Resistance to UV-induced apoptosis in Chinese-hamster ovary cells overexpressing phosphatidylserine synthases

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Externalization of PtdSer (phosphatidylserine) is an important event in signalling removal of apoptotic cells. In contrast with previous work [Yu, Byers, Ridgway, McMaster and Cook (2000) *Biochim. Biophys. Acta* **1487**, 296–308] with U937 cells showing that specific stimulation of PtdSer biosynthesis during apoptosis was caspase dependent, PtdSer biosynthesis in CHO (Chinese-hamster ovary)-K1 increased 2.5-fold during UV-induced apoptosis but was not reversed by a caspase inhibitor, Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone). Also, in CHO-K1 cells, stimulation of synthesis was less specific for PtdSer as similar levels of stimulation were observed for sphingomyelin biosynthesis. Involvement of PtdSer synthase isoforms was tested in CHO-K1 cells overexpressing PSS I (PtdSer synthase I) and PSS II. Both types of transformed cells showed resistance to UV-induced apoptosis based on the decreased levels of caspase 3 activation and morphology changes; externalization of PtdSer was reduced with UV treatment even though expression of endogenous scramblase increased slightly. Serine-labelling experiments showed that PSS I- or PSS II-expressing

cells had higher basal levels of PtdSer biosynthesis compared with vector control cells. When cells were exposed to UV light to induce apoptosis, PtdSer biosynthesis was further stimulated 1.5- and 2-fold in PSS I- and PSS II-expressing cells respectively compared with UV-treated vector cells. Caspase activation was not required, as Z-VAD-FMK did not change PtdSer synthesis. Although enhanced PtdSer synthesis was supposed to facilitate apoptosis, cells overexpressing PSS I and II were actually resistant to UV-induced apoptosis. Whereas enhanced PtdSer synthesis was associated with apoptosis, potential anti-apoptotic effects were observed when excess activity of these synthetic enzymes was present. This suggests a tightly regulated role for PtdSer synthesis and/or an important dependence on compartmentation of PSS enzymes in association with scramblase facilitated enrichment of this phospholipid at the cell surface.

Key words: apoptosis, phosphatidylserine (PtdSer), phosphatidylserine synthase I (PSS I), PSS II, scramblase, ultraviolet light.

INTRODUCTION

Phosphatidylserine (PtdSer), one of the major anionic phospholipids, constitutes approx. 10 % of the total phospholipids of mammalian cells and is generally confined to the inner layer of the plasma membrane [1]. Aminophospholipid translocase, an inward lipid transporter specific for PtdSer and PtdEtn (phosphatidylethanolamine), is responsible for maintaining lipid asymmetry under normal conditions [2–4]. During some cellular events, including platelet activation [5,6], aging of red blood cells [7] or physiological cell death [8,9], PtdSer is actively translocated to the outer leaflet. This externalization is catalysed by members of a phospholipid scramblase family [10,11] involved in Ca²⁺-stimulated flip-flop of phospholipid between lipid bilayers, which is not specific for a phospholipid type. One member of ATP-binding-cassette protein family, ABC1, has been implicated in promoting PtdSer-specific outward movement during apoptosis as a putative floppase [12,13].

PtdSer externalization plays important physiological roles. PtdSer expressed on the surface of activated platelets is an assembly site for coagulation complexes and regulates blood clotting. Exposed PtdSer serves as a marker for apoptotic and senescent cells and signals non-inflammatory removal of such cells through phagocytosis by macrophages [14–17]. Outward translocation of PtdSer and concomitant inward movement of SM (sphingomyelin), also results in membrane protrusions and shedding of membrane vesicles from apoptotic cells [18,19]. Given

the important and multiple roles of PtdSer externalization, regulation of PtdSer mobilization may have important implications for therapeutic intervention.

On the basis of our previous studies of PtdSer synthesis and externalization in U937 cells [20], we hypothesized that PtdSer externalization during apoptosis may alter *de novo* PtdSer biosynthesis as a result of mobilization and shedding of PtdSer at the plasma membrane. *De novo* synthesis of PtdSer occurs at the ER (endoplasmic reticulum) and mitochondria-associated membranes through base exchange of serine with the head groups of existing phospholipids catalysed by PSS I (PtdSer synthase I) and PSS II [21–23]. The two isoforms have different substrate specificities; PSS I utilizes phosphatidylcholine, whereas PSS II converts PtdEtn into PtdSer [24–27]. In CHO (Chinese-hamster ovary)-K1 cells, feedback control, as PtdSer accumulates, appears to regulate serine base-exchange reactions to maintain constant levels of PtdSer [28,29]; however, mechanisms by which cells sense PtdSer levels remain unclear. PtdSer is also the main precursor for PtdEtn in CHO-K1 cells [30]. Newly synthesized PtdSer is transported to mitochondria where PtdSer decarboxylase catalyses the conversion of PtdSer into PtdEtn [31]. In U937 cells, PtdSer biosynthesis is enhanced along with PtdSer externalization after stimulation of apoptosis by a variety of stimuli, and blockage of externalization and apoptosis with broad-spectrum caspase inhibitors leads to abrogation of enhanced PtdSer formation [20]. In the present study, we show that PtdSer biosynthesis is also stimulated in CHO-K1 cells after UV-induced apoptosis

Abbreviations used: CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PSS I, PtdSer synthase I; SM, sphingomyelin; TBS, Tris-buffered saline; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

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but is regulated through a caspase-independent pathway. Overexpression of PSS I or PSS II in CHO-K1 cells indicated that these enzymes are involved in up-regulating PtdSer synthesis in UV-induced apoptosis, but this increase in their activities is not coupled with caspase activation. Furthermore, increased capacity for PtdSer synthesis appears to have a protective effect to reduce UV-induced apoptosis in these cells.

