Lipid Antioxidant, Etoposide, Inhibits Phosphatidylserine Externalization and Macrophage Clearance of Apoptotic Cells by Preventing Phosphatidylserine Oxidation*

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Apoptosis is associated with the externalization of phosphatidylserine (PS) in the plasma membrane and subsequent recognition of PS by specific macrophage receptors. Selective oxidation of PS precedes its externalization/recognition and is essential for the PS-dependent engulfment of apoptotic cells. Because etoposide is a potent and selective lipid antioxidant that does not block thiol oxidation, we hypothesized that it may affect PS externalization/recognition without affecting other features of the apoptotic program. We demonstrate herein that etoposide induced apoptosis in HL-60 cells without the concomitant peroxidation of PS and other phospholipids. HL-60 cells also failed to externalize PS in response to etoposide treatment. In contrast, oxidant (H2O2)-induced apoptosis was accompanied by PS externalization and oxidation of different phospholipids, including PS. Etoposide potentiated H2O2-induced apoptosis but completely blocked H2O2-induced PS oxidation. Etoposide also inhibited PS externalization as well as phagocytosis of apoptotic cells by J774A.1 macrophages. Integration of exogenous PS or a mixture of PS with oxidized PS in etoposide-treated HL-60 cells reconstituted the recognition of these cells by macrophages. The current data demonstrate that lipid antioxidants, capable of preventing PS peroxidation, can block PS externalization and phagocytosis of apoptotic cells by macrophages and hence dissociate PS-dependent signaling from the final common pathway for apoptosis.

A common feature of the apoptotic program is phospholipid signaling aimed at the generation of "eat me" signals on the surface of the apoptotic cell that make it recognizable by phagocytes (1). This apoptotic signaling is mediated through the loss of plasma membrane phospholipid asymmetry and the concomitant externalization of phosphatidylserine (PS) (2). PS-dependent signaling is coupled to the final common pathway of apoptosis, i.e. the caspase-driven dismantling of the cell, thus allowing for effective phagocytosis and clearance of cell corpses. The importance of phagocytosis of apoptotic cells for prevention of spillage of cellular contents and resultant tissue disruption and inflammation has been emphasized in numerous studies in recent years (3, 4); however, the specific mechanisms that govern PS externalization and recognition during apoptosis remain to be elucidated.

We have recently shown that PS externalization during apoptosis is preceded by its selective oxidation, likely catalyzed by cytochrome c released from mitochondria (5, 6). We have also demonstrated that oxidized PS (PS-OX) may serve as an "eat me" signal for macrophage receptor(s), thus facilitating recognition and PS-dependent engulfment of apoptotic cells. (7, 8) Based on these observations, we hypothesized that PS oxidation acts as an essential component of the signaling pathway that is required for PS externalization and the safe clearance of apoptotic cells by macrophages (5, 8, 9, 10). Our hypothesis predicts that lipid antioxidants capable of blocking PS oxidation will inhibit PS externalization and/or recognition of apoptotic cells by phagocytes. An important feature of such a lipid antioxidant, however, is that it should block phospholipid oxidation without affecting other redox-sensitive mechanisms.

Our previous work has established that a phenolic antitumor drug, etoposide (a topoisomerase II inhibitor), acts as a powerful lipid antioxidant but does not protect thiols against oxidation because of a relatively high reactivity of etoposide phe- noxyl radicals toward SH-groups (11). Etoposide has been reported to cause DNA damage and induce apoptosis accompanied by ROS generation (12, 13). It is conceivable that the fastidious lipid antioxidant traits of etoposide may dissociate PS-dependent signaling pathways of apoptosis from the final common pathway for apoptosis by inhibiting PS oxidation. However, etoposide effects on phospholipid peroxidation, as...
they relate to PS signaling pathways of apoptosis, have not been documented to date.

In the present study, we used etoposide as a tool to dissect the mechanism of PS signaling during apoptosis. We found that etoposide fails to trigger PS oxidation and externalization in HL-60 cells. Moreover, etoposide was able to block oxidation of all major phospholipids, including PS, during oxidant (H$_2$O$_2$)-induced in these cells. Oxidation correlated with the etoposide-dependent inhibition of PS externalization and phagocytosis of apoptotic HL-60 cells by J774A.1 macrophages. The etoposide-mediated dissociation of PS signaling during apoptosis could be overcome by the integration into HL-60 cells of exogenous PS, and even more so upon integration of PS-OX, thus emphasizing the essential role of PS oxidation in the clearance of apoptotic cells. These results demonstrate that etoposide can modulate PS-dependent externalization and recognition of apoptotic cells by macrophages by means of regulation of PS oxidation.

