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A Role for Oxidative Stress in Apoptosis: Oxidation and Externalization of Phosphatidylserine Is Required for Macrophage Clearance of Cells Undergoing Fas-Mediated Apoptosis


Exposure of phosphatidylserine (PS) on the surface of apoptotic cells has been suggested to serve as an important recognition signal for macrophages. In this work we show that triggering of the death receptor Fas on Jurkat cells results in the generation of reactive oxygen species with oxidation and externalization of PS but not of the other major aminophospholipid, phosphatidylethanolamine. These cells were readily ingested by several classes of macrophages, whereas Raji cells, which are defective for Fas-induced PS exposure, remained unengulfed. However, when Raji cells were incubated with the thiol-reactive agent N-ethylmaleimide to induce PS exposure in the absence of other features of apoptosis, these cells were also engulfed by macrophages. Phagocytosis of Fas-triggered Jurkat cells was inhibited by superoxide dismutase and catalase, which prevent oxidation of PS while allowing PS to remain externalized on these cells. Moreover, liposomes containing oxidized PS (PS-OX) were more potent inhibitors of phagocytosis than those containing its nonoxidized counterpart. Finally, enrichment of the plasma membrane of Jurkat or Raji cells, or myeloid leukemic HL-60 cells, with exogenous PS resulted in phagocytic cell clearance, and this process was further enhanced when PS was substituted for by PS-OX. Taken together, our data suggest that the presence of PS-OX in conjunction with nonoxidized PS on the cell surface is an important signal for macrophage clearance of apoptotic cells. The Journal of Immunology, 2002, 169: 487–499.

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and was blocked by the antiapoptotic protein Bcl-2 (16), thus implying that oxidative modification of PS is an integral part of the apoptotic program. However, elucidation of the specific function of PS oxidation during apoptosis is difficult when phospholipids undergo massive oxidation, as is the case during oxidant-induced apoptosis. Therefore, a model of apoptosis-specific oxidation of phospholipids is necessary to determine whether oxidation of PS plays a role in its externalization and/or subsequent recognition by macrophages. For the present studies, we used two different Fas-sensitive human tumor cell lines, the EBV- transformed B cell Raji and the leukemic T cell Jurkat. These cells, which are shown here to differ in terms of Fas-induced PS externalization, provided an opportunity to test the hypothesis that exposure of PS is required for phagocytosis of apoptotic cells. In addition, we asked whether PS is oxidized in these two cell types in response to Fas ligation and what role, if any, this oxidative modification of PS may play in the recognition of apoptotic cells.

Materials and Methods

Reagents and cell culture

Agnostic anti-Fas Ab (clone CH-11) was obtained from Medical & Biological Laboratories (Nagoya, Japan). The fluorogenic peptide substrate aspartate-glutamate-valine-aspartate (DEVD)-7-amino-4-methyl-coumarin (AMC) and the broad range caspase inhibitor benzoyloxycarbonyl-valine-alanine-aspartate-fluoromethylketone (zVAD-fmk) were obtained from Peptide Institute (Osaka, Japan) and Enzyme Systems Products (Dublin, CA), respectively. cis-Parinaric acid (cis-PnA), 2-methyl-6-(4-methoxy-phenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, hydrochloride (MCLA), dihydroethidium (DHE), and the Amplex Red hydrogen peroxide assay kit were from Molecular Probes (Eugene, OR). HPLC grade solvents were from Fisher Scientific (Pittsburgh, PA). Fluorescamine, superoxide dismutase (SOD), catalase, tert-butyl hydroperoxide, and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (St. Louis, MO). The leukemic T cell line Jurkat, the EBV-transformed B cell line Raji, and the myeloid leukemic cell line HL-60 from the European Collection of Cell Cultures (Sailbury, U.K.) were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). The human monocytic leukemia cell line THP-1 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Alabaster, AL). Results are expressed as the ratio of derivatized to undervatized aminophospholipid based on spectrophotometrical analysis of total phosphorous content.

