in the cell, and the Bcl-2 family of proteins has a regulatory role in MPT pore opening. Thus, the effect of CS on the

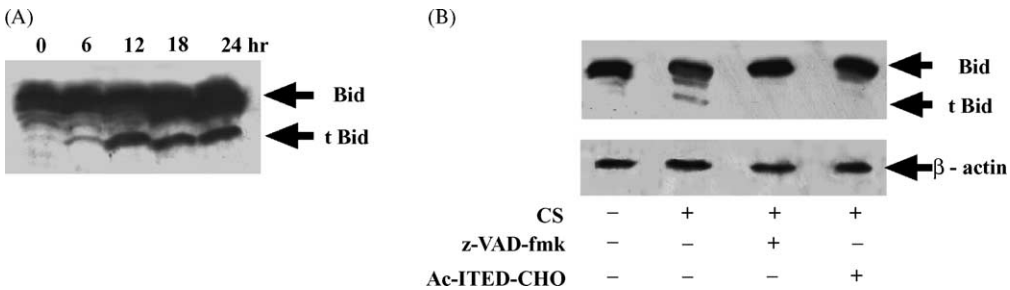

Fig. 6. Effect of CS on the processing of Bid and its sensitivity to z-VAD-fmk and Ac-ITED-CHO. (A) Cells were incubated in the presence or absence of 50 μM CS for 6, 12, 18, and 24 hr. (B) Cells were preincubated in the presence or absence of 75 μM z-VAD-fmk or Ac-ITED-CHO for 1 hr and subsequently with or without 100 μM CS for 18 hr. As a control for protein loading, a western blot analysis of actin was also carried out. 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, truncated Bid.