**EXPERIMENTAL PROCEDURES**

**Chemicals—cis-Parinaric acid (cis-PnA) and 2-methyl-6-(4-methoxy-phenyl)-3,7-dihydroimidazol[1,2-a:pyrazin-3-one hydrochloride (MCLA) were obtained from Molecular Probes (Eugene, OR). HPLC grade solvents, fluorescamine, N-ethylmaleimide (NEM), fetal bovine serum, glutathione, Hoechst 33342, human serum albumin, phenylmethylsulfonyl fluoride, and superoxide dismutase were purchased from Sigma. RPMI 1640 medium, Dulbecco’s modified Eagle’s medium, phosphate-buffered saline (PBS), penicillin, streptomycin, and gentamicin were purchased from Invitrogen. ThioGlo™ 1 was obtained from Calbiochem (San Diego, CA). NBD-phosphatidyleserine (NBD-PS), 1-palmitoyl-2- arachidonyl-sn-glycero-3-(phospho-l-serine), NBD-phosphatidylcho- line (NBD-PC), 1-palmitoyl-2 arachidonyl-sn-glycero-3-phosphocholine were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Cell Culture—**HL-60 human promyelocytic leukemia cells (American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 12.5% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere (5% CO$_2$ plus 95% air) at 37 °C. Cells from passages 25–40 were used for the experiments. The density of cells at collection time was 0.5 × 10$^6$ cells/ml. HL-60 cells were incubated in the presence of etoposide (10 μM or 50 μM) and/or H$_2$O$_2$ in fetal bovine serum-free RPMI 1640 medium without phenol red for 2 h at 37 °C. H$_2$O$_2$ (25 μM) was added four times (every 30 min of incubation). In the case of combination of etoposide with H$_2$O$_2$, etoposide was added 15 min before the addition of H$_2$O$_2$. Macrophages J774A.1 (from the American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml genta- mycin sulfate in a humidified atmosphere (5% CO$_2$ plus 95% air) at 37 °C.

**Apoptotic Nuclear Morphology—**HL-60 cells were incubated in the presence of etoposide and/or H$_2$O$_2$ for 2 h at 37 °C. At the end of the incubation, cells were washed and re-suspended in PBS. Hoechst 33342 (2 μg/ml) was added, and cells were examined under fluorescent microscopy. Results are 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).

**Caspase-3 Activity—**The activity of caspase-3 was determined by using a commercially available kit (Molecular Probes). At specified time intervals, aliquots of etoposide- and/or H$_2$O$_2$-stimulated HL-60 cell suspensions were taken, cells were washed twice with PBS and lysed for 30 min in lysis buffer (Molecular Probes). The suspensions were centrifuged at 4 °C, and supernatants were collected as lysates. For measure- ment of caspase activity, 50 μl of lysates were combined with 50 μl of reaction buffer containing 2 μM EDTA, 10 μM dithiothreitol, 0.2% CHAPS, 0.2 mM Asp-Glu-Val-Asp 7-amino-4-meth- ylycoumarin (DEVD-AMC, a fluorogenic peptide substrate), pH 7.4, and incubated for 30 min at 25 °C. After incubation, fluorescence was measured in a Packard Fusion™ Multifunctional plate reader (PerkinElmer Life Sciences) using excitation at 365 ± 50 nm and emission at 465 ± 55 nm. The protein concentration in cell lysates was measured using the Bio-Rad assay.

**MCLA-enhanced Chemiluminescence—**HL-60 cells (2 × 10$^6$ cells/ml) were pre-warmed in PBS containing 0.5 mM CaCl$_2$, 1 mM MgCl$_2$, and 30 mM glucose for 5 min at 37 °C. After that, etoposide (50 μM) was added, and cells were monitored for 30 min at 37 °C using Luminescence Analyzer 633 (Coral Biomedical Inc., San Diego, CA) set at continuous mixing in the presence of 4 μM MCLA. Assays were performed in the absence and in the presence of superoxide dismutase (50 units/ml) added 5 min prior to the addition of etoposide. The total amount of O$_2$ produced was estimated as the area under the curve (mV × s) upon etoposide stimulation and after subtracting values obtained in the presence of superoxide dismutase. Data were collected and analyzed with the Multiuse PC software version 2.0.2 for Luminoskan 1251 Carousel (Labsystems).

**Assay of GSH—**GSH content in the cells was determined fluorometri- cally using ThioGlo™ 1 as previously described (14). Briefly, cells treated with etoposide and/or H$_2$O$_2$ for 2 h at 37 °C were collected and suspended in PBS. Cells were then washed and lysed in cell lysates prepared by freezing and thawing cells. Immediately after the addition of ThioGlo™ 1 to the cell lysates, fluorescence was measured in a Packard Fusion™ Multifunctional plate reader (PerkinElmer Life Sciences) using excitation of 390 ± 15 nm and emission of 515 ± 30 nm.

**Annexin V Staining of Externalized PS—**PS exposure was deter- mined by flow cytometric detection of annexin V staining using a protocol outlined in the annexin V-FITC apoptosis detection kit (BioVi- sion Research Products, Mountain View, CA). Briefly, HL-60 cells (0.5 × 10$^6$) exposed to etoposide and/or H$_2$O$_2$, washed once with PBS and re-suspended in binding buffer were stained with annexin V (0.5 μg/ml) for 5 min at room temperature. After staining, cells were immediately analyzed using a FACSscan flow cytomter (BD Biosciences) with simultaneous monitoring of green fluorescence (530 nm, 30 nm band-pass filter) for annexin V-FITC and red fluorescence (long-pass emission filter that transmits light >650 nm) associated with propidium iodide. 10,000 events were collected and analyzed using the LYSIS™ II software (BD Biosciences).

**Derivatization of Externalized PS with Fluorescamine—**Labeling of externalized PS with fluorescamine (a probe that reacts with lipids containing primary amino groups) was performed as described previ- ously (8). Briefly, HL-60 cells (3 × 10$^6$) exposed to etoposide and/or H$_2$O$_2$ were suspended in labeling buffer (150 mM NaCl, 5 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM glucose) for 5 min at room temperature. After staining, cells were immediately analyzed using a FACSscan flow cytomter (BD Biosciences) with simultaneous monitoring of green fluorescence (530 nm, 30 nm band-pass filter) for annexin V-FITC and red fluorescence (long-pass emission filter that transmits light >650 nm) associated with propidium iodide. 10,000 events were collected and analyzed using the LYSIS™ II software (BD Biosciences).

