

Mechanisms Underlying Production and Externalization of Oxidized Phosphatidylserine in Apoptosis: Involvement of Mitochondria

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The present study was performed by using selective inhibitors of caspase-8 and caspase-3 functioning upstream and downstream from mitochondria, respectively to determine whether mitochondria are involved in the mechanisms underlying production and externalization of oxidized phosphatidylserine (PSox) during Fas-mediated apoptosis. Treatment with anti-Fas antibody induced caspase-3 activation, chromatin condensation, release of cytochrome c (cyt c) from mitochondria into the cytosol as well as production of PSox and its exposure to the cell surface in Jurkat cells. Inhibition of caspase-8 by pretreatment with Z-IETD-FMK, a membrane permeable selective caspase-8 inhibitor reduced mitochondrial cyt c release, the amount of PSox not only within but also on the surface of Jurkat cells, caspase-3 activation, and apoptotic cell number after treatment with anti-Fas antibody. In contrast, Z-DEVD-FMK, a membrane permeable selective caspase-3 inhibitor was unable to inhibit cyt c release, and the amount of PSox both within and on the surface of the cells after anti-Fas antibody, although it suppressed caspase-3 activation and apoptosis. Thus, these results strongly suggest that mitochondria play an important role in production of PSox and subsequent its externalization during apoptosis.

Key words: apoptosis; caspase inhibitor; cytochrome c; mitochondria; oxidized phosphatidylserine

Phospholipids in plasma membrane are distributed asymmetrically between two leaflets (Bretscher, 1972), and the choline-containing phospholipids, phosphatidylcholine and sphingomyelin, reside mainly in the outer (extracellular) leaflet, whereas the aminophospholipids, phosphatidylserine (PS) and, to a lesser extent, phosphatidylethanolamine (PE), are confined to the inner (cytosolic) leaf-

let (Verkleij and Post, 2000). This asymmetry is considered to be established and maintained by at least three lipid translocators: i) ATP-dependent flippases, members of type 4 P-type ATPases, also referred to as aminophospholipid translocases, that are specific for aminophospholipids, with a preference for PS over PE, and catalyze the inward translocation of aminophospholipids (Folmer et

Abbreviations: AMC, 7-amino-4-methyl-coumarin; BHT, butylated hydroxytoluene; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; CL, cardiolipin; CLOx, oxidized CL; cyt c, cytochrome c; DTT, dithiothreitol; DISC, death-inducing signaling complex; FCS, fetal calf serum; HPTLC, high-performance thin-layer chromatography; LPE, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine; MOMP, mitochondrial outer membrane permeabilization; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMSF, phenylmethyl-sulfonyl fluoride; PS, phosphatidylserine; PSox, oxidized PS; ROS, reactive oxygen species; SAGOOH, 1-stearoyl-2-arachidonylglycerol hydroperoxide; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride

al., 2009); ii) another ATP-dependent translocator floppases, members of ATP-binding cassette transporters, which catalyze the efflux of phospholipids from the inner to the outer leaflet with little selectivity for polar head group of the phospholipids (Contreras et al., 2010) and iii) energy-independent scramblases that are non-selective and catalyze bidirectional transbilayer movement of phospholipids Ca^{2+} -dependently but ATP-independently (Beyers and Williamson, 2010). It is generally accepted that PS predominantly localized in the inner leaflet migrates to the outer leaflet of the plasma membrane during apoptosis, and is recognized by macrophages as an “eat-me” signal, resulting in successful clearance of apoptotic cells (Verhove et al., 1995). This PS egress during apoptosis seems to be associated with the inhibition of flippases as well as the activation of scramblases in the plasma membrane (Fadell et al., 2010).

The generation of reactive oxygen species (ROS) and subsequent oxidative stress are commonly involved in the initiation and/or the execution phases of apoptosis (Banki et al., 1999; Li et al., 1999). Mitochondrial permeability transition and the release of cytochrome c (cyt c) from mitochondria into the cytosol during apoptosis are related to excessive ROS generation (Cai and Jones, 1998; Matura et al., 2005). Recent studies have reported almost simultaneous oxidation and externalization of an anionic phospholipid PS during apoptosis (Matura et al., 2003; Kagan et al., 2004). Selective oxidation of another anionic phospholipids such as cardiolipin (CL) and phosphatidylinositol (PI) has also been induced during apoptosis (Matura et al., 2003; Kagan et al., 2004; Matura et al., 2005).