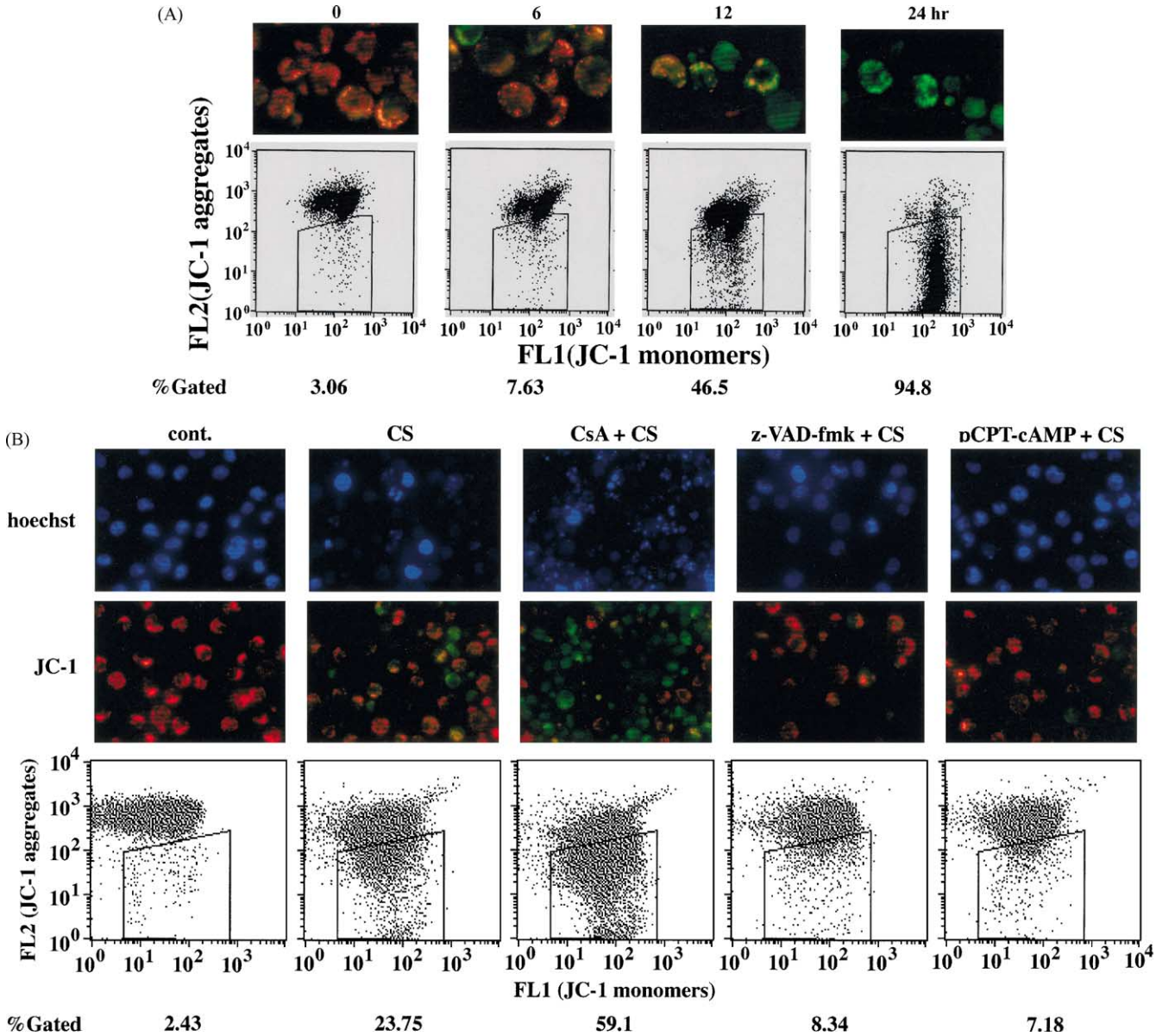

Fig. 7. Effect of CS on mitochondrial membrane potential in HL-60 cells and its sensitivity to z-VAD-fmk and CsA. (A) Cells were incubated in the presence of 50 μM CS for the indicated number of hours. Measurement by flow cytometry took placed at 0, 6, 12, and 24 hr. (B) Cells were preincubated in the presence or absence of 75 μM z-VAD-fmk or 1 μM CsA or 100 μM pCPT-cAMP for 1 hr and subsequently with or without 100 μM CS for 9 hr. After treatment, cells were stained with MitoSensor or JC-1 in PBS. % Gated = the percentage of depolarized cells. Cells stained with MitoSensor were observed under fluorescence microscopy. Similar results were obtained in three separate experiments.

distribution and expression of Bcl-2 and Bax in HL-60 cells was examined. Expression of both proteins was unaffected after treatment with CS for 12–24 hr. However, an increased amount of Bax but not of Bcl-2 in the mitochondrial fraction was observed after treatment with 50 μM CS for 12 hr (Fig. 8). This result indicates that an increased amount of Bax might induce Cyt. *c* release after treatment with CS.

3.9. Changes in $[Ca^{2+}]_i$ of HL-60 cells induced by CS

It has been shown that Ca^{2+} may induce MPT in the presence of inorganic phosphate, which in turn induces the release of Cyt. *c*, thereby initiating apoptosis. Therefore, apoptosis initiated by Ca^{2+} and changes of $[Ca^{2+}]_i$ in HL-60 cells following treatment with CS were monitored as changes in Fura-2 fluorescence intensity. $[Ca^{2+}]_i$ in HL-60 cells was not changed by the addition of CS at a concentration of 100 μM (Fig. 9), or even at a concentration of 150 μM (data not shown). The effect of CS was completely different from that of VES.

3.10. Effect of cAMP on the CS-induced DNA fragmentation of HL-60 cells

Since on the basis of recent studies it was proposed that phospho-Akt has an important role in the activation of

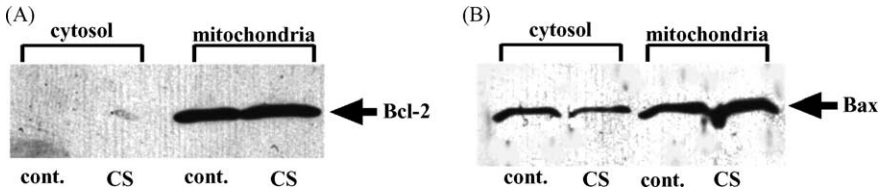

Fig. 8. Effect of CS on the distribution of Bcl-2 and Bax in mitochondrial and cytosolic fractions. Experimental conditions were as described in Fig. 6. Distribution of Bcl-2 and Bax in mitochondrial and cytosolic fractions was detected by western blotting at 12 hr in the presence or absence of 50 μM CS. Similar results were obtained in three separate experiments.