Lipid peroxidation

The ability of cells to metabolically incorporate the fluorescent, oxidant-sensitive fatty acid, cis-PnA, into total cellular phospholipids was exploited to measure lipid peroxidation in selected phospholipids of live cells. The details of this assay have been described elsewhere (17). Briefly, cells were labeled with cis-PnA (2 μg/ml final concentration) in RPMI 1640 medium without phenol red and bovine serum at a density of 1 × 10⁶ cells/ml. cis-PnA-labeled cells were washed and resuspended in a buffer containing 115 mM NaCl, 1 mM MgCl₂, 5 mM Na₂HPO₄, 10 mM glucose, and 25 mM HEPES (pH 7.4) at 1 × 10⁶ cells/ml. Aliquots were taken for phospholipid analyses representing the amount of cis-PnA incorporated immediately after labeling. Cells were then treated with anti-Fas Abs (250 ng/ml) or NEM (5 mM) for 2 h at 37°C, centrifuged, and lysed in 0.5 ml of ice-cold methanol containing butylated hydroxyltoluene (0.1 mg). Total lipids were immediately separated by HPLC as previously described (16). The amount of cis-PnA fluorescence in individual phospholipid classes was normalized to the amount of inorganic phosphorous content of each individual phospholipid class, as determined from parallel HPTLC.

Caspase-3-like enzyme activity

Cleavage of the caspase-3-like enzyme substrate DEVD-AMC was estimated in a fluorometric assay as described previously (18). Briefly, cells were pelleted and frozen on microtiter plates at 1 × 10⁵ cells per well. Substrate (50 μM) was dissolved in a standard reaction buffer (100 mM HEPES, 10% sucrose, 5 mM DTT, and 0.1% 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid (CHAPS)) at 1 mg/ml and was added to each well. Enzyme-catalyzed release of AMC was measured every 70 s during a 30-min period in a Fluorocam II plate reader (Labsystems, Stockholm, Sweden) using 355-nm excitation and 460-nm emission wavelengths. Fluorescence units were converted to picomoles of AMC using a standard curve generated with free AMC. Data from duplicate samples were analyzed by linear regression and are displayed as picomoles of AMC release per minute.

Assessment of nuclear apoptotic morphology

Cells (1 × 10⁵) were harvested after apoptosis induction, washed, resuspended in paraformaldehyde (4% in PBS), and spun down onto glass slides. Cytospin preparations were air dried, then rehydrated in D₂O and stained with 10 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) in the dark for 10 min. Slides were rinsed in D₂O, dried in the dark, and mounted with coverslips using glycerol (50:50, v/v). Apoptotic nuclei were scored under a fluorescence microscope, and a minimum of 200 nuclei were counted per slide.

Assays of ROS generation

Production of superoxide was assessed by oxidation of DHE to ethidium. Cells were incubated with 5 μM DHE in tissue culture medium for 45 min at 37°C and then washed, resuspended in PBS, and submitted to flow cytometric analysis using a FACScan flow cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences). Cellular debris and necrotic cells were excluded based on forward and side scatter characteristics. Alternatively, superoxide was measured by MCLA ECL. Jurkat cells

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(1 × 10^6 cells) were collected by centrifugation, washed in PBS (pH 7.4), and resuspended in 1 ml of prewarmed (37°C) PBS (pH 7.4) containing 0.5 mM CaCl_2, 1 mM MgCl_2, and 30 mM glucose. Anti-Fas Abs were added to cells, and the chemiluminescence signal was continuously recorded for 15 min in the Luminescent Analyzer 633 (Coral Biomedical, San Diego, CA) set at 37°C and continuous mixing. MCLA (4 μM) was used for monitoring the chemiluminescence due to superoxide production. When the effect of SOD plus catalase (50 U/ml each) or zVAD-fmk (10 μM) was tested, cells were preincubated with the respective inhibitor for 5 min at 37°C before addition of the apoptotic stimulus. For some experiments, SOD plus catalase were heat-inactivated for 30 min at 80°C before incubation with cells. The area under the curve (millivolts × seconds) was taken as the total amount of superoxide detected, and values were expressed as fold increase above control. Hydrogen peroxide levels were determined using Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), the oxidation of which yields a fluorescent product in the presence of hydrogen peroxide and HRP (19). Briefly,cells (3 × 10^7/100 μl) were incubated in PBS containing 50 μM Amplex Red and 1 U/ml HRP in the presence or absence of anti-Fas stimulation for 2 h at 37°C. Measurements were conducted at 530/590 nm (A_{530}/A_{590}) using a Cytoflour Model 2350 fluorescence microplate reader (Millipore, Bedford, MA).