**Assay of Phospholipid Peroxidation—**cis-PnA was incorporated into HL-60 cells as described previously (17). Briefly, HL-60 cells were incubated in serum-free RPMI medium 1640 without phenol red in the presence of cis-PnA (1 μg cis-PnA/10$^6$ cells) for 2 h at 37 °C. At the end of incubation, cells were washed with PBS containing human serum albumin (fatty acid-free, 0.5 mg/ml) to remove an excess of unbound cis-PnA. cis-PnA-labeled cells were treated with etoposide (10 or 50 μM) for 2–4 h. Cells were then washed twice with PBS, and cells in these cells were collected and placed in a heated reaction mixture (0.5 μM Hepes buffer containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 10 mM glucose, pH 7.4). H$_2$O$_2$ (25 μM) was added every 30 min during the initial 2 h of incubation. At the end of the incubation, lipids were extracted by the Folch procedure (15). The lipid extracts were separated by normal-phase HPLC using a 5-μm microsorb MV reverse-phase instrument C18 column (4.6 × 250 mm; Robertshaw) at a flow rate of 1 ml/min at 37 °C in a pH 6.0. The separations were performed by using a Shimadzu LC-600 high-performance liquid chromatography system with an in-line configuration of RF 10 AXL fluorescence detector. Fluorescence of cis-PnA was measured at 420 nm emission after excitation at 324 nm. Data were processed and stored in digital form with Shimadzu Multiwin 2 software. Lipid phospholipid content was determined by a method described in this study.
HL-60 cells (4 x 10^6) treated with etoposide (50 μM, 2 h at 37 °C) in the presence or absence of H_2O_2 were centrifuged (400 x g, 10 min) and washed once in incubation buffer (136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2, 5 mM glucose, 10 mM HEPES, pH 7.5). Cell pellets were resuspended in incubation buffer (5 x 10^6 cells/ml) containing 500 μM phenylmethylsulfonyl fluoride, transferred to a microfuge tube, and placed in ice-water for 10 min. NBD-labeled PS (ethanol solution) was added to cells (final concentration, 10 μM) and incubated for 10 min at 4 °C. Labeled cells were centrifuged and resuspended at the same density in incubation buffer with phenylmethylsulfonyl fluoride. Cell suspensions were placed in a 28 °C water bath to initiate internalization, and 75-μl aliquots of cell suspension were removed at various time intervals (1–20 min) and placed into 2.5 ml of incubation buffer including the reducing agent and 10 mM sodium dithionite. Fluorescence (excitation = 470 nm, emission = 540 nm) was then recorded (within 30–60 s). Samples from the last time point were also placed in incubation buffer without dithionite to obtain total fluorescence intensity (FL_total). Internalized fluorescence at various times (FL_t) was normalized as a percent of the total fluorescence by the following equation: % internalized = (FL_t FL_0)/(FL_total FL_0) x 100. To inhibit APT activity, HL-60 cells were incubated in the presence of NEM (10 μM) for 10 min at 37 °C. At the end of incubation, cells were washed twice with PBS, and APT activity was determined as described above.

**Phagocytosis of HL-60 Cells by J774A.1 Macrophages—J774A.1 macrophages were seeded into an eight-well chamber slide (5 x 10^5 cells/well) and cultured overnight in DMEM medium. Before adding target cells (control or etoposide and/or H_2O_2-treated HL-60 cells), stimulated macrophages were washed with serum-free RPMI medium 1640 without phenol red and fluorescently labeled with Cell Tracker™ Orange (5 μg/ml, 10 min at 37 °C) and subsequently washed again (three times) with PBS. Fluorescently labeled cells (5 x 10^5 cells/well) were co-cultured with macrophages for 1 h at 37 °C. After incubation, unbound target cells were washed three times with RPMI medium 1640 and three times with PBS; well contents were fixed with a solution of 2% formaldehyde in PBS containing Hoechst 33342 (1 μg/ml) for 30 min at room temperature. The cells were examined under a Nikon Eclipse TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu charge-coupled device camera (C4742-95-12NBR) and analyzed using the Metamaging Series™ software version 4.6 (Universal Imaging Corp., Downingtown, PA). Macrophages that had a side-by-side connection with target cells (binding) and/or internalized target cells (engulfment) were considered phagocytosis-positive. A minimum of 300 macrophages were analyzed per experimental condition. Results are expressed as the percentage of the phagocytosis-positive macrophages.

**Preparation of PS- and PS-OX-containing Liposomes—**PS was oxidized by incubation with a water-soluble azo-initiator of peroxide radicals, 2,2'-azo-bis-(2-aminopropane) hydrochloride. PS in chloroform was dried under nitrogen and PBS was added to achieve the final concentration of 5 mM. In preliminary experiments, the lipid was incubated with 2,2'-azo-bis-(2-aminopropane) hydrochloride (50 μM) at 37 °C for 4 h and then extracted with chloroform/methanol (2:1, v/v). Oxidation was assessed by measuring the absorbance of PS hydroperoxides with conjugated dienes at 234 nm. Approximately 30% of PS molecules were estimated to be oxidized after a 4-h incubation, whereas 70% remained non-oxidized; this mixture of PS species is hereafter referred to as oxidized PS (PS-OX). Small unilamellar liposomes containing 50% phosphatidylcholine (PC) and 50% PS (nonoxidized PS or PS-OX) were produced as described by Fadak et al. (21). Individual phospholipids, stored in chloroform, were dried under nitrogen. PBS was added to the lipid mixture to obtain a phospholipid concentration of 1 mM, and the lipid mixture was vortexed and sonicated for 3 min on ice. All liposomes were used immediately after preparation.

