Appearance of Phosphatidylserine on Apoptotic Cells Requires Calcium-mediated Nonspecific Flip-Flop and Is Enhanced by Loss of the Aminophospholipid Translocase*

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Phosphatidylserine (PS), ordinarily sequestered in the plasma membrane inner leaflet, appears in the outer leaflet during apoptosis, where it triggers non-inflammatory phagocytic recognition of the apoptotic cell. The mechanism of PS appearance during apoptosis is not well understood but has been associated with loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids of various classes. The human leukemic cell line HL-60, the T cell line Jurkat, and peripheral blood neutrophils, undergoing apoptosis induced either with UV irradiation or anti-Fas antibody, were probed in the cytofluorograph for (i) surface PS using fluorescein isothiocyanate-labeled annexin V, (ii) PS uptake by the aminophospholipid translocase using \textit{N,N,O,O}-tetraacetic acid (NBD)-labeled PS, (iii) nonspecific uptake of phospholipids (as a measure of transbilayer flip-flop) using NBD-labeled phosphatidylcholine, and (iv) the appearance of hypodiploid DNA. In all three types of cells undergoing apoptosis, the appearance of PS followed loss of aminophospholipid translocase and was accompanied by nonspecific phospholipid flip-flop. Importantly, however, in the absence of extracellular calcium, the appearance of PS was completely inhibited despite DNA fragmentation and loss of aminophospholipid translocase activity, the latter demonstrating that loss of the translocase is insufficient for PS appearance during apoptosis. Furthermore, while both the appearance of PS and nonspecific phospholipid uptake demonstrated identical extracellular calcium requirements with an \textit{ED}_{50} of nearly 100 \textmu M, the magnitude of PS appearance depended on the level of aminophospholipid translocase activity. Taken together, the data strongly suggest that while nonspecific flip-flop is the driving event for PS appearance in the plasma membrane outer leaflet, aminophospholipid translocase activity ultimately modulates its appearance.

The appearance of phosphatidylserine in the outer leaflet of the plasma membrane appears to be a universal phenomenon in cells undergoing apoptosis, or programmed cell death (1). Importantly, outer leaflet PS likely serves as a signal in tissues in cells undergoing apoptosis, or programmed cell death (1).


d and see “Discussion”), a distinctly different response than the pro-inflammatory events following tissue necrosis. While phosphatidylserine (PS) is actively transported from the outer to the inner leaflet by the aminophospholipid translocase (3–5), the mechanism(s) by which PS appears in apoptosis is not well understood. Verhoven 	extit{et al.} (3) reported that PS appearance was accompanied by both loss of aminophospholipid translocase activity and enhanced nonspecific transbilayer movement of phospholipids, perhaps due to activation of a “scramblase.” Hence, they proposed that PS may become detectable in the outer leaflet due to the combination of these two events. However, to date, the relative contribution of these two events has not been clarified. Additionally, it is not known what role, if any, other changes occurring during apoptosis (\textit{e.g.} membrane blebbing and vesiculation) may play in the appearance of PS in the outer membrane leaflet. We hypothesized that the appearance of PS on the surface of the cell undergoing apoptosis would result primarily from enhanced calcium-dependent transbilayer movement of phospholipids (flip-flop) across the plasma membrane. Here we present data that demonstrates that (i) the loss of aminophospholipid translocase activity alone does not result in PS appearance, (ii) the appearance of PS likely results from calcium-dependent phospholipid flip-flop that is nonspecific for head group, (iii) while loss of aminophospholipid translocase activity is insufficient to result in PS appearance, loss of its function is probably a necessary event, and (iv) the appearance of PS at the cell surface can be dissociated from nuclear changes (DNA fragmentation) during apoptosis.

MATERIALS AND METHODS

Cell Culture and Isolation

The human leukemia cell line HL-60 and the human T cell line Jurkat were obtained from ATCC (Rockville, MD). Both HL-60s and Jurkats were cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 20% fetal bovine serum and maintained at 37 °C in a 5% \textit{CO}_2, humidified atmosphere and harvested while in log phase of growth at approximately 1.5 × 10^6 cells/ml for HL-60s, and 1 × 10^6 cells/ml for Jurkat. The cells were washed once in phosphate-buffered saline (pH 7.4) and resuspended as described below. Human peripheral blood neutrophils were isolated by the plasma Percoll density gradient method from healthy donors as described previously (6).