**GSH levels**

Intracellular glutathione (GSH) was measured as previously described (20). Briefly, 0.5 × 10^6 cells were washed in PBS and centrifuged for 5 min at 700 × g. The supernatant was discarded and the cell pellet was resuspended in 50 μl of PBS. An equal volume of 8 mM monobromobimane (pH 8) was added and the sample was incubated for 5 min at room temperature in the dark. The protein was precipitated by adding 2.5 μl of 100% trichloroacetic acid to the samples. Samples were then centrifuged at 10,000 × g for 5 min and the low-m.w. thiols were analyzed by HPLC.

**Preparation of PS- and PS-OX-containing liposomes**

PS (1-palmitoyl-2-arachidonoyl-sn-glycero-3-[phospho-cytidylyl]-serine]) was oxidized by incubation with a water-soluble azo-initiator of peroxyl radicals, 2,2′-azo-bis-(2-amidinopropane) hydrochloride. PS in chloroform was dried under nitrogen and PBS was added to achieve the final concentration of 5 mM. The lipid was incubated with 2,2′-azo-bis-(2-aminopropane) hydrochloride (50 mM) at 37°C for 4 h and then extracted with chloroform: methanol (2:1, v/v). Oxidation was assessed by measuring the absorbance hydroperoxides with conjugate dienes at 234 nm. Approximately 16% of PS molecules were estimated to be oxidized after a 4-h incubation, while ~84% remained nonoxidized, and 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 Fadok et al. (21). Individual phospholipids, stored in chloroform, were dried under nitrogen. PBS was added (1000 nmol total lipid per milliliter of PBS), and the lipid mixture was vortexed and sonicated for 3 min on ice. All liposomes were used immediately after preparation.

**Phagocytosis assays**

Target cells (typically 30 × 10^6) were labeled with 50 μM of the fluorescent dye S(6)-carboxytetramethyl-rhodamine N-hydroxy-succimide ester (Sigma-Aldrich) or 0.5 μM CellTracker Orange (Molecular Probes, Eugene, OR) in serum-free medium for 15 min at 37°C and cultured at 10^6 cells/ml in the presence or absence of apoptotic stimuli. In some experiments, SOD (50 U/ml) plus catalase (50 U/ml), heat-inactivated SOD plus catalase, or zVAD-fmk were added 30 min before the induction of apoptosis. Upon harvesting, cells were washed twice and resuspended in medium, and 10^6 viable or apoptotic cells were added to macrophages in 24-well tissue culture plates. After incubation at 37°C for 1 h (for J774A.1...
macrophages) or 3 h (for THP-1 macrophages or HMDMs), nonphagocytosed cells were washed off with several washes in cold PBS and the remaining cells were fixed in 4% paraformaldehyde. Phagocytosis was evaluated by counting macrophages in visual light and thereafter counting macrophage-engulfed cells under UV illumination using an inverted fluorescence microscope at ×400 magnification. At least 300 macrophages per experimental condition were counted. Phagocytosis data are reported as the percentage of phagocytes positive for uptake. For competitive inhibition studies, J774A.1 macrophages were preincubated with different amounts of liposomes containing either PS or PS-OX at 37 °C for 30 min. Fas-induced Jurkat cells or tert-butyl hydroperoxide-treated HL-60 cells were added to macrophages after liposomes were removed and incubated at 37 °C for 1 h before determination of phagocytosis positive for uptake. To integrate PS or PS-OX into nonapoptotic cells, these cells were preincubated for 5 min at 37 °C with NEM (10 μM) and then washed, after which cells were incubated for another 30 min at 37 °C together with different amounts of liposomes containing either PS or PS-OX. Nonincorporated liposomes were removed by washing the cells twice with serum-free medium. Cells with either PS or PS-OX inserted into the plasma membrane were subsequently added to J774A.1 macrophages and incubated at 37 °C for 1 h. Phagocytosis was determined as above.