EXPERIMENTAL

Materials

Anti-c-Myc mAb was purchased from ClonTech. Anti PL-scramblase (Ab-1; PL stands for phospholipid) was from Oncogene Research Products (San Diego, CA, U.S.A.). Anti-human PARP [poly(ADP-ribose) polymerase] pAb was from Santa Cruz Biotechnology. Anti-ACTIVE®-caspase 3 pAb was from Promega. LIPOFECTAMINE™ 2000 was obtained from Life Technologies. PI (propidium iodide) was obtained from Sigma and Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) was purchased from Calbiochem. L-[³H(G)]serine was from Mandel Scientific (Guelph, ON, Canada) and Annexin-V-FLUOS staining kit was from Roche Molecular Biochemicals.

Cell culture

Strain CHO-K1 was obtained from the A.T.C.C. Cells were maintained in a 5 % CO₂ atmosphere in DMEM (Dulbecco's modified Eagle's medium; Life Technologies), supplemented with 5 % (v/v) foetal bovine serum (CANSERA, Etobicoke, ON, Canada) and 300 µM proline.

Induction of apoptosis by UV irradiation

Cells grown in regular growth medium were rinsed with and reseeded in fresh DMEM with different modifications. Cells were exposed to a germicidal lamp providing predominantly 254 nm UV-C light (Philips TUV G30T8 30 W bulb) for 10 min and subsequently cultured for different times.

Cloning of PSSs into pcDNA3.1/Myc-His(+) expression vector

Full cDNA sequences of PSS I (GenBank® accession number A41680) and PSS II (GenBank® accession number BAA20355) were provided by Dr O. Kuge (National Institute of Health, Japan) [21,32]. *Xho*I and *Apa*I sites were engineered into the 5'- and 3'-ends of the full coding sequence of PSS I by PCR amplification with primers 1 (5'-GACCTCGAGATGGCGTCGTGCGTGGGGAGCCGG-3') and 2 (5'-GCAGGGCCCTTCTTCCAACTCCATTGGTGAC-3') respectively. Similarly, *Eco*RI and *Sac*II sites were added to the 5'- and 3'-ends of the full coding sequence of PSS II by PCR amplification with primers 1 (5'-GACGAATTCATGCGGAGGGCCGAGCGCAGAGTC-3') and 2 (5'-GCACCGCGGTGAGGCGGCTGAGGCCCTCCTT-3') respectively. PCR-amplified DNA fragments were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen) and checked by DNA sequencing. PSS I was subcloned into the pcDNA3.1/Myc-His(+) A expression vector (Invitrogen) to generate the expression plasmid, pcDNA-PSS I. PSS II was subcloned into the pcDNA3.1/Myc-His(+) B expression vector (Invitrogen) to generate the expression plasmid, pcDNA-PSS II.

Transient transfection of cells

CHO-K1 cells, grown to 80–90 % confluence, were transfected with pcDNA-PSS I or pcDNA-PSS II constructs (1 µg of plasmid

DNA) using LIPOFECTAMINE™ 2000 reagent according to the manufacturer's instructions. Transfected cells were grown for 24 h before analysis.

Stable expression cell lines

CHO-K1 cells were transfected with pcDNA-PSS I, pcDNA-PSS II or empty vectors using LIPOFECTAMINE™ 2000. Cells were switched to growth medium 1 day after transfection containing 600 µg/ml G418 sulphate (Life Technologies) and the selection medium was refreshed every 48 h. Stable clones were obtained by dilution, subcloning and characterized by immunofluorescence and Western blotting. Cells overexpressing PSS I and PSS II were maintained in growth medium containing 350 µg/ml of G418.