**Treatment of HL-60 Cells with Liposomes—**Naïve or etoposide-treated (50 μM for 2 h at 37 °C) apoptotic HL-60 cells were incubated with NEM (10 μM, 10 min at 37 °C) to inhibit APT. At the end of the incubation, cells were centrifuged and NEM was washed out. NEM-treated cells were incubated in the presence of PC + PS or PC + PS + PS-OX liposomes (2.5–100 μM) for 30 min at 37 °C. After that, cells were washed with PBS labeled with Cell Tracker™ Orange, and phagocytosis assays were performed as described above.

**Statistics—**The results are presented as mean ± S.E. values from at least three experiments, and statistical analyses were performed by either paired or unpaired Student’s t test or one-way ANOVA. The statistical significance of differences was set at p < 0.05.

**RESULTS**

**Oxidant- and Non-oxidant-induced Apoptosis in HL-60 Cells—**To determine the conditions resulting in triggering of apoptosis in HL-60 cells by etoposide, H_2O_2, or a combination of etoposide plus H_2O_2, we studied the appearance of typical nuclear fragmentation as well as caspase-3 activation. Microscopic examination of nuclear morphology showed that, at 50 μM (2 h at 37 °C), etoposide triggered apoptosis in 25.8 ± 6.3% of cells (Fig. 1A). No significant increase in the number of apoptotic cells at this time point was observed when 10 μM of etoposide was employed (data not shown). Under the conditions used, H_2O_2 (25 μM added every 30 min during incubation for 2 h at 37 °C) effectively induced apoptosis in 30.6 ± 3.5% cells. Notably, a combination of etoposide and H_2O_2 yielded further enhancement of apoptosis (46.2 ± 9.9% of apoptotic cells after exposure to 50 μM etoposide plus H_2O_2 (25 μM, four additions over 2 h).

As can be seen from the time course of caspase-3 activation, both etoposide and H_2O_2 induced pronounced responses (Fig. 1B). Even at a low concentration of 10 μM, etoposide caused a significant activation of caspase-3 at 2 h after the exposure (data not shown). At 50 μM, the effect was detectable as early as
Etoposide Inhibits PS Oxidation/Externalization

Fig. 2. Production of superoxide and oxidation of glutathione during apoptosis in HL-60 cells. A, superoxide production induced by etoposide in HL-60 cells. MCLA-enhanced chemiluminescence was monitored in the presence of etoposide (50 μM) for 30 min at 37 °C. Data are mean ± S.E. (n = 3); *, p < 0.05 versus control. B, effect of etoposide and/or H2O2 on glutathione content in HL-60 cells. Cells were stimulated in the presence of etoposide (50 μM) and/or H2O2 (25 μM × 4) and glutathione was measured 2 h after stimulation using ThioGlo™ 1, as described under “Materials and Methods.” Data are shown as mean ± S.E. (n = 9); *, p < 0.05 versus control.

1.5 h after exposure and further increased by 2 h. Similarly, H2O2 caused a marked activation of caspase-3 at both 1.5 and 2 h. The effects of the combination of etoposide plus H2O2 were additive at 1.5 h and saturated at 2 h. Caspase-3 was completely inhibited when a pan-caspase inhibitor, z-VAD-fmk (50 μM), was present in the incubation system during the exposure to etoposide, H2O2, or a combination of etoposide plus H2O2 (data not shown).

Etoposide Treatment of HL-60 Cells Prevents H2O2-induced Oxidation of PS—Execution of the apoptotic program triggered by both oxidant and non-oxidant stimuli may be associated with the production of reactive oxygen species (ROS) and ensuing oxidative stress (22). Therefore, we determined ROS production and oxidative stress in our model. To determine whether apoptosis was accompanied by the production of superoxide, we used a superoxide-specific enhancer of chemiluminescence, MCLA. The results in Fig. 2A show that superoxide production was significantly increased in HL-60 cells exposed to etoposide (50 μM) for 2 h at 37 °C as compared with untreated cells. To assess the degree of oxidative stress, we measured the level of intracellular GSH in HL-60 cells. We found that after etoposide (50 μM) stimulation for 2 h at 37 °C, the GSH content significantly decreased to 63.6 ± 2.0% of its initial level (Fig. 2B). Similarly, H2O2 (25 μM × 4, 2 h) caused a pronounced depletion of GSH. Finally, when HL-60 cells were exposed to a combination of etoposide and H2O2, an even stronger depletion of GSH was evident. This suggests that etoposide is not likely to interfere with thiol-dependent redox signaling pathways of apoptosis.