Statistics
Data are expressed as mean ± SEM. Changes in variables for different assays were analyzed by either Student’s t test (single comparisons) or one-way ANOVA for multiple comparisons. If ANOVA revealed significant changes between samples, multiple unpaired Student’s t tests were performed. Differences among means were considered significant when $p < 0.05$.

Results
Cell type-specific externalization of PS, but not of PE, during Fas-mediated apoptosis
Incubation of the two human cell lines Jurkat and Raji with agonistic anti-Fas Abs (250 ng/ml) resulted in apoptosis as evidenced by a time-dependent increase in caspase-3-like enzyme activity (determined by the in vitro cleavage of the fluorescent peptide substrate, DEVD-AMC) and the occurrence of characteristic nuclear condensation (Fig. 1, a and b). However, when these cells were subjected to flow cytometric analysis using fluorescently labeled annexin V, PS exposure was detected only in Jurkat cells (Fig. 1c), in line with our previous observations (22). These apoptotic events, including the exposure of PS in Jurkat cells, were completely abrogated by the pan-caspase inhibitor, zVAD-fmk (10 μM). Annexin V staining is most commonly used to detect the percentage of cells that have externalized PS, rather than to provide a precise quantitative measurement of the amount of PS on the cell surface. Furthermore, use of annexin V provides no information regarding the possible externalization of the other major

![FIGURE 2. Selective externalization of PS in apoptotic Jurkat cells. a, Typical two-dimensional HPTLC of total lipid extracts from control (a1 and a2) and Fas-induced Jurkat cells (a3 and a4). HPTLC chromatograms were visualized using a Fluor-S MultiImager (Bio-Rad, Hercules, CA) equipped with a UV light source after treatment with fluorescamine (a1 and a3) or equipped with a VIS light source after staining by exposure to iodine vapors (a2 and a4). NL, Neutral lipids; FFA, free fatty acids; Sph, sphingomyelin; PI, phosphatidylinositol; Dpg, diphosphatidylglycerol; mPE, modified PE by fluorescamine; mPS, modified PS by fluorescamine. Also shown is a comparison of the amount of PS vs PE accessible to fluorescamine derivatization on the surface of Jurkat cells (b) or Raji cells (c) after treatment with anti-Fas Abs (250 ng/ml) for 2 h in the presence or absence of zVAD-fmk (10 μM). The percentage of total PS and PE modified by fluorescamine was determined by phosphorous analysis of each modified phospholipid spot following two-dimensional HPTLC as described in Materials and Methods. Data are expressed as mean ± SEM ($n = 3$). n.d., Not detectable.](http://www.jimmunol.org/doi/pdf/10.4049/jimmunol.9901135)
aminophospholipid, phosphatidylethanolamine (PE). Therefore, we labeled externalized PS and PE using fluorescamine (Fig. 2a), a cell-impermeable fluorescent reagent capable of reacting with primary amines (15). As shown in Fig. 2b, no detectable PS was found on the outer surface of control cells. In contrast, ~9.8% of total cellular PS was available for fluorescamine derivatization in Jurkat cells after exposure to anti-Fas Ab. The amount of fluorescamine-modified PE was 7.1 ± 1.3% and 6.7 ± 0.8% of total PE.