Incubation Conditions

HL-60s were plated at 2 × 10^6 cells/ml in either 35-mm dishes or 12-well plates in RPMI with calcium (550 \textmu M) or without calcium (nominally present at 620 nM) from Life Technologies, Inc. (Gaithersburg, MD) supplemented with 0.5% bovine serum albumin, with or without 5 \mu M cytochalasin D. Neutrophils were plated at 5 × 10^6 cells/ml in 12-well plates in RPMI with or without calcium with 0.25% HBS, Hepes-buffered saline; FITC, fluorescein isothiocyanate; BAPTA, 1,2-bis(O-aminophenoxy)ethane-N,N',N",N"-tetraacetic acid.

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‡ The abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; NBD, \textit{N,N,O,O}-tetraacetic acid (NBD)-labeled PS, (iii) nonspecific uptake of phospholipids (as a measure of transbilayer flip-flop) using NBD-labeled phosphatidylcholine, and (iv) the appearance of hypodiploid DNA. In all three types of cells undergoing apoptosis, the appearance of PS followed loss of aminophospholipid translocase and was accompanied by nonspecific phospholipid flip-flop. Importantly, however, in the absence of extracellular calcium, the appearance of PS was completely inhibited despite DNA fragmentation and loss of aminophospholipid translocase activity, the latter demonstrating that loss of the translocase is insufficient for PS appearance during apoptosis. Furthermore, while both the appearance of PS and nonspecific phospholipid uptake demonstrated identical extracellular calcium requirements with an \textit{ED}_{50} of nearly 100 \textmu M, the magnitude of PS appearance depended on the level of aminophospholipid translocase activity. Taken together, the data strongly suggest that while nonspecific flip-flop is the driving event for PS appearance in the plasma membrane outer leaflet, aminophospholipid translocase activity ultimately modulates its appearance.

The appearance of phosphatidylserine in the outer leaflet of the plasma membrane appears to be a universal phenomenon in cells undergoing apoptosis, or programmed cell death (1). Importantly, outer leaflet PS likely serves as a signal in tissues for the noninflammatory engulfment of apoptotic cells (Ref. 2 and see “Discussion”), a distinctly different response than the pro-inflammatory events following tissue necrosis. While phosphatidylserine (PS) is actively transported from the outer to the inner leaflet by the aminophospholipid translocase (3–5), the mechanism(s) by which PS appears in apoptosis is not well understood. Verhoven 	extit{et al.} (3) reported that PS appearance was accompanied by both loss of aminophospholipid translocase activity and enhanced nonspecific transbilayer movement of phospholipids, perhaps due to activation of a “scramblase.” Hence, they proposed that PS may become detectable in the outer leaflet due to the combination of these two events. However, to date, the relative contribution of these two events has not been clarified. Additionally, it is not known what role, if any, other changes occurring during apoptosis (\textit{e.g.} membrane blebbing and vesiculation) may play in the appearance of PS in the outer membrane leaflet. We hypothesized that the appearance of PS on the surface of the cell undergoing apoptosis would result primarily from enhanced calcium-dependent transbilayer movement of phospholipids (flip-flop) across the plasma membrane. Here we present data that demonstrates that (i) the loss of aminophospholipid translocase activity alone does not result in PS appearance, (ii) the appearance of PS likely results from calcium-dependent phospholipid flip-flop that is nonspecific for head group, (iii) while loss of aminophospholipid translocase activity is insufficient to result in PS appearance, loss of its function is probably a necessary event, and (iv) the appearance of PS at the cell surface can be dissociated from nuclear changes (DNA fragmentation) during apoptosis.
human serum albumin. Apoptosis was induced by UV irradiation at 254 nm for 10 min (neutrophils) or 5 min (HL-60s). Both cell types were then incubated at 37 °C in a 5% CO₂ humidified atmosphere for 2 h (HL-60) or 4 h (neutrophils) or as indicated to allow the apoptotic phenotypes to develop. Apoptosis was also induced in neutrophils by the addition of mouse anti-human Fas IgM (Upstate Biotechnology, Lake Placid, NY) (400 ng/ml) with continued exposure for 4 h. Jurkat cells were incubated at 2 × 10⁶ cells/ml in Hepes-buffered saline (see below) for 3 h with mouse anti-human Fas IgM (100 ng/ml), or apoptosis was induced by UV irradiation at 254 nm for 10 min. In experiments in which calcium was added back, the cells were initially incubated in RPMI without calcium, and then Ca(NO₃)₂ added to the concentrations which calcium was added back, the cells were initially incubated in RPMI without calcium, and then Ca(NO₃)₂ added to the concentrations which calcium was added back, the cells were incubated in 26160 medium for 10 min (neutrophils) or 5 min (HL-60) and at the times indicated, and the cells replaced in culture until the end of the incubation period. As stated in the text, in certain experiments, cells were incubated with the following agents: BAPTA/AM (10 μM) was purchased from Calbiochem (San Diego, CA); dl-α-tocopherol (0.06–2 mM) from Roche Vitamins and Fine Chemicals (Nutley, NJ); N-acetylcysteine (5 or 10 mM), diithiothreitol (1–10 mM), ascorbic acid (0.06–2 mM), and EGTA (2 mM) were purchased from Sigma. At the end of the incubation period, cell cultures were subdivided and samples simultaneously stained for surface PS, phospholipid uptake, and DNA degradation as described below.