We revealed that H_2O_2 is important not only for PS oxidation but also for its externalization during apoptosis using catalase overexpressing HP100 cells (Matura et al., 2004). We also found that there is oxidized PS (PSox) both within and on the surface of Jurkat cells undergoing apoptosis after treatment with anti-Fas antibody (Matura et al., 2005).

It has been suggested that peroxidase activity of cyt c is closely related to PS oxidation and

externalization (Kagan et al., 2004). Therefore, we determined whether departure of cyt c from mitochondria is required for production of PSox as well as its externalization during apoptosis using two selective caspase inhibitors functioning upstream or downstream from mitochondria in the present study.

Materials and Methods

Materials

Fluorescamine was purchased from Sigma Chemical (St. Louis, MO). Anti-Fas antibody (Clone CH-11) was purchased from Medical and Biological Laboratories (Nagoya, Japan). Fetal calf serum (FCS) was purchased from PAA Laboratories GmbH (Linz, Austria). Membrane-permeable caspase-8 inhibitor (Z-IETD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) were purchased from R & D Systems (Minneapolis, MN). Methanol (HPLC-grade), acetone (HPLC-grade), acetic acid, iodine, ninhydrin spray, butylated hydroxytoluene (BHT) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Chloroform (HPLC-grade) was purchased from Kanto Kagaku (Tokyo, Japan). 7-Amino-4-methyl-coumarin (AMC) and acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) were purchased from Peptide Institute (Osaka, Japan). RPMI 1640 medium with phenol red was obtained from Nissui Pharmaceutical (Tokyo). RPMI 1640 medium without phenol red was obtained from Cosmo Bio (Tokyo). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). High-performance thin-layer chromatography (HPTLC) plates (10 × 10 cm, Silica Gel 60) were obtained from Merck (Darmstadt, Germany). 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LPE) was purchased from Avanti Polar Lipids (Alabaster, AL). 1-Stearoyl-2-arachidonylglycerol hydroperoxide (SAGOOH) was synthesized as described previously (Takekoshi et al., 1995). All other chemicals used were of analytical grade.

Cell cultures

Jurkat T cell line was obtained from Riken Cell Bank (Tsukuba, Japan), and grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin) at 37 °C in a humidified incubator with 5% CO₂. All the experiments were performed using RPMI 1640 medium containing 10% FCS. Cells were seeded at a concentration of 2×10^5 cells/mL and logarithmic growth was maintained by passaging every 2 or 3 days.

Determination of apoptotic cells

Apoptotic cells were assessed by nuclear morphology as described previously (Matsura et al., 2002, 2004). Jurkat cells (1.0×10^6 cells/mL) were incubated with anti-Fas antibody at 37 °C, washed with phosphate-buffered saline (PBS), and then fixed with 1% glutaraldehyde overnight. Samples were centrifuged, resuspended in PBS, stained with Hoechst 33342 (1 mM), and then mounted on a glass slide, and observed under fluorescence microscope. Results were expressed as the percentage of the cells showing characteristic nuclear morphological features of apoptosis (nuclear condensation and fragmentation) relative to the total number of counted cells (≥ 200 cells per each point).

Measurement of caspase-3 activity

The activity of caspase-3 was determined as described previously (Matsura et al., 2002, 2004). Briefly, after treatment of Jurkat cells (1×10^6 cells/mL) with anti-Fas antibody (200 ng/mL), cells were collected, washed in PBS, and lysed for 20 min on ice in lysis buffer containing 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 1 mM phenylmethyl-sulfonyl fluoride (PMSF) and 5 mM dithiothreitol (DTT). The suspensions were centrifuged at 4 °C and the supernatants were collected as lysates. For measurement

of caspase activity, 10 µg of lysate diluted to 20 µL with lysis buffer was mixed with 20 µL of $2 \times$ ICE buffer containing 40 mM HEPES-KOH (pH 7.4), 20% (v/v) glycerol, 1 mM PMSF and 4 mM DTT supplemented with 40 µM Ac-DEVD-AMC (a fluorogenic peptide substrate) and incubated for 60 min at 37 °C. After 60 min, 160 µL of distilled water was added and the fluorescence was measured in a CytoFluor II (Applied Biosystems, Foster City, CA) fluorescence microplate reader using excitation at 360 ± 40 nm and emission at 460 ± 40 nm. One unit of caspase activity was defined as the amount of enzyme required to release 1 pmol AMC per minute at 37 °C.

The protein concentration of cell lysates was measured by the method of Bradford (Bradford, 1976).