Western blotting

Cells were washed with cold TBS (Tris-buffered saline). For PARP preparations, cell extracts were collected by lysing 1×10^7 cells in 200 µl of sample buffer [62 mM Tris/HCl, pH 6.8/1.25 % SDS/3.3 % (v/v) 2-mercaptoethanol/12.5 % (v/v) glycerol/0.05 % Bromophenol Blue]. For PSS or phospholipid scramblase preparations, total protein extracts were prepared by lysing cells in 0.5 ml lysis buffer [1 % (w/v) Triton X-100, 40 µl/ml 5× protease inhibitor cocktail (Roche Molecular Biochemicals) in TBS] followed by incubation on ice for 10 min. The cell lysate was centrifuged at 15 000 *g* for 10 min at 4 °C. Protein in a 200 µl aliquot was precipitated with 1 ml cold acetone, and samples redissolved in SDS sample buffer were incubated at 37 °C for 30 min before loading on to a polyacrylamide gel. The protein concentration of each sample was determined using a micro bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.). Samples were resolved by SDS/PAGE (8 % gel for PARP, 10 % for Myc-tagged PSS I and PSS II and 15 % for phospholipid scramblase) and transferred on to PVDF membrane (Millipore) according to the manufacturer's instructions. For PARP antibody blotting, the membrane was incubated at 22 °C for 1 h with hybridization solution containing anti-human PARP (1:4000) in 5 % skim milk-TTBS (0.04 % Tween 20 in TBS). The blot was rinsed with TBS and incubated for 45 min with goat anti-rabbit horseradish peroxidase-coupled secondary antibody (1:10 000) at 22 °C in 5 % skim milk-TTBS. For detection of Myc-tagged proteins, the membrane was incubated with hybridization solution containing anti-c-Myc mAb (1:2000) according to the manufacturer's instructions. The blot was then incubated with goat anti-mouse horseradish peroxidase-coupled secondary antibody (1:10 000). For phospholipid scramblase antibody blotting, the membrane was incubated at 4 °C for 16 h with hybridization solution containing anti-scramblase (1:100) and 30 min with goat anti-rabbit secondary antibody (1:10 000) in buffers mentioned above. Enhanced chemiluminescence (ECL®; Amersham Biosciences) was used to detect relevant proteins according to the manufacturer's instructions. Cells for immunofluorescence and confocal microscopy were grown on glass coverslips. After incubation, cells were fixed with formaldehyde (3 %, v/v) and permeabilized with 0.05 % Triton X-100 in PBS for 10 min at –20 °C. For detection of c-Myc-tagged protein, cells were incubated with anti-c-Myc mAb (1:500, v/v) in PBS containing 1 % BSA (PBS-BSA) for 1 h and rinsed with PBS-BSA. FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes) was added (2 µg/ml) and incubated for 45 min. The activation of caspase 3 in PSS-expressing cells was detected by incubating overnight with anti-ACTIVE®-caspase 3 pAb (1:500, v/v) at 4 °C followed by staining with FITC-conjugated goat anti-rabbit IgG at 22 °C. Cells were then incubated

with anti-c-Myc mAb (1:500, v/v) in PBS-BSA for 1 h at 22 °C and rinsed with PBS-BSA. Texas Red-conjugated goat anti-mouse secondary antibody (Molecular Probes) was added (2 µg/ml) and incubated for 45 min. In some cases, after staining for active caspase 3, PI was added 30 min after incubation with the secondary antibody detection and incubated for another 15 min to stain the nuclei. Cells were rinsed twice with PBS-BSA. Coverslips were mounted in 2.5 % (v/v) 1,4-diazabicyclo[2,2,2] octane and 90 % glycerol in 50 mM Tris/HCl (pH 9.0) on glass slides. FITC staining was visualized by excitation at 488 nm, and Texas Red and PI staining was visualized by excitation at 543 nm using a Zeiss inverted laser-scanning confocal microscope LSM-510. Superimposed images were obtained with LSM-510 Image software.

Assays for PtdSer externalization

Cells grown on glass coverslips were rinsed with PBS. Annexin-V-FITC staining was performed according to the manufacturer's instructions. After rinsing coverslips with binding buffer to remove unbound annexin-V, cells were fixed with 4 % (w/v) paraformaldehyde for 15 min and rinsed twice with PBS. Coverslips were mounted on glass slides. Green fluorescent images were acquired with a Zeiss LSM-510.

Lipid analyses

In most experiments, after incubation of cells with [³H]serine for various time periods, the culture medium was removed and saved. Cells were rinsed twice with 1 ml cold PBS and both washes were combined with the original culture medium. Cells were harvested by scraping in 3.5 ml methanol/water (5:4, v/v). Wild-type CHO-K1 cells were incubated for 24 h after UV irradiation, and were collected by scraping and centrifugation at 600 g for 10 min and the culture medium containing microvesicles was removed and saved. Cell pellets were rinsed twice with 2 ml cold PBS and the wash was combined with the culture medium. Cell pellets were resuspended in 3.5 ml methanol/water (5:4, v/v). Lipids were extracted from cell pellets and the medium using a modified Folch procedure [33,34]. Radioactivity in lipid extracts was quantified using a liquid-scintillation counter. Phospholipids were separated using TLC with a solvent system of chloroform/ethanol/triethylamine/water (4:5:4:1, by vol.) and quantified with a Bioscan 200 Imaging Scanner. Total phospholipid mass was determined by measuring the phosphorus content of lipid extracts [35]. Total phospholipid biosynthesis was represented by combining the radioactivity of phospholipids from cell pellets and from the medium and then normalizing relative to total phosphorus mass. Results from 3 or 4 experiments were expressed as means ± S.E.M., and statistical differences were calculated using Student's *t* test.