Several experiments were conducted to determine if the calcium flux alone was sufficient to induce annexin binding and a loss of aminophospholipid translocase activity. In these experiments, HL-60 cells (without apoptosis induction) were harvested, washed as above, and then treated with the indicated concentrations of ionomycin (Calbiochem, San Diego, CA) and incubated at 37 °C in a 5% CO₂, humidified atmosphere 30 min and stained as described below.

Flow Cytometry

PS Detection—Cells bearing PS in the plasma membrane outer leaflet were identified as those binding FITC-labeled annexin V using an Apoptosis Detection Kit (R&D Systems, Minneapolis, MN). The binding of FITC-labeled annexin V to phosphatidylserine on the surface of apoptotic cells closely correlates with the appearance of nuclear and cytoplasmic condensation by light microscopy (7) and the appearance of hypodiploid DNA (see below). Briefly, 10⁵ cells were pelleted, resuspended in 100 μl of Hepes-buffered saline, and 100 ng of FITC-labeled annexin V and 500 ng of propidium iodide were added. The cells were incubated 15 min at room temperature, then the samples were transferred to ice and the sample volume brought to 0.5 ml. Analysis was done on a Becton Dickinson (San Jose, CA) FACScan or FACScalibur flow cytometer, and the results were analyzed with PC Lysis software. Annexin positive cells were determined as described in the Apoptosis Kit by setting quadrants to separate viable cells from PI permeant cells, and non-apoptotic cells from those staining highly for the FITC-labeled annexin V probe. Percent apoptosis was determined from the cells staining greater than the control population threshold. Mean fluorescence of the PI impermeant cells was simultaneously determined.