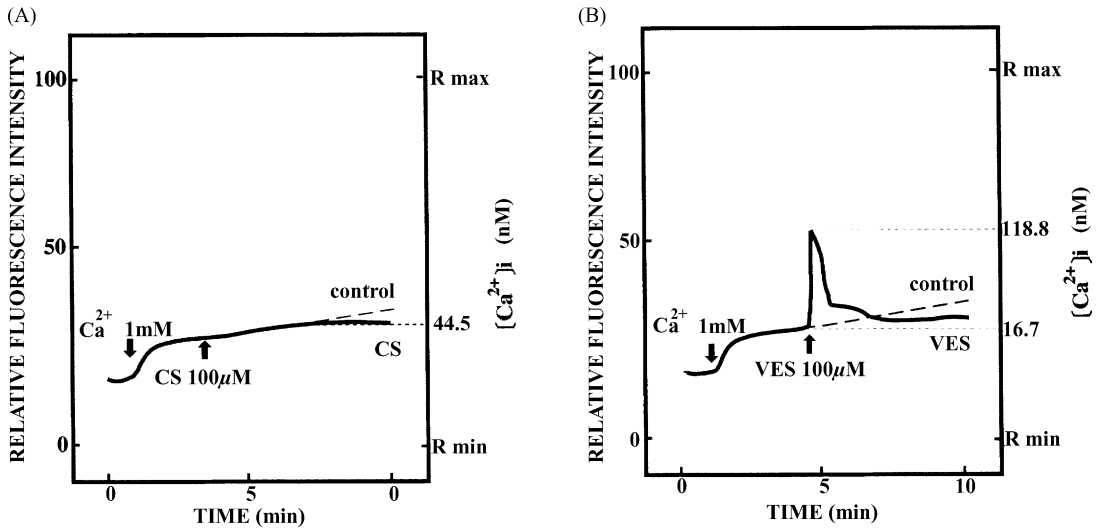

Fig. 9. Effect of CS on the Ca^{2+} status in HL-60 cells. HL-60 cells (1×10^7/mL) were loaded with 5 μM Fura-2 in KRP solution containing 10 mM glucose at 37°. The fluorescence intensity of Fura-2-loaded cells was monitored by a calcium analyzer (Jasco CAF 110) at 37°. Then, at the indicated times,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

caspase-8 [13,14], the effect of pCPT-cAMP on CS-induced DNA fragmentation was examined. The CS-induced DNA fragmentation was suppressed completely by preincubation of the cells with cAMP (Fig. 10). Furthermore, CS-induced depolarization of mitochondria was suppressed by pCPT-cAMP (Fig. 7B). These results indicate that CS might inhibit the phosphorylation of Akt and stimulate the activation of caspase-8.

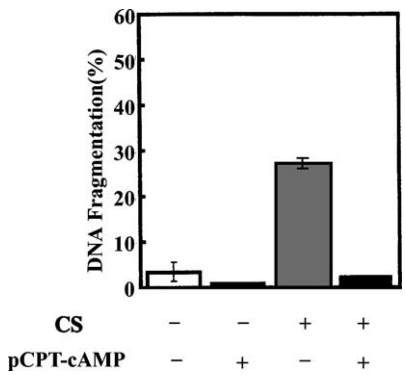

Fig. 10. Suppression of CS-induced DNA fragmentation of HL-60 cells by cAMP. Experimental conditions were the same as described in Fig. 3. Cells were preincubated for 1 hr with 100 μM pCPT-cAMP before the addition of 50 μM CS for 18 hr. Values are means ± SD derived from three separate experiments.

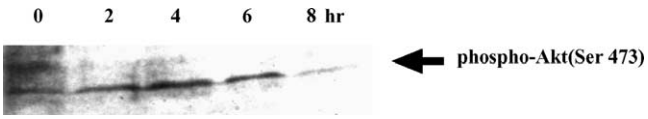

Fig. 11. Suppression of phospho-Akt content in HL-60 cells by CS. Experimental conditions were the same as described in Fig. 3. At various times after treatment with 50 μM CS, phospho-Akt in HL-60 cells was detected by western blot analysis with anti-phospho-Akt antibody. Similar results were obtained in three separate experiments.