Synthase activity

After the removal of the culture medium, cell lysate was prepared by scraping cells in 0.5 ml ice-cold suspension buffer (250 mM sucrose/10 mM Hepes buffer, pH 7.5) and sonication for 30 s on ice. Samples were centrifuged at 600 g for 2 min at 4 °C. The supernatant from cell extracts (100 µl) was added to an equal volume of prewarmed assay mixture (5 mM CaCl₂/50 mM Hepes buffer, pH 7.5/1 µCi [³H]serine) and incubated at 37 °C for 20 min [36]. Reactions were terminated by adding 1.5 ml of methanol/water (5:4, v/v) and 2 ml of chloroform. Samples were mixed thoroughly and centrifuged to facilitate phase separation. Lipid was extracted using modified Folch procedure and total

Figure 1 Effects of induction of apoptosis on metabolism of serine-derived phospholipids

(A) CHO-K1 cells were treated with or without UV light and cultured for the time periods indicated. Proteins were extracted and separated (20 µg) using SDS/PAGE. Immunoblotting was performed with anti-human PARP pAb as described in the Experimental section. (B) CHO-K1 cells were incubated in serine-free DMEM. After treating cells with or without UV light, they were incubated with 12 µCi [³H]serine for time periods indicated and harvested as described in the Experimental section. Lipids were extracted and separated by TLC, and radioactivities in PtdSer, PtdEtn and SM were normalized relative to total phospholipid phosphorus. Results are means ± S.E.M. for six samples.

radioactivity was determined using the liquid-scintillation counter. Protein concentration was measured using a micro bicinchoninic acid kit.

RESULTS

Metabolism of serine-derived phospholipids after UV irradiation

CHO-K1 cells are susceptible to UV-induced apoptosis [37,38]. After exposure to UV light, CHO-K1 cells developed apoptotic morphology and biochemical changes such as cleavage of PARP within 8–12 h (Figure 1A). Synthesis of serine-derived phospholipids was monitored during the process of UV-induced apoptosis using [³H]serine. A time-dependent increase in PtdSer formation was observed in cells exposed to UV irradiation (2.5-fold increase by 18 h), whereas the level of PtdEtn derived from decarboxylation of PtdSer decreased slightly (Figure 1B). As UV irradiation did not increase serine uptake, the stimulation of PtdSer formation probably resulted from direct regulation of the serine base-exchange reaction at the ER or mitochondria-associated membranes through activities of PSSs; a decrease in PtdSer decarboxylation or an increase in intracellular levels of serine was not responsible. Incorporation of serine into SM was also stimulated in a time-dependent manner after UV irradiation (Figure 1B).

Membrane blebbing into microvesicles is a typical morphological feature of apoptotic cells at late stages of cell death. When cells were separated from cell-free medium containing microvesicles at 24 h after UV irradiation, PtdSer and SM recovered from vesicles were 6-fold higher than control levels without UV treatment (Table 1), whereas a 1.5-fold increase was observed in the levels of serine-derived PtdEtn in vesicles. Thus, in contrast with U937 cells, biosynthesis of SM is also stimulated along with the PtdSer in CHO-K1 cells after UV-induced apoptosis and both

Table 1 Effects of UV-induced apoptosis on distribution of serine-derived phospholipids in cellular and vesicular fractions of CHO-K1 cells

CHO-K1 cells were treated with or without UV light and cultured for 24 h in serine-free DMEM with 12 μ Ci [3 H]serine. Cells were collected by centrifugation and lipids were extracted from cell pellets and vesicles as described in the Experimental section. Lipids were separated by TLC and radioactivities in PtdSer, PtdEtn and SM were normalized relative to total phospholipid phosphorus. Results are means $\pm$ S.E.M. for six samples. Changes were also expressed as fold increase in UV-treated cells compared with untreated cells using averages from six samples. The level of significance was assessed with a Student's *t* test for unpaired data.

	[3 H]Serine incorporation into phospholipids (1×10^3 d.p.m./nmol phosphorus)		
	PtdSer	PtdEtn	SM
Cells			
Control	0.87 ± 0.02	0.64 ± 0.01	0.39 ± 0.01
UV	$1.65 \pm 0.01^*$	$0.34 \pm 0.01^*$	$0.62 \pm 0.02^*$
Fold change	1.9	0.5	1.6
Vesicles			
Control	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
UV	$0.46 \pm 0.02^*$	$0.12 \pm 0.01^*$	$0.55 \pm 0.02^*$
Fold change	5.8	1.5	6.1

* $P < 0.001$ versus control.

phospholipids were transported to apoptotic vesicles at higher levels.

Blockage of PARP cleavage by a caspase inhibitor, Z-VAD-FMK, indicated that UV-induced apoptosis was inhibited (Figure 2A). However, stimulation of PtdSer biosynthesis was not reversed by the inhibition of apoptosis progression by Z-VAD-FMK (Figure 2B), indicating that activation of caspases is not required for the up-regulation of PtdSer formation. This observation contrasts with the caspase-dependent PtdSer stimulation we observed using U937 cells [20]. A slight increase in SM biosynthesis was observed in the presence of Z-VAD-FMK (Figure 2C).