Phospholipid Uptake—Phospholipid uptake was carried out in Hepes-buffered saline (HBS), 137 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5 mM glucose, 10 mM Hepes (pH 7.4), with or without CaCl₂ as indicated. Calcium concentrations of buffers were confirmed by the use of the calcium determination kit (Sigma) utilizing Arsenazo III reagent, and by the indo-1 method (8, 9) and was 1 mM unless otherwise stated. NBD-labeled phospholipids (Avanti Polar-Lipids, Inc., Alabaster, AL) were prepared by drying 1 μg/sample of 1-palmitoyl-1-[6-[7-nitro-2,1,3-benzoxadiazol-4-yl]amino] caproyl]-sn-glycero-3-phosphocholine (NBD-PC) or 1 μg of 1-palmitoyl-1-[6-[7-nitro-2,1,3-benzoxadiazol-4-yl]amino] caproyl-sn-glycero-3-phosphoserine (NBD-PS) in a glass tube. The lipids were then resuspended in 20 μl of HBS containing 200 μM phenylmethylsulfonyl fluoride with 0.25% bovine serum albumin (or “lipopolysaccharide-free” human serum albumin when used in conjunction with neutrophils or Jurkats). Previous studies have shown that these NBD-labeled probe lipids are readily solubilized in aqueous media containing albumin and will partition into the plasma membrane outer leaflet (3, 10, 11). Following the incubation period, cells were harvested, washed once, and then resuspended in HBS (1 × 10⁶ cells/ml). The cells (5 × 10⁵ in 50 μl) were incubated with 1 μl of the lipid suspension and 5 μl of 50 μg/ml propidium iodide for 10 min at room temperature. Albumin extraction of the plasma membrane outer leaflet to remove

FIG. 1. Time course of plasma membrane changes in (A) HL-60s and (B) neutrophils undergoing apoptosis following UV irradiation. Representative cytofluorograph histograms demonstrate the appearance of PS (FITC-labeled annexin V binding), aminophospholipid translocase activity (NBD-PS uptake), and nonspecific phospholipid flip-flop (NBD-PC uptake).
Intracellular Calcium Concentration Determination

Intracellular calcium concentration in HL-60s was determined by the method of Lennon et al. (18). Briefly, the cells were incubated with 1.5 μM fura-2-acetoxymethylester (Molecular Probes, Eugene OR) for 30 min at 37 °C in a 3% CO₂ water bath with occasional agitation to keep the cells in suspension. The cells were then washed in HBS or RPMI salt solution with or without calcium, and resuspended at 2 × 10⁶ cells/ml in 3 ml at 37 °C in a cuvette in a SLM 8000 C spectrofluorometer. Excitation and emission wavelengths were 336 and 353 nm, respectively. Following recording of the fluorescence corresponding to basal intracellular calcium, or for 15 min following calcium addition in the repletion experiments, Triton X-100 (final concentration 0.1% (v/v)) was added to lyse the cells and determine the maximum fluorescence on release of the dye into 1.2 mM calcium. Minimum fluorescence was achieved by addition of EGTA (final concentration 10 mM) in 4 mM Tris.

RESULTS

The detection of outer leaflet PS was temporally associated with both loss of aminophospholipid translocase activity and enhancement of nonspecific phospholipid flip-flop in all cell types and with both apoptotic stimuli. As shown for UV-irradiated HL-60s and neutrophils, cells undergoing apoptosis were found to be positive for PS in the outer leaflet after a latent period of approximately 1 h (Figs. 1 and 2). Ultimately, in these cultures 60 ± 4% of HL-60s and 73 ± 6% of neutrophils stained positively for FITC-labeled annexin V (%) by 2 and 3 h incubation, respectively. Apparent initial stimulation of PS followed in all cases by decline. The appearance of PS followed loss of aminophospholipid translocase activity as measured by uptake of NBD-PS. Recognizing the requirement of extracellular calcium in the enhancement of transbilayer movement of plasma membrane phospholipids (10, 14, 19, 20) and a potential role in the inhibition of the aminophospholipid translocase (4, 21), we asked whether extracellular calcium is necessary for the appearance of PS. As shown in Fig. 3, omission of calcium (nominally present at 620 nm) from the incubation medium resulted in complete lack of detectible outer leaflet PS in both UV-irradiated HL-60s and anti-Fas-treated Jurkat cells (and...
neutrophils, not shown) which was comparable to control (non-apoptotic) cells. This was in marked contrast to cells undergoing apoptosis in the presence of calcium. Importantly, dissociation of PS appearance in the outer leaflet from loss of aminophospholipid translocase activity (loss of PS uptake) was demonstrated, in that PS uptake was completely unchanged whether calcium was added (550 μM) or nominally present (620 nM) (Fig. 4). The data also clearly dissociated the appearance of PS from the nuclear changes of apoptosis since calcium deprivation did not block the development of hypodiploid DNA (Fig. 4).