3.11. Inhibition of phospho-Akt in HL-60 cells by CS

To gain further insight into the regulation of caspase-8 by Akt, the effect of CS on the phosphorylation of Akt was examined by western blotting using an anti-phospho-Akt antibody. The amount of phosphorylated Akt in HL-60 cells was decreased by treatment with 50 μM CS in a time-dependent manner (Fig. 11). The rate of decrease in phospho-Akt was paralleled by an increase in DNA fragmentation after treatment of the cells with CS.

4. Discussion

The findings of the present study clearly demonstrate that CS, but not cholesterol and succinate, inhibits cell

growth, modulates mitochondrial functions, releases Cyt. *c* into the cytosol, and activates caspase-3, thereby inducing apoptosis of HL-60 cells. Kinetic analysis revealed that an increase in tBid as well as an increased distribution of Bax in mitochondria might enhance the release of Cyt. *c* into the cytosol. These results suggest that CS induced apoptosis of HL-60 cells by modulating mitochondrial membrane functions, thereby activating the caspase cascade pathway via mitochondrial Cyt. *c* release, which may be induced by caspase-8-cleaved Bid and by increased Bax in mitochondria. In these studies, new data were obtained to support the involvement of this pathway in HL-60 cell apoptosis. The validity of caspase-8-dependent formation of tBid was established by its sensitivity to z-VAD-fmk and Ac-ITED-CHO. Depolarization of the intracellular mitochondrial membrane potential was also suppressed by z-VAD-fmk but was stimulated significantly by CsA. The classic MPT is dependent upon the $[Ca^{2+}]_i$, is energy dependent, and is accompanied by mitochondrial swelling and depolarization [10]. On the contrary, it has been reported recently that the nonclassic MPT, which is insensitive to CsA, occurs without swelling and depolarization of the mitochondria [29]. Thus, CS induced the nonclassic type of MPT and such depolarization might be a result of caspase activation although this does not initiate Cyt. *c* release [30]. Furthermore, recent studies have indicated the regulation of caspase-8 activity through Akt [13,14]. In this study, we found that CS inhibited the phosphorylation of Akt and that membrane permeable cAMP, pCPT-cAMP, strongly suppressed the CS-induced DNA fragmentation. Similar results were also obtained using VES (data not shown). These results indicate that the inhibition of the phosphorylation of Akt might act as an initial step in the apoptosis pathway induced by CS and VES.

The $[Ca^{2+}]_i$ is an important factor in the initiation of the signal for apoptosis in cells [11,12]. However, in contrast to the transiently increased $[Ca^{2+}]_i$ induced by VES [4], no such change was observed with CS. In this context, preliminary experiments showed that α-tocopherol is unable to mobilize Ca^{2+}. These results suggest that the $[Ca^{2+}]_i$ might not have the ability to regulate the signal for apoptosis by CS. Furthermore, the MPT of isolated mitochondria and Cyt. *c* distribution were not affected by CS at a concentration of 100 μM (data not shown). These results suggest that CS may not directly trigger the release of Cyt. *c* from mitochondria into the cytosol as observed with VES.

It has been shown that apoptosis occurs spontaneously in freshly isolated human neutrophils. This spontaneous apoptosis was associated with translocation of Bax to the mitochondria and subsequent caspase-3 activation, but not with changes in the expression of Bax [31]. In this study, we also found that CS-induced apoptosis of HL-60 cells was associated with translocation of Bax to the mitochondria and activation of caspase-3 without triggering differentiation of the cells to granulocytes. Thus, this mechanism of apoptosis might exist in both spontaneous granulocyte apoptosis and CS-induced apoptosis of HL-60 cells.

The proapoptotic activity of VES appears to involve inhibition of PKCα, since phorbol myristate acetate (PMA) prevented apoptosis triggered by VES [2]. The molecular mechanism of inhibition of PKCα by VES may depend on increased protein phosphatase 2A (PP2A) as well as α-tocopherol as reported by Azzi and coworkers [32,33]. On the contrary, PKC might not be involved in CS-induced apoptosis since PMA has no effect on CS-induced apoptosis of HL-60 cells (data not shown). These results indicate that PKC and PP2A may not play a critical role in the mechanism of CS-induced apoptosis.