Flow cytometry of PS externalization

Annexin V binding to cells was determined using a commercially available Annexin V-FITC Apoptosis Detection Kit (Medical & Biological Laboratories, Nagoya, Japan) and flow cytometry as previously described (Matsura et al., 2005). Briefly, after treatment of cells (1×10^6 cells/mL) with anti-Fas antibody, cells were recovered and washed once with pre-warmed RPMI 1640 medium without phenol red. Cells were incubated with fluorescein isothiocyanate-conjugated annexin V (0.5 µg/mL) for 5 min and then were collected by centrifugation and washed with binding buffer. Propidium iodide (0.6 µg/mL) was added, and cells were immediately analyzed with an Epics cytofluorometer (Beckman Coulter, Fullerton, CA).

Determination of cyt c release

Jurkat cells were treated with 200 ng/mL anti-Fas antibody, harvested and washed three times with ice-cold PBS. Cells were resuspended in isotonic buffer containing 10 mM HEPES, 0.3 M mannitol, 0.1% bovine serum albumin, 5 mM EDTA and 1 mM PMSF supplemented with 0.1 mM digitonin (5×10^7 cells/mL), left on ice for 5 min and imme-

diately centrifuged at $8,500 \times g$ for 5 min at 4°C . The supernatant collected was used as the cytosolic fraction. The cyt c amount in the cytosolic fraction was measured by Western blotting using cyt c (6H2) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1000) as described previously (Matsura et al., 2005). The densitometric quantitative analysis was performed by using NIH Image.

Fluorescamine labeling of externalized aminophospholipids and measurement of phosphorus

Labeling of externalized aminophospholipids, PS and PE with membrane-impermeable fluorescamine, a probe for visualizing lipids that contain primary amino groups, was carried out by methods previously described (Matsura et al., 2005). Briefly, Jurkat cells (1×10^6 cells/mL) treated with anti-Fas antibody (200 ng/mL) for 8 h at 37°C were suspended in labeling buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM NaHCO_3 , 5 mM glucose, and 20 mM HEPES/KOH, pH 8.0). Cells were gently agitated in the presence of fluorescamine (200 mM) for 30 s. The reaction was stopped by the addition of 40 mM Tris-HCl, pH 7.5. Cells were counted again and collected by centrifugation, and lipids in 8×10^7 cells were extracted by the Folch procedure (Folch et al., 1957) and analyzed by HPTLC. To protect lipids from autoxidation during the extraction procedure, the antioxidant BHT (1.3 mM) was added to each sample. Aliquots of the cells (1×10^7) were stored at -80°C until phosphorus measurement. The phosphorus content of lipids was determined by the method of Chalvardjian and Rudnicki (Chalvardjian and Rudnicki, 1970).

Detection of externalized and oxidized PS by HPTLC

Externalized and oxidized PS was determined as described previously (Matsura et al., 2005). Briefly, lipid extracts redissolved in chloroform:methanol (2:1, v/v) were applied to the HPTLC plate under an

N_2 stream. The phospholipid classes in the extracts were analyzed by two-dimensional HPTLC using a solvent system of chloroform:methanol:water (55:35:4, v/v/v) in the first direction and chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5, v/v/v/v/v) in the second. The phospholipids were visualized by exposure to iodine vapor. The identity of each phospholipid was established by comparison with the R_f values of authentic standards (Phospholipid Kit; DOOSAN Serdary Research Laboratories, Yongin City, Korea). Fluorescamine-modified PS and PE were localized by exposure of HPTLC plates to UV light with a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA). LPE (2 nmol) was used as an internal standard for fluorescamine-modified phospholipids. We photographed the plate, scanned PS, PE and LPE modified by fluorescamine (referred to as mPS, mPE and mLPE, respectively) in control and anti-Fas antibody-treated cells, and quantified the ratios mPS/mLPE in both cells by using NIH Image. In addition, we measured the phosphorus content in the total lipids extracted from both control and Fas-ligated cells (8×10^7 cells) and expressed the content of mPS as the following relative density: $10^4 \times \text{mPS/mLPE}/\text{phosphorus}$ of 8×10^7 cells.

To determine PSox on the cell surface, colorimetric spray detection with TMPD was carried out (Matsura et al., 2005). After chromatography, the plate was dried under an N_2 stream and sprayed immediately with freshly prepared 1% (w/v) TMPD in methanol:water:acetic acid (50:50:1, v/v/v). Synthetic SAGOOH was used as an external standard as described previously (Matsura et al., 2005). We quantified the ratio of TMPD-visualized phospholipid/TMPD-visualized SAGOOH in control and anti-Fas antibody-treated cells by using NIH Image and expressed the content as the following relative density: $10^4 \times \text{TMPD-visualized phospholipid}/\text{TMPD-visualized SAGOOH}/\text{phosphorus}$ of 8×10^7 cells. Then, the same plate was subjected to UV light exposure to determine whether the PSox was externalized. Thereafter, the content of PSox exposed on the cell surface was expressed as the following relative ratio: (TMPD-visualized mPS/

TMPD-visualized SAGOOH)/(mPS/mLPE). Additionally, to determine unmodified PS, i.e., PS within the cells, the same plate was sprayed with ninhydrin, another probe that reacts with primary amino groups.