Although elevation of intracellular calcium has been shown to cause apoptosis in HL-60s (22, 23), it has been shown to inhibit apoptosis of neutrophils (24). Thus it was necessary to determine intracellular calcium concentration during apoptosis. Confirming the work of Lennon et al. (18), we found that over the time course of culture in calcium-containing media, HL-60s undergoing apoptosis demonstrated no elevation of intracellular calcium as measured by the intracellular calcium indicator fura-2 (Table I). Since measures of steady state intracellular calcium do not eliminate the possibility of calcium flux across the plasma membrane, we explored the requirement of extracellular calcium further by UV irradiating neutrophils and HL-60s in RPMI without calcium, culturing for 1 h, and then adding different concentrations of calcium. As shown for HL-60s, incubation under calcium depleting conditions resulted in a mean intracellular calcium concentration of 44 nm, which was followed by rapid (steady state levels achieved within 2 min) repletion of extracellular calcium when extracellular calcium was added (Fig. 5). Using this deplete-replete protocol, as shown in Fig. 6, both the appearance of PS and uptake of NBD-PC (i.e. phospholipid flip-flop) had nearly identical requirements for extracellular calcium. The appearance of PS as determined by mean fluorescence of FITC-labeled annexin V binding was found to be half-maximal (EC50) at 105 μM (± 12 μM S.E., n = 4) (Fig. 6A). Similarly, the calcium dependence of PC uptake was nearly identical and determined to be half-maximal (EC50) at 94 μM (±20 μM S.E., n = 4) (Fig. 6B), closely linking the calcium requirement of both PS and PC flip-flop.

Taken together, these results strongly suggest that flux of extracellular calcium during apoptosis, even in the absence of discernible elevation of intracellular calcium, is absolutely required for the appearance of outer leaflet PS during apoptosis and confirms that outer leaflet PS results from nonspecific flip-flop of phospholipids. These data do not, however, address whether calcium flux is sufficient for the appearance of PS. To address this possibility, HL-60s were treated with ionomycin (25 nM to 2 mM) which resulted in the rapid development of intracellular calcium concentration up to 1 μM, nearly 10 times that seen during apoptosis. However, the appearance of outer leaflet PS did not occur and PS uptake was unchanged over 30 min following treatment with ionomycin (Fig. 7). Thus calcium flux alone is insufficient to mimic the membrane events of apoptosis. Furthermore, these data suggest the hypothesis that if the aminophospholipid translocase is functioning, PS is not detected in the outer leaflet despite calcium flux and enhanced phospholipid flip-flop (17). In support of this hypothesis, it was noted in the course of these experiments that prolonged incubation (3 h) of HL-60s in the presence of ionomycin resulted in development of the full apoptotic phenotype as has been shown previously (23) with the appearance of PS, loss of NBD-PS uptake, enhanced NBD-PC uptake, and DNA degradation (data not shown).

To determine the relative importance of aminophospholipid translocase activity compared with nonspecific phospholipid
flip-flop in the appearance of PS, attempts were made to prolong aminophospholipid translocase activity during apoptosis. Since the aminophospholipid translocase has been shown to be susceptible to oxidant injury (25) a variety of agents were used to preserve activity. Dithiothreitol, ascorbic acid, N-acetylcysteine, and tocopherol (see “Materials and Methods”) alone, and in combination, were used without efficacy to preserve aminophospholipid translocase activity. As calcium has also been reported to inhibit aminophospholipid translocase activity (albeit at high concentrations (4)), intracellular (BAPTA/AM) and extracellular calcium (EGTA) were chelated. Loss of PS uptake was unchanged even in the presence of chelators demonstrating that loss of aminophospholipid activity is calcium independent.