To establish the extent to which oxidation of phospholipids was associated with the execution of the apoptotic program induced by etoposide, H2O2, and a combination of etoposide and H2O2, we used a cis-PnA-based technique for quantitative assessment of oxidation of different classes of phospholipids in live cells (17, 23). To this end, cellular phospholipids were metabolically labeled with cis-PnA, an oxidation-sensitive fluorescent fatty acid containing four conjugated double bonds (17). After incubation of cis-PnA-loaded cells with etoposide and/or H2O2, total lipid extracts were prepared and separated by fluorescence HPLC. Typical chromatograms are presented in Fig. 3A. Four peaks corresponding to the major classes of cis-PnA-labeled phospholipids were identified in control HL-60 cells as phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC), respectively (Fig. 3A, a). Treatment of cells with H2O2 (25 μM × 4, 2 h) resulted in a marked oxidation of all major phospholipid classes, including PS, as evidenced by decreased intensities of fluorescence responses (Fig. 3A, b). Etoposide alone at both 10 μM (data not shown) and 50 μM did not cause any significant oxidation of phospholipids. In fact, etoposide exerted an antioxidant effect against H2O2-induced phospholipid peroxidation. Protective effects of etoposide were already apparent at 10 μM (data not shown) and were complete at 50 μM (Fig. 3B). As expected for live cells, phospholipid oxidation involved only a relatively small fraction of total membrane phospholipids such that HPTLC analysis of “gross” phospholipid composition did not reveal any significant alterations...
after stimulation of apoptosis in HL-60 cells with either H2O2 or etoposide (data not shown).

**Etoposide Inhibits H2O2-induced PS Externalization in HL-60 Cells**—We were further interested in determining the extent to which inhibition of PS oxidation was associated with changes in its externalization during apoptosis in HL-60 cells. To quantify PS exposure, we used two different assays: (i) flow cytometric analysis of fluorescently labeled annexin V to evaluate the number of cells with externalized PS, and (ii) derivatization of externalized aminophospholipids by a non-permeable reagent for primary amines, fluorescamine, to determine the amounts of PS on the cell surface. Using annexin V-FITC labeling, we found no significant increase in the number of annexin V-positive cells after treatment with etoposide alone at both 10 μM (data not shown) or 50 μM (2 h), as compared with non-treated controls (<2.5% cells with exposed PS on their surface; Fig. 4). Treatment of HL-60 cells with H2O2 (2 h at 37 °C) resulted in a significant increase in the number of cells with externalized PS (up to 14.5%). Most importantly, HL-60 cells treated with H2O2 in the presence of etoposide (50 μM) responded with a significant (although incomplete) decrease of annexin V binding such that only ~7% cells remained annexin V-positive. At 10 μM, etoposide did not significantly reduce the number of H2O2-induced annexin V-positive cells (data not shown). Increasing the treatment time of HL-60 cells with etoposide (50 μM) to 4 and 6 h, however, resulted in a significantly increased number of cells with externalized PS (up to 8.1 ± 1.3 and 11.3 ± 0.4%, respectively) (Fig. 5A). After 4 and 6 h of exposure of HL-60 cells to H2O2, 22.4 ± 2.5 and 30.8 ± 2.4% were annexin V-positive. At these later time points, etoposide did not decrease H2O2-induced externalization of PS, although it remained lower than the expected additive sum (30.5 and 42.5% at 4 and 6 h, respectively). Most importantly, the dependence of PS externalization on its oxidation held true for these later time-points as well. In particular, elevated levels of PS externalization were associated with PS oxidation revealed by decreased contents of cis-PnA-PS in HL-60 cells treated with etoposide alone, H2O2 alone, or a combination of H2O2 plus etoposide at both 4 and 6 h. Although etoposide did not protect PS against H2O2-induced oxidation at either 4 or 6 h, in both cases the oxidation levels were lower than the expected additive effects of etoposide alone and H2O2 alone. Our incubation conditions (50 μM etoposide and/or H2O2 25 μM × 4) were not associated with any significant accumulation of necrotic (PI-positive/annexin V-positive) cells during 2, 4, or 6 h exposures as compared with their content in control samples (1.5–2.0%).

To quantitate the amounts of externalized PS on cell surfaces, we treated the cells with fluorescamine, a cell-impermeable fluorescent reagent, extracted phospholipids, and resolved these by HPTLC (Fig. 6A). Exposure to iodine vapors revealed all major phospholipid classes (PC, PE, sphingomyelin, PI, diphosphatidylglycerol, PS) as well as neutral lipids and free fatty acids (Fig. 6A, FFA, panels 2, 4, 6). Importantly, four spots, two for each of the aminophospholipids, PE and PS, respectively, could be detected on HPTLC plates. Comparison of the iodine-revealed spots (Fig. 6A, panels 2, 4, 6) with those revealed by fluorescence of fluorescamine-labeled aminophospholipids on the plates (Fig. 6A, panels 1, 3, 5) demonstrated that only one of the two spots in each of the PE- and PS-couples was fluorescent, i.e., labeled with fluorescamine. These spots corresponded to PE and PS available to fluorescamine modification on the cell surface and were designated as phosphatidylethanolamine modified by fluorescamine and phosphatidylserine modified by fluorescamine, respectively. Using measurements of P1 in the spots, we further quantitated the amounts of externalized PS (Fig. 6B). In control HL-60 cells, the content of PS exposed on the cell surface did not exceed 5% of its total amount (8.1 ± 2.8 pmol/106 cells). After 2 h of exposure to H2O2, HL-60 cells harbored significantly higher levels of externalized PS, which reached 10% of its total amount. Etoposide alone (at 50 μM) caused a slight enhancement of PS externalization that did not, however, significantly differ from the control. In the presence of etoposide, the effect of H2O2 was inhibited. Hence, at a 2-h exposure to 50 μM etoposide, the amounts of externalized PS were not significantly different from the control but were different from those detected after exposure to H2O2 alone. The amounts of PE on cell surfaces accessible for fluorescamine derivatization were not significantly different in control cells as compared with the cells treated with etoposide, H2O2, and the combination of both stimuli (data not shown).