**FIGURE 3.** Caspase-dependent oxidative stress in Jurkat cells upon Fas ligation. 

a, Superoxide production in Jurkat cells as assessed by MCLA ECL. Cells were treated with 250 ng/ml anti-Fas Abs in the absence or presence of either SOD plus catalase (50 U/ml each) or zVAD-fmk (10 μM). Chemiluminescence was monitored for 15 min and integral values (millivolts × seconds) were measured. Values (mean ± SEM) are shown as the fold increase above control (n = 4), ∗, p < 0.01 vs each of the other conditions. b, Representative real-time measurement of MCLA ECL in Jurkat cells treated as outlined above, c, MCLA ECL detection in Raji cells incubated for 2 h in the presence or absence of anti-Fas Abs (250 ng/ml). No significantly enhanced superoxide production was detected upon Fas triggering (mean ± SEM; n = 3). d, Jurkat or Raji cells were treated with anti-Fas Abs (250 ng/ml) for the indicated times and then incubated with the fluorescent probe DHE before flow cytometric analysis. The percentage of cells with superoxide production (upper right quadrants) is indicated for each sample. Data shown are representative of three independent experiments. e, Amplex Red-based detection of hydrogen peroxide in Jurkat cells incubated as in a. Heat-inactivated SOD plus catalase were included as a negative control. Results are reported as nanomolar hydrogen peroxide based on a calibration curve of hydrogen peroxide concentrations. Data shown are mean ± SEM (n = 3–4), ∗, p < 0.001 anti-Fas vs control; **, p > 0.05 anti-Fas vs heat-inactivated SOD plus catalase by one-way ANOVA. f, The level of intracellular GSH was estimated by HPLC in Jurkat and Raji cells incubated in the presence or absence of anti-Fas Abs (250 ng/ml) for 2 h. Data are expressed as mean ± SEM (n = 3).
before and after Fas ligation, respectively. However, in Raji cells, PS remained sequestered in the inner leaflet after induction of apoptosis and the amount of PE on the cell surface was unchanged by Fas stimulation (Fig. 2c). The Fas-induced increase in fluorescently-modified PS in Jurkat cells was prevented by zVAD-fmk (10 μM), while caspase inhibition had no effect on the level of PE on the cell surface (Fig. 2b). These data demonstrate the selective externalization of PS during the course of Fas-induced apoptosis in Jurkat cells but not in Raji cells.

**Caspase-dependent oxidative stress in Fas-triggered Jurkat cells**

To determine whether Fas-mediated execution of the apoptotic program was accompanied by the production of ROS we used a superoxide-specific enhancer of chemiluminescence, MCLA. As seen in Fig. 3a, Fas-triggered apoptosis in Jurkat cells was, indeed, associated with the production of superoxide. Moreover, ROS production was completely blocked by zVAD-fmk (10 μM) and significantly suppressed by the combination of SOD and catalase (50 U/ml each) (Fig. 3, a and b). In contrast, no significant increase in superoxide production was detected in Fas-triggered Raji cells (Fig. 3c). Similarly, a time-dependent increase in superoxide production was seen in Jurkat cells based on the oxidation of DHE to its fluorescent derivative, ethidium, while Fas-triggered Raji cells failed to generate ROS (Fig. 3d). Moreover, using the highly sensitive Amplex Red-based assay, we observed SOD plus catalase-inhibitable hydrogen peroxide production in Jurkat cells; as expected, heat-inactivated antioxidant enzymes were unable to block the generation of hydrogen peroxide (Fig. 3e). We have previously demonstrated that there is a decrease in intracellular GSH during Fas-mediated apoptosis in Jurkat cells (20). To further assess the degree of oxidative stress, we measured the level of intracellular GSH in Jurkat and Raji cells. After Fas triggering for 2 h, GSH levels dropped to <60% of control levels in Jurkat cells, while levels in Raji cells remained essentially unchanged (Fig. 3f). These results indicate that Fas ligation results in a typical repertoire of apoptotic responses including the caspase-dependent generation of ROS in Jurkat cells but not in Raji cells.