Stable expression of PSS I or PSS II

To study the involvement of PSS activity in regulating PtdSer formation during UV-induced apoptosis, we established stable cell lines overexpressing c-Myc-tagged PSS I and PSS II. Stable expression of PSS I gave a prominent protein band at 42 kDa (Figure 3A), consistent with PSS I being a highly hydrophobic protein that migrates faster in SDS/PAGE than predicted by the theoretical mass of 55 kDa [25]. Stable expression of PSS II gave a protein band close to its calculated mass (55 kDa) with smeared bands migrating at high molecular mass probably due to extensive polymerization [39]. Immunofluorescence of these cells with anti-Myc antibody showed that both PSS I and PSS II localized to ER (Figure 3B). *In vitro* serine base-exchange activities were 2–3-fold higher in CHO-K1 cells stably overexpressing PSS I or PSS II when compared with that in control cells transfected with empty vector (Figure 3C). Several mixed clonal populations of PSS I and PSS II cell lines were initially characterized with regard to susceptibility to UV light-induced apoptosis. Analysis of the original set of PSS I and PSS II overexpressing clones resulted in the observation that expression levels of PSS I and PSS II correlated strongly with the degree of resistance to apoptosis. One cell line for both PSS I and PSS II overexpressing cells was chosen for further in-depth mechanistic analysis of this general phenomenon.

Figure 2 Effects of Z-VAD-FMK on apoptosis and on PtdSer and SM biosynthesis

(A) CHO-K1 cells were treated with or without UV light and incubated for 12 h with 100 μ M Z-VAD-FMK. PARP cleavage was detected as described in the Experimental section. (B, C) After treatment of cells with or without UV light, cells were incubated with 12 μ Ci [3 H]serine in serine-free DMEM with or without 100 μ M Z-VAD-FMK for 12 h. Cells were harvested and lipids were extracted and separated by TLC. Radioactivity in PtdSer (B) or SM (C) was determined. Results are means $\pm$ S.E.M. for six samples. The level of significance (versus cells irradiated with UV only) was assessed using Student's *t* test for unpaired data.

Resistance to apoptosis in cells expressing PSS I or PSS II

When cells overexpressing PSS I or PSS II were irradiated with UV light, they showed less significant apoptotic morphology such as cell shrinkage, refractile appearance and rounding compared with UV-treated vector control cells (Figure 4, left panels), indicating possible resistance towards UV-induced apoptosis rendered by expression of PSS I or PSS II. PtdSer externalization after UV light exposure, measured as green-fluorescent annexin-V binding, was less in cells overexpressing PSS I or PSS II (Figure 4, right panels) than in control cells exposed to UV light. Endogenous levels of scramblase, measured by Western-blot analysis and quantified by gel scanning (Figure 5), was not significantly different ($P > 0.1$) for vector controls and cells overexpressing PSS I or PSS II in the absence of UV treatment at 6 or 10 h. At 6 h after exposure to UV light, scramblase expression slightly decreased ($P < 0.05$) in cells exposed to UV compared with control vector cells, whereas cells overexpressing PSS I or PSS II showed no such changes after UV treatment. Cells overexpressing PSS I or PSS II had slightly higher ($P < 0.02$) levels of scramblase when compared with control cells after all cells were exposed to UV; however, similar changes were not detected at 10 h. Under control conditions, both PSS I- and

Figure 3 Expression of PSS I or PSS II and detection of serine base-exchange activity *in vitro*

(A) G418-resistant clones were selected in CHO-K1 cells transfected with empty expression vectors, pcDNA-PSS I or pcDNA-PSS II constructs. Results from one clone with the best expression rate are shown. Proteins were extracted and separated (20 μ g) by SDS/PAGE. Myc-PSS I and PSS II were detected by Western blotting with anti-c-Myc mAb. (B) Immunofluorescence detection of c-Myc-tagged proteins was performed on cells stably expressing PSS I or PSS II and vector controls as described in the Experimental section. Images were obtained using confocal microscopy. Scale bar, 10 μ m. (C) Cell extracts from PSS I- and PSS II-expressing cells and vector cells were prepared and assayed for serine base-exchange activities as described in the Experimental section. Results are means $\pm$ S.E.M. for six samples.

PSS II-expressing cells, as well as the control line, showed little response to an antibody raised against an epitope only appearing in activated caspase 3 (Figure 6A, lack of green fluorescence in left panels). After exposure to UV light, extensive activation of caspase 3 occurred in vector cells (70%) after 12 h incubation, but less so in PSS I- (33%) or PSS II-expressing cells (45%) (Figure 6B). Nuclear condensation and fragmentation developed later in UV-treated vector cells, whereas relatively normal nuclear morphology remained in most cells expressing high levels of PSS I; PSS II cells showed less resistance to apoptotic nuclear changes (results not shown).