APT, an ATP-dependent enzyme, is responsible for the maintenance of the asymmetric distribution of PS in viable cells through its efficient internalization of PS molecules appearing on the surface of the plasma membrane (2). Because inhibition of PS oxidation by etoposide was accompanied by blockade of PS externalization, we next determined whether etoposide...
Fig. 6. Externalization of PS in HL-60 cells revealed by fluorescamine. HL-60 cells were treated with etoposide (50 μM) and/or H2O2 (25 μM × 4) for 2 h at 37°C. At the end of incubation, phospholipid externalization was analyzed by fluorescamine-associated fluorescence and HPTLC of externalized PS. A, typical two-dimensional HPTLC of total lipid extracts from control (1 and 2), etoposide-treated (3 and 4), and H2O2-exposed (5 and 6) cells. HPTLC chromatograms were visualized by using a Fluor-S Multimager (Bio-Rad) with a UV light source after treatment with fluorescamine. B, amounts of externalized PS (fluorescamine-modified) on the surface of HL-60 cells after stimulation with etoposide and/or H2O2. The percentage of total PS modified by fluorescamine was determined by phosphorous analysis of each modified phospholipid spot after two-dimensional HPTLC, as described under “Materials and Methods.” Data are expressed as mean ± S.E. (n = 3); *, p < 0.05 versus control; **, p < 0.05 versus H2O2.

and/or H2O2 affected APT activity as it relates to PS externalization in HL-60 cells. To this end, we utilized a fluorescent phospholipid substrate for APT, NBD-PS. NBD-PS was incorporated into the outer leaflet of plasma membrane of HL-60 cells, and its internalization by APT was monitored over time and presented as a percentage of internalized NBD-PS (Fig. 7A). Control (non-treated) HL-60 cells displayed a high level of APT activity (43.4 ± 4.4 pmol NBD-PS/min/10^6 cells) resulting in complete internalization of exogenously added NBD-PS within ~15 min of incubation. In contrast, almost all NBD-PC remained on the external surface over the 20-min incubation period, indicating that internalization was specific for amorphous phospholipids (data not shown). Pretreatment of cells with NEM (10 μM, 10 min at 37°C) resulted in an essentially complete inhibition of APT (Fig. 7A). H2O2 (25 μM × 4, 2 h) reduced the ability of cells to internalize NBD-PS by ~2-fold (Fig. 7A), and etoposide did not affect H2O2-induced APT inhibition (Fig. 7B). Accordingly, the rate of NBD-PS internalization was 1.6-fold higher in control cells than in H2O2-exposed cells (both in the absence and in the presence of etoposide; Fig. 7B). No significant changes in the ability of HL-60 cells to internalize NBD-PS were observed after exposure to etoposide alone (2 h at 37°C). Thus, etoposide did not protect APT against H2O2-induced inactivation.

Etoposide Inhibits Macrophage Engulfment of H2O2-treated HL-60 Cells—Because phagocytosis relies on the recognition of externalized PS on the surface of apoptotic cells (1, 21), which in turn may depend upon PS oxidation (7, 8), we further studied the effects of etoposide and/or H2O2 on phagocytosis of HL-60 cells by J774A.1 macrophages (Fig. 8A). Relatively low levels of phagocytosis-positive macrophages (~2.0–2.5%) were detected when control (non-treated) HL-60 cells were incubated for 1 h with J774A.1 macrophages. Etoposide-treated HL-60 cells were phagocytosed at a slightly (albeit significantly, ~1.3-fold) higher level than control cells. In contrast, H2O2-treated HL-60 cells were readily ingested by J774A.1 macrophages, yielding a 2.1-fold increase in the number of phagocytosis-positive cells. Remarkably, etoposide (at 50 μM) caused inhibition of phagocytosis of HL-60 cells treated with H2O2 (Fig. 8A) insofar as the level of phagocytosis-positive macrophages was not different from that detected in the presence of etoposide alone.

If inhibition of PS oxidation and externalization by etoposide is responsible for the inability of macrophages to recognize apoptotic cells, then enrichment of the surface of etoposide-triggered apoptotic HL-60 cells with PS and/or PS-OX should reinstate their phagocytosis by macrophages. To test this, we integrated PS or a mixture of PS+PS-OX into the plasma membrane of both control and etoposide-treated HL-60 cells by incubating them with liposomes containing PS or PS+PS-OX (50 μM) and subsequently co-cultured these cells with J774A.1 macrophages. It should be noted that target HL-60 cells were pretreated with NEM (10 μM, 10 min) to inhibit APT to prevent internalization of exogenously added PS or PS+PS-OX (see Fig. 8A). Expectedly, this pretreatment resulted in some externalization of endogenous PS (data not shown) and consequently in an increased background level of phagocytosis-positive macrophages (Fig. 8B). Nevertheless, we found that phagocytosis of HL-60 cells was significantly stimulated by both PS and PS+PS-OX (Fig. 8B). Notably, PS-OX turned out to be mark-
edly more effective in stimulating engulfment of both etoposide-pretreated as well as non-pretreated HL-60 cells by J774A.1 macrophages (Fig. 8B). When control (non-pretreated with etoposide) HL-60 cells were enriched with different amounts of PS or PS/OX from 6 to 105 pmol/10^6 cells integrated into the outer leaflet of plasma membrane, a higher effectiveness of PS-OX compared with PS (2.0- to 5.0-fold) as a phagocytosis signal for J774A.1 macrophages was observed at each of the concentration used.