Statistical evaluations

Data were expressed as mean \pm SE. Changes in variables for different assays were analyzed either by Student's *t* test (single comparisons) or by one-way ANOVA for multiple comparisons. Differences were considered to be significant at $P < 0.05$.

Results

Inhibition by caspase inhibitors of Fas-mediated apoptosis and caspase-3 activation

According to the results of our previous experiments (Matsura et al., 2005), we induced apoptosis by treatment of Jurkat cells with 200 ng/mL anti-Fas antibody for 8 h at 37 °C. To determine the concentration of caspase inhibitors required to inhibit Fas-mediated apoptosis in Jurkat cells, we treated the cells with Z-IETD-FMK, a membrane-permeable caspase-8 inhibitor, or Z-DEVD-FMK, a membrane-permeable caspase-3 inhibitor at concentrations ranging from 0.5 to 5 μ M 30 min before addition of anti-Fas antibody. Z-IETD-FMK inhibited Fas-mediated apoptosis in a dose dependent manner, and the numbers of apoptotic cells were significantly reduced to 14% of cells exposed to anti-Fas antibody alone by treatment with 5 μ M Z-IETD-FMK (Fig. 1A). Pretreatment of the cells with Z-IETD-FMK also inhibited Fas-mediated caspase-3 activation dose-dependently (Fig. 1B). Treatment with 5 μ M Z-IETD-FMK significantly inhibited caspase-3 activity by 46% compared with anti-Fas antibody alone (Fig. 1B). Z-DEVD-FMK pretreatment similarly inhibited Fas-mediated apoptosis in a dose-dependent manner. Treatment with 5 μ M of Z-DEVD-FMK suppressed apoptotic cell number (Fig. 2A) and caspase-3 activity (Fig.

2B) by 87% and 54%, respectively, compared with anti-Fas antibody alone.

Given these results, we used 5 μ M as the concentration of caspase inhibitors in the following experiments.

Effect of caspase inhibitors on PS externalization in Jurkat cells following Fas ligation

We next examined whether there is difference in the effect of caspase-8 inhibitor and caspase-3 inhibitor functioning upstream and downstream from mitochondria, respectively, on PS externalization during Fas-mediated apoptosis. As shown in Fig. 3,

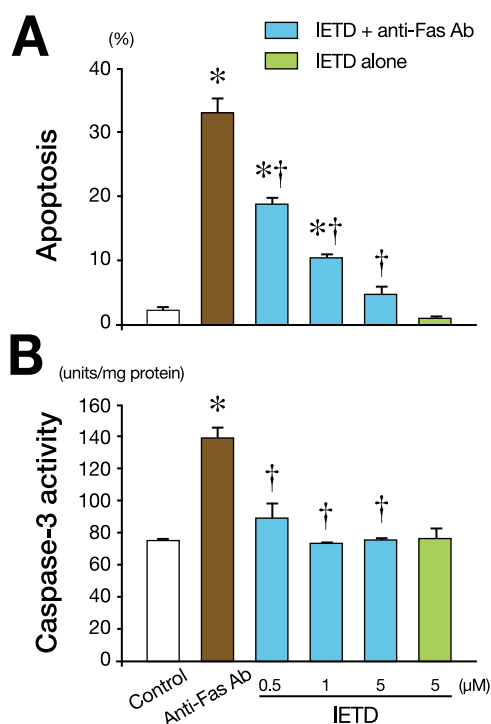


Fig. 1. Effect of caspase-8 inhibitor on Fas-mediated apoptosis and caspase-3 activation in Jurkat cells. Cells were incubated with anti-Fas antibody (200 ng/mL) in 10% fetal calf serum containing RPMI 1640 for 8 h at 37 °C under a 5% CO₂/95% air atmosphere. Z-IETD-FMK, a caspase-8 inhibitor at the indicated concentrations was added 30 min before anti-Fas antibody treatment. The ratio of apoptotic cells (A) was determined by examining nuclear morphology after Hoechst 33342 staining. Caspase-3 activities (B) were measured with a fluorescent substrate as described in Materials and Methods. Data points represent the means \pm SE ($n = 3$). * $P < 0.01$ versus control, † $P < 0.01$ versus anti-Fas antibody alone.