Another possible mechanism of CS-induced apoptosis is the modulation of Fas signaling [6] and increased AP-1 activity by the activation of c-Jun amino-terminal kinase and c-Jun [34–36]. VES also readily associates with lipoproteins *in vitro* and *in vivo*, which act as efficient VES carriers, thereby reducing cell proliferation rates and increasing apoptosis in some cancer cells [5]. Preliminary experiments in this laboratory showed that CS-induced apoptosis was not inhibited by cycloheximide and actinomycin D (data not shown). Thus, no such mechanism appears to be involved in CS-induced apoptosis.

From these results, the following pathway of apoptosis induced by CS was considered to be a possibility. CS suppressed Akt activity thereby activating caspase-8, which stimulated the generation of tBid and induced the release of Cyt. *c* independent of the classic MPT mechanism. Cyt. *c* release was also stimulated in association with increases in Bax during apoptosis.

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References

[1] Simmonds AC, Rooney EK, Lee AG. Interactions of cholesterol hemisuccinate with phospholipids and $[Ca^{2+}$–$Mg^{2+}]$-ATPase. Biochemistry 1984;23:1432–41.
[2] Neuzil J, Weber T, Schroder A, Lu M, Ostermann G, Gellert N, Mayne GC, Olejnicka B, Negre-Salvayre A, Sticha M, Coffey RJ, Weber C. Induction of cancer cell apoptosis by α-tocopheryl succinate: molecular pathways and structural requirements. FASEB J 2001;15:403–15.
[3] Neuzil J, Weber T, Gellert N, Weber C. Selective cancer cell killing by α-tocopheryl succinate. Br J Cancer 2001;848:87–9.
[4] Yamamoto S, Tamai H, Ishisaka R, Kanno T, Arita K, Kobuchi H, Utsumi K. Mechanism of α-tocopheryl succinate-induced apoptosis of promyelocytic leukemia cells. Free Radic Res 2000;33:407–18.
[5] Pussinen PJ, Lindner H, Glatter O, Reicher H, Kostner GM, Wintersperger A, Malle E, Sattler W. Lipoprotein-associated α-tocopheryl-succinate inhibits cell growth and induces apoptosis in human MCF-7