In the absence of specific inhibitors or enhancers of the aminophospholipid translocase, as an alternative approach we sought to exploit both the calcium deplete-replete model used above and the latency period prior to complete loss of the aminophospholipid translocase to determine the effect of aminophospholipid translocase function on the appearance of PS. For these experiments, HL-60s were UV irradiated and incubated in RPMI without calcium to inhibit PS appearance until subsequent calcium (550 μM) addition at either 1 or 2 h. One hour was chosen since there is residual PS uptake at this time by a functioning aminophospholipid translocase (37 ± 17% of control), while at 2 h there is little translocase activity (10 ± 2% of control) remaining (Fig. 8). At both times NBD-PC uptake as a measure of nonspecific phospholipid flip-flop is equivalent. We hypothesized that if aminophospholipid translocase activity is important in limiting the appearance of PS by returning PS to the inner leaflet, then given equivalent calcium-induced nonspecific phospholipid flip-flop, we would expect to detect less outer leaflet PS at 1 h than at 2 h following UV irradiation. In support of this hypothesis, the appearance of PS (measured as both percent of FITC-labeled annexin V positive cells and mean fluorescence) following calcium addition at 1-h post-UV irradiation was significantly less than that at 2 h post-UV irradiation (Fig. 8). The increased appearance of PS at 2 h was not attributable to increased “leakiness” of the cells since the rise in intracellular calcium was more robust when calcium was added back at 1 h than at 2 h following UV irradiation. In support of this hypothesis, the appearance of PS (measured as both percent of FITC-labeled annexin V positive cells and mean fluorescence) following calcium addition at 1-h post-UV irradiation was significantly less than that at 2 h post-UV irradiation (Fig. 8). The increased appearance of PS at 2 h was not attributable to increased “leakiness” of the cells since the rise in intracellular calcium was more robust when calcium was added at 1 h than at 2 h post-UV irradiation (data not shown). Notably, regardless of when calcium was added back (1 or 2 h), if cells were assayed with FITC-labeled annexin V at 3 h post-UV irradiation, all demonstrated identical and maximal appearance of outer leaflet PS and DNA degradation (data not shown).

**DISCUSSION**

Calcium is thought to play a role in many model systems of apoptosis (26, 27). For example, in thymocytes, an increase in intracellular calcium is thought to activate a calcium/magnesium-dependent endonuclease which leads to DNA fragmentation,
and in many cells, elevation of intracellular calcium with the use of ionophores and/or thapsigargin can induce apoptosis (22, 23, 26, 28). Along these lines, the oncogene bcl-2 associated with inhibition of apoptosis, may act in part, by controlling extrusion of calcium from mitochondria and endoplasmic reticulum (26). Calcium-dependent intracellular targets relevant to the process of apoptosis likely include endonucleases (26), transglutaminases (29, 30), and importantly, the proteolytic cascades involving calpain (31) and the caspases (32–34). In-transglutaminases (29, 30), and importantly, the proteolytic degradation and loss of the aminophospholipid translocase activity alone was insufficient to result in the appearance of PS, we have not been able to ascertain the degree to which loss of this activity is necessary for the appearance of PS. Our attempts to preserve activity with antioxidants were unsuccessful. Since a candidate aminophospholipid translocase has recently been cloned (39), genetic tools to modulate its activity will likely be available in the near future. Our data using UV-irradiated HL-60s do suggest that residual activity (37%) of the aminophospholipid translocase can attenuate the appearance of PS in the outer leaflet (Fig. 8). The diminished PS detection at the earlier time point (1 h) may be likened to the model of the activated neutrophil where outer leaflet PS is not detected in the presence of aminophospholipid translocase activity despite enhanced nonspecific transbilayer movement (17). Alternatively, in the thrombin-stimulated platelet, modest outer leaflet PS can be demonstrated despite enhanced aminophospholipid translocase activity, presumably due to PS externalization from flip-flop in excess of PS internalization by the aminophospholipid translocase (15). We also note that while the appearance of outer leaflet PS in HL-60s, Jurkats, and neutrophils undergoing apoptosis was accompanied by both loss of aminophospholipid translocase activity and enhanced nonspecific phospholipid uptake, there were some quantitative differences in the degree of loss of aminophospholipid translocase activity and the enhancement of nonspecific flip-flop. Thus, it is quite probable that future studies will show differences in the relative contribution of these processes to the appearance of PS and phospholipid flip-flop in different cells undergoing apoptosis or activation.

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Appearance of Phosphatidylserine on Apoptotic Cells

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