**DISCUSSION**

**Etoposide-mediated Inhibition of PS Oxidation Externalizes PS-dependent Signaling from Other Features of the Apoptotic Program**—The exposition of PS on the surface of apoptotic cells has been identified as a common “eat-me” signal that interacts with the so-called PS-receptor or other PS-binding molecules on the phagocytic cell membrane (24–26). However, the specific mechanisms involved in the egress of PS, normally sequestered in the inner leaflet of the plasma membrane, remain poorly characterized. We have reported previously that oxidation of PS precedes its externalization during both oxidant- and non-oxidant-induced apoptosis (10). These findings are suggestive of a causative link between oxidative modification of PS and its subsequent translocation. One of the experimental approaches to proving such a link is to use antioxidants capable of blocking phospholipid oxidation and associated PS externalization. It is important to note, however, that disruption of mitochondrial electron transport and subsequent ROS production are essential components of apoptosis that may be critically involved in redox-sensitive apoptosis signaling pathways (22, 27). Indeed, the ability of antioxidants to interrupt the execution of the apoptotic program has been reported by several laboratories (28–30). Therefore, the antioxidant of choice should have specific redox properties that make it effective in protecting phospholipids, but not other targets, against oxidative modifications during apoptosis. As a phenolic compound, etoposide can act as an effective donor of reducing equivalents for reactive radicals participating in the initiation and propagation of lipid oxidation, i.e. etoposide can act as a radical scavenger to yield

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**Fig. 7.** Effect of etoposide and H2O2 on APT activity in HL-60 cells. NBD-PS was incorporated into the outer leaflet of the plasma membrane of control HL-60 cells and HL-60 cells treated with etoposide (50 μM) and/or H2O2 (25 μM × 4), and the rate of NBD-PS internalization was calculated. A, internalization of NBD-PS in HL-60 cells. NBD-PS was incorporated into the outer leaflet of plasma membrane of control HL-60 cells, HL-60 cells treated with H2O2 (25 μM × 4), or treated with N-ethylmaleimide (10 μM, 10 min), and APT-mediated internalization of NBD-PS was monitored over time and presented as a percentage of internalized NBD-PS. ○, control; ●, H2O2 (25 μM × 4); □, H2O2 (25 μM × 4); ▣, H2O2 (25 μM × 4); ◼, NEM (10 μM). B, effect of etoposide and/or H2O2 on the rate of NBD-PS internalization in HL-60 cells. Data are depicted as pmol of NBD-PS/10^6 cells/min (mean ± S.E. (n = 5); * p < 0.001 versus control; **, p < 0.01 versus H2O2 or etoposide + H2O2.

**Fig. 8.** Phagocytosis of apoptotic HL-60 cells by J77A.1 macrophages. A, effect of etoposide on J77A.1 macrophage engulfment of H2O2-treated HL-60 cells. HL-60 cells were treated with etoposide (50 μM) and/or H2O2 for 2 h and subsequently analyzed for phagocytic engulfment as described under “Materials and Methods.” Data are expressed as mean ± S.E. (n = 8); *, p < 0.05 versus control (non-treated HL-60 cells); **, p < 0.01 versus H2O2-treated HL-60 cells. B, phagocytosis of HL-60 cells enriched with PS- or PS-OX-containing liposomes. Control (non-apoptotic) or etoposide-treated (50 μM) apoptotic HL-60 cells were pre-incubated with NEM (10 μM for 10 min at 37 °C), incubated with liposomes containing different mixtures of phospholipids at 50 μM (PC and PS (50:50%), PC and PS plus PS-OX (50:50%)), and subsequently analyzed for phagocytic engulfment by J774A.1. Data are mean ± S.E. (n = 6); *, p < 0.05 versus control.
Etoposide Inhibits PS Oxidation/Externalization

Etoposide, a P-type ATPase, maintains the asymmetrical distribution of PS in cells (2). Inhibition of APT has been suggested as playing an important role in PS externalization during apoptosis (40, 41). However, the link between APT inactivation and the execution of the apoptotic program remains unclear. It has been suggested that APT is not a substrate for caspases (42), indicating that alternative mechanisms may be involved in the inhibition or inactivation of this enzyme. In particular, depletion of ATP may contribute to APT inhibition during apoptosis (34, 43). The current data indicate that etoposide inhibits PS externalization through its effect on PS oxidation, rather than by means of a direct inhibition of APT. This is supported by our results demonstrating that etoposide did not prevent H$_2$O$_2$-induced inhibition of APT. Moreover, because etoposide alone did not affect APT activity in HL-60 cells but triggered production of ROS and caspase activation, one may assume that its prevention of phospholipid peroxidation is likely responsible, at least in part, for the lack of APT inhibition. It is tempting to speculate that oxidized PS may contribute to APT inactivation and this effect is minimized in the presence of etoposide. It is possible that oxidized PS acts as an essential regulator of APT activity and PS externalization in intrinsic apoptotic pathways, but it is relatively less important in extrinsic mitochondria-independent (type I) apoptosis.