and HBL-100 breast cancer cells. Biochim Biophys Acta 2000;1485: 129–44.
[6] Israel K, Yu W, Sanders BG, Kline K. Vitamin E succinate induces apoptosis in human prostate cancer cells: role for Fas in Vitamin E succinate-triggered apoptosis. Nutr Cancer 2000;36:90–100.
[7] Neuzil J, Svensson I, Weber T, Weber C, Brunk UT. α-Tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation. FEBS Lett 1999;445:295–300.
[8] Skulachev VP. Why are mitochondria involved in apoptosis? FEBS Lett 1996;397:7–10.
[9] Reed JC. Cytochrome *c*: can't live with it—can't live without it. Cell 1997;91:559–62.
[10] Zoratti M, Szabo I. The mitochondrial permeability transition. Biochim Biophys Acta 1995;1241:139–76.
[11] Petersen A, Castilho RF, Hansson O, Wieloch T, Brundin P. Oxidative stress, mitochondrial permeability transition and activation of caspase in calcium ionophore A23187-induced death of cultured neurons. Brain Res 2000;857:20–9.
[12] Andreyev A, Fiskum G. Calcium induced release of mitochondrial cytochrome *c* by different mechanisms selective for brain vs. liver. Cell Death Differ 1999;6:825–32.
[13] Rytömaa M, Lehmann K, Downward J. Matrix detachment induces caspase-dependent cytochrome *c* release from mitochondria: inhibition by PKB/Akt but not Raf signalling. Oncogene 2000;19:4461–8.
[14] Zhang L, Himi T, Morita I, Murota S. Inhibition of phosphatidylinositol-3 kinase/Akt or mitogen-activated protein kinase signaling sensitizes endothelial cells to TNF-alpha cytotoxicity. Cell Death Differ 2001;8:528–36.
[15] Yabuki M, Inai Y, Yoshioka T, Hamazaki K, Yasuda T, Inoue M, Utsumi K. Oxygen-dependent fragmentation of cellular DNA by nitric oxide. Free Radic Res 1997;26:245–55.
[16] Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971;55:283–90.
[17] Kariya S, Ogawa Y, Yoshida S, Yabuki M, Imajo Y, Utsumi K. X-irradiation enhances the expression of Bcl-2 in HL-60 cells: the resulting effects on apoptosis and radiosensitivity. Int J Mol Med 1999;3:145–52.
[18] Arita K, Utsumi T, Kato A, Kanno T, Kobuchi H, Inoue B, Akiyama J, Utsumi K. Mechanism of dibucain-induced apoptosis in promyelocytic leukemia cells (HL-60). Biochem Pharmacol 2000;60:905–15.
[19] Yamamoto M, Saeki K, Utsumi K. Isolation of human salivary polymorphonuclear leukocytes and their stimulation-coupled responses. Arch Biochem Biophys 1991;289:76–82.
[20] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[21] Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1 but not $DiOC_6(3)$ or rhodamin 123, is a reliable fluorescent probe to assess $\Delta\psi$ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.
[22] Yabuki M, Kariya S, Ishisaka R, Yasuda T, Yoshioka T, Horton AA, Utsumi K. Resistance to nitric oxide-mediated apoptosis in HL-60 variant cells is associated with increased activities of Cu, Zn-superoxide dismutase and catalase. Free Radic Biol Med 1999;26:325–32.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[24] Slee EA, Zhu H, Chow SC, MacFarlane M, Nicholson DW, Cohen GM. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32. Biochem J 1996;315:21–4.
[25] Deas O, Dumont C, MacFarlane M, Rouleau M, Hebib C, Harper F, Hirsch F, Charpentier B, Cohen GM, Senik A. Caspase-independent cell death induced by anti-CD2 or staurosporine in activated human peripheral T lymphocytes. J Immunol 1998;161:3375–83.
[26] Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl-2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell 1998;94:481–90.
[27] Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491–501.
[28] Kluck RM, Esposti MD, Perkins G, Renken C, Kuwana T, Bossy-Wetzel E, Goldberg M, Allen T, Barber MJ, Green DR, Newmeyer DD. The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. J Cell Biol 1999;147:809–22.
[29] Sultan A, Sokolove PM. Palmitic acid opens a novel CsA-insensitive pore in the inner mitochondrial membrane. Arch Biochem Biophys 2001;386:37–51.
[30] Xia T, Jiang C, Li L, Wu C, Chen Q, Liu S-S. A study on permeability transition pore opening and cytochrome *c* release from mitochondria, induced by caspase-3 *in vitro*. FEBS Lett 2002;510:62–6.
[31] Maianski NA, Mul FP, van Buul JD, Roos D, Kuijpers TW. Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils. Blood 2002;99:672–9.
[32] Ricciarelli R, Tasinato A, Clement S, Ozer NK, Boscoboinik D, Azzi A. α-Tocopherol specifically inactivates cellular protein kinase Cα by changing its phosphorylation state. Biochem J 1998;334:243–9.
[33] Ricciarelli R, Azzi A. Regulation of recombinant PKCα activity by protein phosphatase 1 and protein phosphatase 2A. Arch Biochem Biophys 1998;355:197–200.
[34] Yu W, Simmons-Menchaca M, You H, Brown P, Birrer MJ, Sanders BG, Kline K. RRR-α-tocopheryl succinate induction of prolonged activation of c-jun amino-terminal kinase and c-jun during induction of apoptosis in human MDA-MB-435 breast cancer cells. Mol Carcinog 1998;22:247–57.
[35] Qian M, Kralova J, Yu W, Bose Jr HR, Dvorak M, Sanders BG, Kline K. c-Jun involvement in Vitamin E succinate induced apoptosis of reticuloendotheliosis virus transformed avian lymphoid cells. Oncogene 1997;15:223–30.
[36] Zhao B, Yu W, Qian M, Simmons-Menchaca M, Brown P, Birrer MJ, Sanders BG, Kline K. Involvement of activator protein-1 (AP-1) in induction of apoptosis by Vitamin E succinate in human breast cancer cells. Mol Carcinog 1997;19:180–90.