Z-IETD-FMK inhibited PS externalization in Jurkat cells after Fas ligation by 40%. In contrast, Z-DEVD-FMK failed to suppress PS externalization.

Effect of caspase inhibitors on release of mitochondrial cytochrome c in Jurkat cells following Fas ligation

To examine effect of caspase inhibitors on the release of cyt c from mitochondria into the cytosol, we measured cyt c in the cytosol of Jurkat cells at 8 h after treatment with anti-Fas antibody by Western blotting. Z-IETD-FMK prevented the efflux of cyt c from mitochondria into the cytosol during Fas-mediated apoptosis in Jurkat cells (Fig. 4). How-

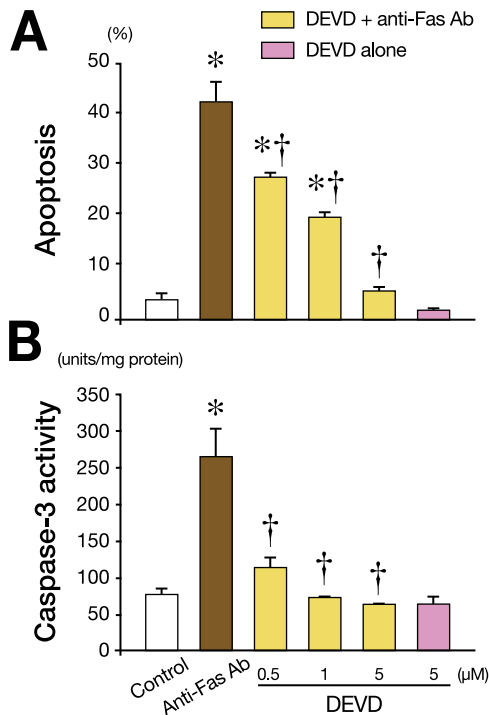


Fig. 2. Effect of caspase-3 inhibitor on Fas-mediated apoptosis and caspase-3 activation in Jurkat cells. Cells were incubated with anti-Fas antibody (200 ng/mL) as described in the legend to Fig. 1. Z-DEVD-FMK, a caspase-3 inhibitor at the indicated concentrations was added 30 min before anti-Fas antibody treatment. The ratio of apoptotic cells (A) was determined by examining nuclear morphology after Hoechst 33342 staining. Caspase-3 activities (B) were measured with a fluorescent substrate as described in Materials and Methods. Data points represent the means \pm SE ($n = 3$). * $P < 0.01$ versus control, † $P < 0.01$ versus anti-Fas antibody alone.

ever, Z-DEVD-FMK was unable to suppress Fas-mediated mitochondrial cyt c release (Fig. 4).

Effect of caspase inhibitors on the amount of PS α transmigrated to the cell surface during Fas-mediated apoptosis

As reported previously (Matsura et al., 2005), we developed the fluorescamine-TMPD method to detect PS α externalized after Fas ligation using two probes on HPTLC chromatogram: TMPD for phospholipid hydroperoxides and fluorescamine for external aminophospholipids. The amount of externalized PS, i.e., mPS increased after treatment of the cells with anti-Fas antibody (200 ng/mL) for 8 h (Fig. 5A). TMPD-visualized mPS, i.e., oxidized mPS also increased after Fas ligation (Fig. 5B). Z-IETD-FMK reduced both levels of mPS and oxidized mPS to control level (Figs. 5A and B). In contrast, Z-DEVD-FMK failed to suppress both levels (Figs. 5A and B). Semi-quantitatively, Fas ligation increased the amount of PS α on the cell surface 1.7 times compared with control (Fig. 5C). Z-IETD-FMK significantly decreased the amount by 38% (Fig. 5C). However, Z-DEVD-FMK did

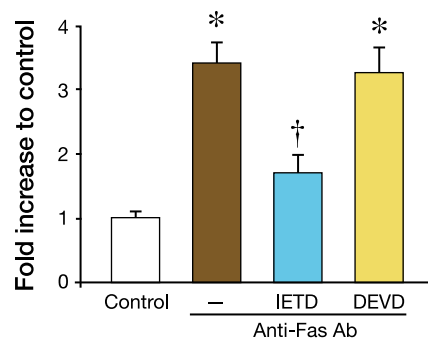


Fig. 3. Effect of caspase inhibitors on phosphatidylserine (PS) exposure to the surface of Jurkat cells after treatment with anti-Fas antibody. Cells were incubated with anti-Fas antibody (200 ng/mL) as described in the legend to Fig. 1. Z-IETD-FMK (5 μ M) or Z-DEVD-FMK (5 μ M) was added 30 min before anti-Fas antibody treatment. At the end of incubation, cells were collected and PS externalization was determined by flow cytometry with an Annexin V-FITC Apoptosis Detection Kit as described in Materials and Methods. Data points represent the means \pm SE ($n = 3$). * $P < 0.01$ versus control, † $P < 0.01$ versus anti-Fas antibody alone (black column).