**SOD plus catalase-inhibitable oxidation of PS upon Fas ligation of Jurkat cells**

We then studied the oxidation of membrane phospholipids during Fas-triggered apoptosis. To this end, phospholipids were metabolically labeled with cis-PnA, an oxidation-sensitive fluorescent fatty acid containing four conjugated double bonds (17). We found that oxidation of PS in Jurkat cells in response to Fas ligation, as determined by the decrease in fluorescence intensity of esterified cis-PnA, was markedly increased, while no apparent oxidation was detectable in any other major class of phospholipids such as PC, PE, and PI (Table I). Furthermore, oxidation of PS was much less pronounced in Fas-triggered Raji cells, in line with the absence of Fas-induced oxidative stress in these cells (Table I). Of note, HPTLC analyses demonstrated that the distribution pattern of the major phospholipids in Jurkat cells remained quantitatively unchanged after treatment with anti-Fas Ab, thus demonstrating that oxidation of PS was not associated with any significant alteration in phospholipid composition (data not shown). Importantly, Fas-induced PS oxidation was completely prevented upon preincubation of cells with zVAD-fmk (10 μM) and was also effectively blocked by the antioxidant enzymes SOD (50 U/ml) plus catalase (10 U/ml) (Table I). Hence, Fas-induced execution of the apoptotic program in Jurkat cells involves oxidation of PS downstream of the activation of caspases.

**Cell surface exposure of PS is critical for macrophage clearance**

To test the importance of PS exposure for phagocytosis, we examined whether apoptotic Jurkat and Raji cells could be phagocytosed to a similar extent. Cells were treated with anti-Fas Abs as indicated and then incubated with the human macrophage-like cell line THP-1, the murine macrophage cell line J774A.1, or primary HMDMs (Fig. 4). Jurkat cells were readily ingested in all cases and phagocytosis was preventable by zVAD-fmk (Fig. 4, a and b), while engulfment of Fas-triggered Raji cells was not seen when THP-1 or J774A.1 cells were used (Fig. 4, a and b) and

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**Table I. Effect of anti-Fas mAb on oxidation of cis-PnA-labeled phospholipids in Jurkat cells**

<table>
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<th>PC</th>
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<td>116.8 ± 6.6**</td>
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* cis-PnA-loaded cells (10⁶ cells/ml) were incubated in L1210 buffer (115 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM NaH₂PO₄, 10 mM glucose, 25 mM HEPES (pH 7.4)) with anti-Fas mAb (250 ng/ml) in the presence or absence of zVAD-fmk (10 μM) or SOD (50 U/ml) plus catalase (10 U/ml) for 2 h at 37°C. Lipids were then extracted and resolved by HPLC. Values are expressed as the percentage of control nanograms of cis-PnA per microgram of lipid phosphorus in individual phospholipid. Data are mean ± SEM. *p < 0.005 vs control. **p < 0.0001 vs anti-Fas.

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**Table II. Effect of anti-Fas mAb on oxidation of cis-PnA-labeled phospholipids in Raji cells**

<table>
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<th>Additions</th>
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<td>111.5 ± 9.2</td>
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* cis-PnA-loaded cells (10⁶ cells/ml) were incubated in L1210 buffer (115 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM NaH₂PO₄, 10 mM glucose, 25 mM HEPES (pH 7.4)) with anti-Fas mAb (250 ng/ml) in the presence or absence of z-VAD-fmk (250 ng/ml) in the presence or absence of z-VAD-fmk (10 μM) for 2 h at 37°C. Lipids were then extracted and resolved by HPLC. Values are given as the percentage of control nanograms of cis-PnA per microgram of lipid phosphorus in individual phospholipid. Data are expressed as mean ± SEM.
occurred only to a minor degree in the case of HMDMs (Fig. 4c). Nonstimulated HMDMs are thought to use an αβ/CD36/thrombospondin-dependent mechanism for phagocytosis, while β-glucan stimulation has been suggested to render these cells PS dependent (23). However, as seen in Fig. 4c, the degree of phagocytosis of Fas-stimulated cells was unaltered by β-glucan stimulation of HMDMs. NEM is a commonly used inhibitor of the aminophospholipid translocase, an enzymatic activity required for the maintenance of plasma membrane phospholipid asymmetry (24). We and others have recently shown that NEM can trigger PS exposure in the absence of other signs of apoptosis (22, 25). Indeed, incubation of Raji cells for 2 h with NEM (5 mM) resulted in the