Etoposide-mediated Inhibition of PS Oxidation Blocks Macrophage Engulfment of HL-60 Cells—Because the externalization of PS on the surface of apoptotic cells acts as an important “eat me” signal for phagocytes, we assumed that etoposide-treated apoptotic HL-60 cells that fail to externalize PS would be less readily engulfed by macrophages. Indeed, we found that etoposide-triggered, early apoptotic cells were poorly engulfed by J774A.1 macrophages. In contrast, apoptosis induced by H$_2$O$_2$ was accompanied by PS externalization, and these cells were successfully eliminated by macrophages. Moreover, the combination of etoposide and H$_2$O$_2$ resulted in a significant inhibition of both H$_2$O$_2$-induced PS externalization and phagocytosis of these cells. Thus, etoposide not only dissociates the common final pathway of apoptosis from the PS signaling pathway essential for phagocytosis, but also blocks this signaling during H$_2$O$_2$-induced apoptosis. Similarly, overexpression of the antiapoptotic protein Bcl-2 prevents PS exposure and macrophage engulfment but does not affect DNA fragmentation or the activation of downstream caspases in Fas-triggered SKW6.4 cells (44). Taken together, these data suggest that the execution of cell death and the clearance of cell corpses are governed, in part, by distinct mechanisms or subprograms.

Our data suggest that etoposide-dependent inhibition of PS oxidation contributes to the failure of macrophages to recognize apoptotic HL-60 cells. In line with these findings, we found that enforced PS externalization in apoptotic HL-60 cells could overcome the etoposide-induced concealment from macrophage recognition. PS-OX integrated into the plasma membrane of etoposide-treated HL-60 cells was an even more potent recognition signal and yielded more effective engulfment by macrophages. These findings concur with our previous studies in which viable Jurkat, Raji, and HL-60 cells were ingested after enrichment with PS and/or PS-OX (8), and further underscore the importance of PS oxidation in PS-dependent signaling pathways of phagocytic clearance of apoptotic cells. Note, etoposide alone, or in combination with other drugs, was recently shown to induce apoptosis and subsequent phagocytosis of Burkitt lymphoma cell lines; this effect was inhibited upon the addition of H$_2$O$_2$ (45, 46). The apparent contradiction between our findings and those of Shacter et al. (45, 46) is likely explained by the relatively high concentration of H$_2$O$_2$ (200 $\mu$M) utilized by these investigators resulting in potent inhibition of apoptosis and accumulation of relatively large amounts of necrotic and “late” apoptotic cells recognizable by macrophages. Indeed, Hampton and Orrenius (47) have shown that H$_2$O$_2$ exerts a dual regulation of caspases, such that low doses (50 $\mu$M) are able to trigger apoptosis, whereas high doses (above 200 $\mu$M) yield oxidative inactivation of these enzymes and subsequent switching of the mode of cell death to necrosis. Notably, our experimental conditions (50 $\mu$M etoposide and/or H$_2$O$_2$ 25 M $\times$ 4) did not yield any significant increase in the number of necrotic cells over an incubation of 2–6 h with etoposide and/or H$_2$O$_2$. When high concentrations of H$_2$O$_2$ were used (250 $\mu$M), a significant accumulation of necrotic HL-60 cells (up to 30%) was observed already after a 2-h exposure, in agreement with the data by Shacter et al. (45, 46).

Since its introduction in 1971, etoposide has become one of the most widely used and effective antitumor drugs in the treatment of different malignancies in both adults and children (48). As an anticancer drug, etoposide is commonly used in combination therapy in both conventional and high-dose regimens (49–55). Although most common conventional regimens result in etoposide plasma concentrations around 2–10 $\mu$M (49–
52), it is not unusual in clinical practice to utilize high dose etoposide (–500 mg/m²) that may yield peak plasma concentrations of 100 μM or higher (53). Utilization of higher doses of etoposide (1500–2400 mg/m²; Refs. 54, 55) can be expected to achieve even higher plasma concentrations. Therefore, the concentration of etoposide used in the current study is well within the range of therapeutic levels achievable during etoposide treatment. One of the unfortunate side effects of etoposide, particularly in children, is therapy-related acute myelogenous leukemias (AML; Refs. 56, 57). Although specific mechanisms responsible for the induction of AML are not fully understood, they include inhibition of topoisomerase II, likely enhanced by myeloperoxidase-catalyzed one-electron metabolism of etoposide in bone marrow progenitor cells (58). The latter is associated with the production of different reactive free radical intermediates potentially leading to direct genotoxic effects as well as non-genotoxic carcinogenesis (31, 39, 59, 60). Apoptosis and safe elimination of transformed cells by phagocytes are of obvious importance for the prevention of pro-carcinogenic effects of etoposide. Our results suggest that impaired PS signaling and elimination of etoposide-triggered apoptotic cells may contribute to the deleterious side effects of this drug, particularly those leading to AML.

To conclude, we have shown that etoposide, an antimtumor agent with unique antioxidant properties, can suppress phospholipid oxidation and PS externalization in HL-60 cells. Moreover, we have shown that PS signaling can be dissociated from other features of the apoptotic program insofar as H₂O₂-induced apoptosis was potentiated, whereas H₂O₂-induced PS oxidation and externalization as well as phagocytosis by J774A1 macrophages were blocked upon incubation of cells with etoposide. Both externalization of PS and oxidation of phospholipids (including PS and PC) have been identified as important determinants of macrophage clearance of apoptotic cells (1, 8, 61, 62). We speculate that oxidation of PS may combine these features of the apoptotic process and facilitate externalization of PS as well as PS-OX, with subsequent recognition of these phospholipid species by neighboring macrophages. Future studies should aim to quantify amounts of PS and its oxidized counterpart on the surface of apoptotic cells as well as to identify the macrophage receptor(s) that are involved in the recognition of PS/PS-OX. A more detailed understanding of the mechanisms that govern the safe removal of apoptotic cells will aid in the design of novel therapeutic strategies in conditions of unscheduled cell death and/or impairment of cell clearance.

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