not inhibit the increase in the amount (Fig. 5C). Furthermore, PS unmodified by fluorescamine, i.e., PS within the cells (referred to as PSin) was determined using ninhydrin staining. Although anti-Fas antibody treatment decreased PSin, Z-IETD-FMK but not Z-DEVD-FMK prevented the decrease in PSin after Fas ligation (data not shown). As mentioned above, the amount of PSin in the cells treated with anti-Fas antibody or anti-Fas antibody plus Z-DEVD-FMK was lesser than that in control cells or the cells treated with anti-Fas antibody plus Z-IETD-FMK. However, the degree of PSin oxidation was stronger in the former cells (Fig. 5B).

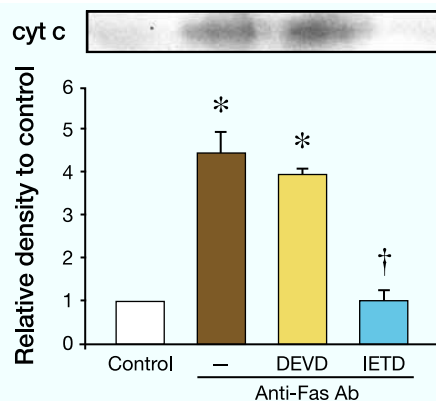


Fig. 4. Effect of caspase inhibitors on cytochrome c (cyt c) release into the cytosol in Jurkat cells after treatment with anti-Fas antibody. Cells were incubated with anti-Fas antibody (200 ng/mL) as described in the legend to Fig. 1. Z-IETD-FMK (5 μ M) or Z-DEVD-FMK (5 μ M) was added 30 min before anti-Fas antibody treatment. At the end of incubation, cytosolic fractions were prepared and analyzed for cyt c content by Western blotting as described in Materials and Methods. The results of densitometric analysis are shown as fold increase relative to control. Data points represent the means \pm SE ($n = 3$). * $P < 0.01$ versus control, † $P < 0.01$ versus anti-Fas antibody alone (black column).