PtdSer synthesis in cells overexpressing PSS I or PSS II

To test the involvement of PSS I and PSS II in regulating PtdSer formation during UV-induced apoptosis, CHO-K1 cells overexpressing the two enzymes were exposed to UV light and synthesis of serine-derived phospholipids, including PtdSer and SM and PtdEtn from PtdSer decarboxylation, was monitored using

[³H]serine. Cells overexpressing PSS I had a slightly higher basal rate of PtdSer formation and approx. 50% higher PtdSer decarboxylation to PtdEtn when compared with vector control cells, whereas no major difference in SM synthesis was observed (Figure 7). When irradiated by UV exposure, PSS I-expressing cells showed earlier stimulation of PtdSer biosynthesis with PtdSer levels increasing above those of UV-treated vector cells by 3 h; the increase in UV-treated vector cells started approx. 8 h after UV irradiation when apoptotic morphology started to develop. After 16–24 h of incubation after UV irradiation, PtdSer levels in UV-treated PSS I-expressing cells remained 1.5–2-fold higher than those in UV-treated vector cells (Figure 7, top panel). Similar to the 50% inhibition of PtdEtn formation observed in UV-treated vector cells relative to their untreated counterparts, UV-treated PSS I-expressing cells also had a 50% decrease in PtdSer decarboxylation to PtdEtn when compared with untreated PSS I-expressing cells (Figure 7, middle panel). PSS I overexpression did not change SM biosynthesis relative to vector cells under control conditions, whereas UV-treated PSS I-expressing cells had a slightly higher rate of SM stimulation when compared with UV-treated vector cells (Figure 7, bottom panel).

When PSS II was overexpressed in CHO-K1 cells, a higher basal level of PtdSer synthesis (1.5-fold) and decarboxylation (2-fold) was observed, whereas SM levels were slightly lowered in cells overexpressing PSS II (Figure 8). UV irradiation induced significant stimulation of PtdSer formation in PSS II-expressing cells; at all incubation times, PtdSer levels in UV-treated PSS II cells were 2-fold higher than those in the UV-treated vector cells (Figure 8, top panel). PtdSer decarboxylation to PtdEtn was not changed appreciably in PSS II-expressing or vector cells after UV irradiation except at 12 h of incubation. UV-treated cells with PSS II expression had a 70% decrease in PtdEtn formation when compared with untreated PSS II-expressing cells (Figure 8, middle panel). Although SM synthesis increased 3-fold in both the UV-treated PSS II-expressing and vector cells, PSS II-expressing cells showed a 60% lower level of the stimulation after UV irradiation when compared with the treated vector cells (Figure 8, bottom panel). Overall, cells expressing PSS I or PSS II showed resistance towards UV-induced apoptosis and PtdSer biosynthesis was enhanced after UV treatment in these cells. Thus UV irradiation seemed to up-regulate the activities of both PSS I and PSS II, promoting serine base-exchange reactions to form PtdSer without changing the rate of PtdSer decarboxylation appreciably.

Caspase-independent PtdSer biosynthesis in PSS I or PSS II cells

Stimulation of PtdSer biosynthesis in apoptotic CHO-K1 cells was not inhibited by the caspase inhibitor, Z-VAD-FMK. Potential caspase dependence of PtdSer biosynthesis in PSS I- and PSS II-expressing cells was further studied by co-incubating cells with Z-VAD-FMK after UV irradiation. Under these conditions, further stimulation of PtdSer biosynthesis mediated by PSS I and PSS II overexpression was completely insensitive to Z-VAD-FMK, although PARP cleavage was blocked (Figure 9).

DISCUSSION

PtdSer externalization is an important cellular response with significant physiological roles during a variety of cellular events. Our studies focused on potential correlations underlying translocation of PtdSer at the plasma membrane and *de novo* biosynthesis of this phospholipid. We considered the possibility

Figure 4 Morphology and PtdSer externalization for cells overexpressing PSS I or PSS II after UV irradiation

CHO-K1 cells overexpressing PSS I, PSS II and vector control clones were grown in DMEM and treated with or without UV light. After 12 h of incubation, phase-contrast photographs (first two columns) were taken at $\times 300$ magnification. Annexin-V binding (green fluorescence) was assayed as described in the Experimental section (columns 3 and 4).

Figure 5 Expression of endogenous phospholipid scramblase in cells overexpressing PSS I and PSS II after UV irradiation

Vector control cells and cells overexpressing PSS I or PSS II were grown and treated with or without UV light. After 6 or 10 h incubations, scramblase expression was assessed by Western-blot analysis and quantified as described in the Experimental section. The upper panels are representative Western blots at 6 and 10 h for the same conditions shown in the lower panel, with the last lane on the right containing protein extracted from U937 leukaemia cells, an additional control for scramblase expression. Quantitative values in the lower panel are means $\pm$ S.E.M. for six separate dishes of cells in three experiments at 6 h and means $\pm$ S.E.M. for 12 separate dishes of cells in five experiments at 10 h.

that PtdSer externalization may influence biosynthesis of new PtdSer because of disturbances in the intracellular distribution of PtdSer. Previously, we established that PtdSer biosynthesis and

externalization in U937 cells were stimulated during apoptosis induced with various stimuli and those specific changes in PtdSer synthesis and movement were regulated through caspase-dependent mechanisms [20]. To see the extent to which the stimulation of PtdSer synthesis and externalization might be generally found in all cells during apoptosis, we further investigated PtdSer biosynthesis during UV-induced apoptosis in CHO-K1 cells. Our studies demonstrated that the apoptotic changes were developed in CHO-K1 cells as a result of UV irradiation. Furthermore, PtdSer biosynthesis was also enhanced after UV irradiation. This enhanced activity and transfer seem to result from the direct stimulation of serine base-exchange reactions instead of the blockage of PtdSer decarboxylation or increased serine uptake. Thus the stimulation of PtdSer formation during apoptosis in both U937 and CHO-K1 cells suggest that altered PtdSer metabolism alone may not be a cell-type-specific response, but rather may accompany PtdSer externalization in many types of apoptotic cells.