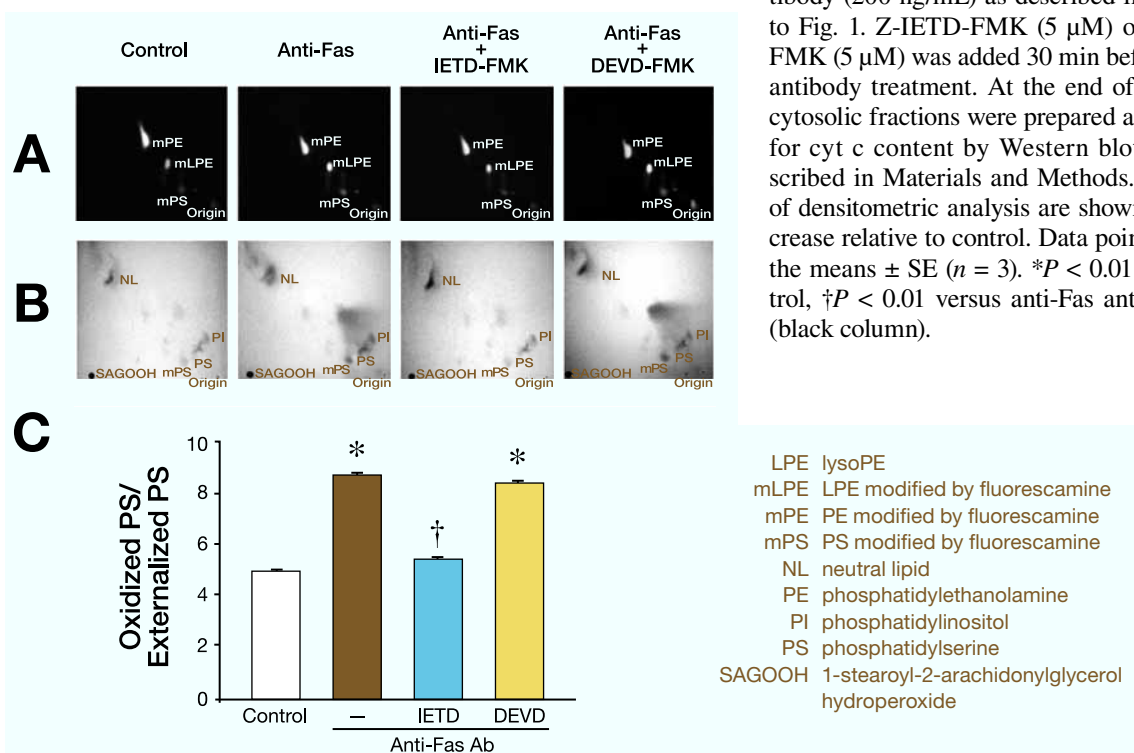


Fig. 5. Effect of caspase inhibitors on oxidation of PS exposed to the surface of Jurkat cells after treatment with anti-Fas antibody. Cells were incubated with anti-Fas antibody (200 ng/mL) as described in the legend to Fig. 1. Z-IETD-FMK (5 μ M) or Z-DEVD-FMK (5 μ M) was added 30 min before anti-Fas antibody treatment. At the end of incubation, total lipids of cells were extracted after a short treatment with fluorescamine and subjected to HPTLC.

A: Fluorescamine-modified PS and PE were localized by exposure of HPTLC plates to UV light. LPE was used as an internal standard.

B: To determine PSox on the cell surface, colorimetric spray detection with TMPD was carried out as described in Materials and Methods. SAGOOH was used as an external standard as described in Materials and Methods. Representative data from three separate experiments are shown.

C: The content of PSox exposed on the cell surface was shown as the relative ratio as described in Materials and Methods. Data points represent the means \pm SE of 3 separate experiments. * $P < 0.01$ versus control, † $P < 0.01$ versus anti-Fas antibody alone (black column).

HPTLC, high-performance thin-layer chromatography; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

Discussion

In the present study, we demonstrated using selective inhibitors of caspase-8 and caspase-3 that mitochondria play an important role in both production and externalization of PSox during apoptosis.

Caspases are a special family of proteases characterized by a cysteine in the catalytic site and an unusual cleavage specificity for aspartic acid residues (Grutter, 2000). To date the human caspase family has 11 members, which are classified into two major subfamilies, the apoptotic and the inflammatory caspases (Siegel, 2006). The apoptotic caspases are further divided into two classes, initiator and executioner caspases (Kantari and Walczak, 2011). Caspase-8 is an initiator caspase and caspase-3 is an executioner one.

There are two signaling pathways for apoptosis, intrinsic (mitochondrial) and extrinsic (receptor-mediated) pathways (Olsson and Zhivotovsky; 2011). The intrinsic apoptosis induced by stresses such as DNA damages, ROS, ultraviolet and anti-cancer drugs, involves disruption of mitochondrial integrity accompanied by the release of cyt c into the cytosol. In the cytosol, released cyt c binds to apoptotic protease-activating factor 1, ATP, and procaspase-9 to form the apoptosome and lead to activation of executioner caspases, which finally induce cell death. In the extrinsic apoptosis, extracellular cognate ligands (e.g., Fas ligand) or agonistic antibodies (e.g., anti-Fas antibody CH-11) stimulate oligomerization of receptor (e.g., Fas), the recruitment of adaptor proteins (e.g., FADD) and initiator procaspase-8 molecules to the cytosolic side of the receptor to form the death-inducing signaling complex (DISC) (Shawgo et al., 2009). Within the DISC, procaspase-8 molecules are activated by dimerization and subsequently undergo auto-processing. Active caspase-8 can directly activate the executioner caspase-3 (type I cells) or cleaves the cytosolic BH3-only protein Bid to truncated Bid (tBid) (type II cells). In turn, tBid translocates to the mitochondria and stimulates mitochondrial outer membrane permeabilization (MOMP), the release

of intermembrane space proteins such as cyt c into the cytosol and apoptosome formation responsible for the activation of executioner caspases including caspase-3. Jurkat cells we used in this study belong to type II cells (Shawgo et al., 2009).

Recent studies have suggested that peroxidase activity of cyt c is closely related to PS oxidation and externalization (Kagan et al., 2004; Byir et al., 2006). Furthermore, it has been reported that PS oxidation in lung epithelial A549 cells undergoing apoptosis after Fas ligation was sensitive to a pan-caspase inhibitor, Z-VAD, which inhibited cyt c efflux from mitochondria but was insensitive to a caspase-3 inhibitor, Z-DQMD, which failed to prevent mitochondrial disruption and cyt c release (Jiang et al., 2004). However, they did not examine the effect of caspase inhibitors on PSox externalization in their study.