In contrast with observations with U937 cells, up-regulation of PtdSer biosynthesis in apoptotic CHO-K1 cells seemed to be less specific for PtdSer as SM formation was enhanced at levels similar to that of the PtdSer. Newly synthesized SM was also transported to apoptotic bodies so that enrichment relative to whole cells was similar to that for the PtdSer. The stimulation of both PtdSer and SM biosynthesis was insensitive to Z-VAD-FMK in CHO-K1 cells, indicating a caspase-independent regulation of phospholipid biosynthesis in these cells. Thus our results indicate that increased PtdSer biosynthesis may be a general phenomenon during apoptosis, but stimulation of PtdSer synthesis may involve distinct regulatory pathways depending on the types of cells involved.

PtdSer formation in mammalian cells is catalysed by at least two PSS isoforms [23]. Stimulation of PtdSer biosynthesis observed during apoptosis could involve the regulation of either PSS I or

Figure 6 Caspase 3 activation after UV irradiation of cells overexpressing PSS I or PSS II

(A) CHO-K1 cells overexpressing PSS I, PSS II and vector control clones were treated with or without UV light and cultured for an additional 12 h. Cells were fixed, permeabilized and incubated with anti-active-caspase 3 pAb followed by anti-c-Myc mAb. Fluorescein-conjugated goat anti-rabbit IgG was used to detect the activated form of caspase 3 (green). Texas Red-conjugated goat anti-mouse IgG was used to detect Myc-PSS I or PSS II (red). Scale bar, 20 μ m. (B) Approx. 1000 cells were counted in each sample to determine cells positive for active caspase 3. Results are means $\pm$ S.E.M. for three samples. The level of significance (versus UV-treated vector cells) was assessed using Student's *t* test for unpaired data.

PSS II activity. PSS I enzyme is directly inhibited by PtdSer, its product, so that overexpression of PSS I may not necessarily lead to increased PtdSer biosynthesis and change of PtdSer mass *in vivo* [28,29]. When PtdSer is externalized to the cell surface

Figure 7 Biosynthesis of serine-derived phospholipids in cells overexpressing PSS I

PSS I (∇ and $\blacktriangledown$) and control ($\circ$ and $\bullet$) cells were seeded in serine-free DMEM. After treatment of cells with ($\blacktriangledown$ and $\bullet$) or without (∇ and $\circ$) UV light, they were incubated for the indicated time periods with 20 μ Ci [3 H]serine. Cells were harvested and lipids were extracted from cells and apoptotic vesicles and separated by TLC. Radioactivities in PtdSer, PtdEtn and SM were determined as described in the Experimental section. Results are means $\pm$ S.E.M. for six samples.

and subsequently removed by shedding of PtdSer-containing microvesicles, cells may be capable of sensing decreased intracellular levels of PtdSer and activate PtdSer biosynthesis through activities of serine base-exchange enzymes. We proposed that if PSS I and PSS II are directly involved, even greater stimulation of PtdSer formation might result from overexpression of these enzymes in CHO-K1 cells undergoing UV-induced apoptosis. Indeed, we found that CHO-K1 cells overexpressing either PSS I or PSS II showed much higher levels of PtdSer biosynthesis in response to UV irradiation, indicating involvement of these enzymes in enhancing PtdSer biosynthesis. In contrast, relatively little change was observed in the metabolism of other major phospholipids derived from serine. We also postulated that increased *de novo* PtdSer biosynthesis due to overexpression of PSS I and PSS II might positively regulate PtdSer externalization and as a result influence the progression of UV-induced apoptosis. Surprisingly, UV-induced stimulation of serine incorporation into PtdSer in cells overexpressing PSS I or PSS II did not result in an increase in PtdSer externalization (on the basis of the

Figure 8 Biosynthesis of serine-derived phospholipids in cells overexpressing PSS II

PSS II (■ and □) and control (● and ○) cells were grown in serine-free DMEM. After treatment of cells with (■ and ●) or without (□ and ○) UV light, they were incubated for the indicated time periods with 20 μ Ci [3 H]serine. Cells were harvested and lipids were extracted from cells and apoptotic vesicles and separated by TLC. Radioactivities in PtdSer, PtdEtn and SM were determined. Results are means $\pm$ S.E.M. for six samples.

annexin-binding experiments, externalization of PtdSer actually decreased slightly in UV-treated cells expressing PSS isoforms relative to vector controls). Also scramblase expression, anticipated to influence PtdSer externalization and cell apoptosis [40], was altered relatively less in cells overexpressing PSS I or PSS II, with or without UV treatment, relative to control cells. Furthermore, apoptosis was negatively regulated, by overexpression of PSS I or PSS II in UV-irradiated cells, shown by a lack of caspase 3 activation and other apoptotic morphological changes. An increase in PtdSer biosynthesis in UV-treated, PSS-expressing cells occurred shortly after UV irradiation (3–4 h) and thus was independent of, or preceded, PtdSer externalization (8 h) as apoptosis was prevented or delayed in these cells. In contrast with caspase-dependent PtdSer stimulation in U937 cells, activation of caspases seems to be uncoupled from PtdSer biosynthesis in CHO-K1 cells and is also not required for regulating the activity of either PSS I or PSS II. PtdSer biosynthesis catalysed by PSS I or PSS II appears to be a caspase-independent event. The increase in PtdSer biosynthesis in cells overexpressing PSS I and PSS II precedes externalization of

Figure 9 Effects of Z-VAD-FMK on PARP cleavage and phospholipid biosynthesis

(A) CHO-K1 cells overexpressing PSS I, PSS II and vector control clones were treated with or without UV light and incubated for 12 h with 100 μ M Z-VAD-FMK. Proteins were extracted and PARP cleavage was detected. (B) PSS I, PSS II and control cells were grown in serine-free DMEM. After treatment of cells with or without UV light, 100 μ M Z-VAD-FMK was added to cells as indicated. Cells were incubated for 8 h (PSS I-expressing cells) or 12 h (PSS II-expressing cells) in the presence of 20 μ Ci [3 H]serine. Cells were harvested and lipid extraction, phospholipids separation and quantification were performed. Radioactivity in PtdSer was determined. Results are means $\pm$ S.E.M. for six samples.

PtdSer, indicating that their activities may be regulated by events upstream of apoptosis rather than being associated with PtdSer externalization. These observations indicate limitations to the possibility of a universal causal relationship between the up-regulation of PtdSer biosynthesis and externalization. The greater stimulation of PtdSer biosynthesis in PSS-expressing cells after UV irradiation appears to be a direct result of high levels of expression of these serine base-exchange enzymes. By extrapolation (but not proven in our studies), PSS I and/or PSS II may be up-regulated in wild-type CHO-K1 cells after UV exposure. PSS I and (or) PSS II catalyse(s) the incorporation of serine into PtdSer in response to UV irradiation in a caspase-independent manner. The mechanisms of stimulation of serine base-exchange enzymes have yet to be identified. Activities of serine base-exchange enzymes are stimulated by Ca^{2+} [41] and depletion of Ca^{2+} from ER stores strongly inhibits PtdSer biosynthesis [42]. Serine base-exchange reactions can be stimulated by cationic amphiphilic chemicals, K^+ -channel blockers and

Ca^{2+} /calmodulin antagonists [43]. Ca^{2+} also stimulates the proteolytic activity of calpains and a number of studies have observed that calpain activation precedes apoptotic cell death. Calpains themselves are generally believed to be unable to activate caspases directly and instead appear to cleave Bcl-2 family members resulting in a net increase in the release of cytochrome *c* from the mitochondria for subsequent apoptosome formation. However, cleavage of PSS I or PSS II was not observed in our studies implying that they are not the direct substrates for either caspases or calpains. The involvement of these various potential stimulators of PtdSer biosynthesis during induced apoptosis may contribute to the increase in new PtdSer formation and merits further in-depth investigation.

Lack of PtdSer externalization and apoptosis in PSS-expressing cells treated with UV light was unexpected. Reasons for this resistance to enhanced apoptosis in PSS I- or PSS II-expressing cells remain unknown but seem to be specific for UV-induced apoptosis. UV irradiation causes cell-cycle arrest and DNA damage. Failure to correct the latter results in the cell death due to activation of caspase-dependent pathways mediated by p53 [44]. It seems probable that upstream events normally associated with UV-induced apoptosis may be inhibited when PSS I or PSS II are overexpressed. Whether this is directly caused by an excessive amount of PSS I or PSS II enzymes or is a result of higher levels of intracellular PtdSer is not clear. PtdSer serves as a cofactor for proteins crucial in cell signalling, such as PKC (protein kinase C) and MARCKS (myristoylated alanine-rich C-kinase substrate) [45,46]. It can be speculated that increased intracellular levels of PtdSer may result in the recruitment of PtdSer-associated proteins and thus influence the progress of apoptosis. High levels of PtdSer inside the cell probably serve as a survival signal. Accordingly, the process of translocation of PtdSer to the cell surface may have positive effects on the progression of apoptosis. Although we speculate that exposure of PtdSer on the cell surface may provide a signal for enhancement of PtdSer biosynthesis, it appears that an increase in intracellular PtdSer levels in response to UV irradiation in conjunction with overexpressing PSS I or PSS II did not elicit the same level of regulation or positive feedback on PtdSer translocation.

In conclusion, our results show that PtdSer biosynthesis is stimulated in CHO-K1 cells in a caspase-independent manner after UV-induced apoptosis. Overexpression of PSS I or PSS II specifically enhances stimulation of PtdSer formation after UV irradiation, indicating that increasing the content of synthase enzymes can up-regulate PtdSer biosynthesis. However, PtdSer formation through PSS I and PSS II activities is uncoupled from caspase activation. Moreover, cells overexpressing PSS I or PSS II showed significant resistance to UV-induced apoptosis. These studies provide a better understanding of the role or limitations of PtdSer biosynthesis and movement in the process of apoptosis. Furthermore, the regulation of PtdSer biosynthesis and its externalization may allow for consideration of novel ways of altering induction or inhibition of apoptosis.

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