Previously we developed fluorescamine-TMPD method to directly determine the presence of PSox on the surface of apoptotic cells (Matsura et al., 2005). This method is semiquantitative but can easily detect PS externalization and oxidation on the same HPTLC plate (i.e., PSox on the cell surface). Additionally, we can detect PSox within the cells on the same HPTLC plate by a combination of staining with ninhydrin, another probe that reacts with primary amino groups. We revealed by these methods that treatment of Jurkat cells with anti-Fas antibody increased PSox within the cells and caused PSox to appear on the cell surface (Matsura et al., 2005).

To illustrate mitochondrial involvement in production and exposure of PSox, we examined by fluorescamine-TMPD method the effect of selective inhibitors of caspase-8 and caspase-3 on production of PSox and its externalization during extrinsic type II cell apoptosis in the present study. As mentioned above, caspase-8 inhibitor can block the apoptotic signal from DISC to mitochondria. By contrast, caspase-3 inhibitor functions downstream from mitochondria (more precisely downstream of apoptosome formation) to suppress characteristic morphological and biochemical changes in apoptosis. Inhibition of caspase-8 by Z-IETD-FMK, a

membrane permeable selective caspase-8 inhibitor reduced mitochondrial cyt c release into the cytosol, the amount of PSox not only within but also on the surface of type II Jurkat cells, caspase-3 activation, and apoptotic cell number after treatment with anti-Fas antibody (CH-11). In contrast, Z-DEVD-FMK, a membrane permeable selective caspase-3 inhibitor was unable to inhibit cyt c release, and the amount of PSox both within and on the surface of the cells after anti-Fas antibody as expected, although it suppressed activation of the executioner caspase-3 and apoptosis. These results strongly suggested that mitochondrial event, especially cyt c departure from mitochondria plays a critical role in production of PSox within the cells and subsequent its appearance on the cell surface during extrinsic apoptosis.

It is considered that a potential mechanism underlying PSox production during apoptosis relies on the specific interactions between positively charged cyt c and negatively charged phospholipids such as PS in the cytosolic leaflet of the plasma membrane and CL in the mitochondria (Kagan et al., 2004, 2009). As for CL, high-affinity binding of CL with cyt c—accompanied by unfolding of the protein—is realized largely through an electrostatic interface between negatively charged phosphates on CL and positively charged lysines on cyt c, as well as through hydrophobic interactions of CL's acyl groups with a hydrophobic domain of the protein (Kagan et al., 2009). PS may also bind and unfold cyt c via analogous mechanisms. It has been reported that the interaction of cyt c with CL causes a marked negative shift of cyt c's redox potential, thus precluding its operation as an electron acceptor from mitochondrial complex III (Kagan et al., 2009). It is proposed that cyt c in cyt c/CL complexes functions as peroxidase to oxidize CL in the presence of ROS (O_2^- and H_2O_2) produced by disrupted mitochondrial electron transport (Kagan et al., 2009; Hüttemann et al., 2011). Since cyt c has lower affinity for oxidized CL (CLOx) than for CL, it gets detached from membranes. Thereafter, cyt c is released into the cytosol following stimulation of MOMP by CLOx (Kagan et al., 2009). cyt c

released into the cytosol forms cyt c/PS complexes in the cytosolic leaflet of plasma membrane as mentioned above and probably oxidizes PS by its peroxidase activity (Matsura et al., 2003). We previously observed using HP100 cells overexpressing catalase derived from HL-60 cells that endogenous H_2O_2 is a key oxidant for cyt c release from mitochondria as well as PS oxidation during apoptosis (Matsura et al., 2004). Taken together, these findings support strongly the notion that interaction of cyt c/anionic phospholipid (CL in mitochondria or PS in plasma membranes) complexes with H_2O_2 is important for PS oxidation via peroxidase activity of cyt c.

In respect to externalization of PSox, recent report demonstrated that flippase can recognize PSox and PS as its substrates, and that PSox may act as a “non-enzymatic scramblase” and stimulate scrambling of PS and/or PSox (Tyurina et al., 2004). However, its precise mechanisms remain to be elucidated. At least, it must be clarified how the affinity of membrane phospholipid translocators including flippases and scramblases for PSox is.

In conclusion, the present study has shown that mitochondria play an essential role in production of PSox and subsequent its transmigration to the cell surface during extrinsic apoptosis by blocking signal transduction from DISC upstream and downstream from mitochondria using selective caspase-8 inhibitor and caspase-3 inhibitor, respectively.

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