![FIGURE 4. PS-dependent phagocytosis of Fas-triggered cells by several types of macrophages. Jurkat and Raji cells were treated with anti-Fas Abs (250 ng/ml) for 2 h (a) or 4 h (b and c) and subsequently analyzed for phagocytic engulfment by J774A.1 cells (a), THP-1 cells (b), or unstimulated vs β-glucan-stimulated HMDMs (c), as described in Materials and Methods. For some experiments, cells were preincubated in the presence of zVAD-fmk (10 μM) for 30 min before induction of apoptosis. Data are expressed as mean ± SEM (n = 3–6). *, Statistically significant differences compared with each of the other groups by one-way ANOVA (p < 0.01).](image)

![FIGURE 5. Macrophage uptake of nonapoptotic, PS-expressing Raji cells. a, Raji cells were incubated for 2 h with the thiol-reactive agent, NEM (5 mM), and PS exposure was assessed using the fluoroscamine-based assay. The amount of PS accessible to fluoroscamine derivatization on the surface of these cells is depicted. No activation of caspase-3-like enzymes was detected upon NEM treatment (data not shown). Data are expressed as mean ± SEM (n = 3). b, NEM-treated Raji cells were cocultured with J774A.1 macrophages and the percentage of phagocytes positive for uptake was scored. Data shown are mean ± SEM (n = 3).](image)
FIGURE 6. PS oxidation is required for efficient phagocytosis of apoptotic Jurkat cells. Jurkat cells were incubated with anti-Fas Abs (250 ng/ml) for 2 h (a and c) or 4 h (b, d and e) in the absence or presence of zVAD-fmk (10 μM), SOD (50 U/ml) plus catalase (50 U/ml), or heat-inactivated antioxidant enzymes, and analyzed for PS exposure (a), caspase-3-like enzyme activity (b), phagocytosis by J774A.1 cells (c), phagocytosis by THP-1 cells (d), or phagocytosis by unstimulated vs β-glucan-stimulated HMDMs (e), as described in Materials and Methods. Data are expressed as mean ± SEM (n = 3–6). * p < 0.01.
externalization of almost 15% of total cellular PS, as determined by fluorescein labeling of these cells (Fig. 5a). PS was oxidized under these conditions (41 ± 7.4% cis-PnA fluorescence vs control; n = 3; p < 0.005), as was PE (37.2 ± 4.6% cis-PnA fluorescence vs control; n = 3; p < 0.001), while activation of caspase-3-like enzymes was undetectable (data not shown). As shown in Fig. 5b, NEM-treated Raji cells were readily engulfed by J774A.1 macrophages. Taken together, these observations suggest that PS exposure is both necessary and sufficient for macrophage recognition of dying cells. In addition, the current data show that execution of apoptosis and clearance of cell corpses are separable events.

**Oxidation of PS potentiates macrophage clearance of Jurkat and Raji cells**

A significant fraction of externalized PS molecules on the surface of Fas-triggered Jurkat cells could represent PS-OX. In other words, not only externalization but also oxidation of at least some PS molecules may be essential for recognition and phagocytosis of apoptotic cells. To test this hypothesis, Jurkat cells were incubated with SOD plus catalase (or heat-inactivated antioxidant enzymes) before addition of anti-Fas Abs to prevent oxidation of PS. The addition of SOD plus catalase did not significantly reduce the percentage of annexin V-positive cells (22 ± 4% in the absence vs 18 ± 4% in the presence of SOD plus catalase; n = 3), and activation of caspase-3-like enzymes also remained unaltered under these conditions (Fig. 6, a and b). However, SOD plus catalase, but not the heat-inactivated enzymes, significantly diminished phagocytosis of Jurkat cells by J774A.1 or THP-1 macrophages (Fig. 6, c and d), and similar results were obtained when primary macrophages were used (Fig. 6e). Of note, SOD plus catalase appeared to have a greater inhibitory effect when HMDMs were β-glucan stimulated (Fig. 6e). To further assess the importance of PS oxidation for phagocyte recognition, phagocytosis of Fas-triggered Jurkat cells by the macrophage cell line J774A.1 was tested in the presence or absence of liposomes containing PC plus nonoxidized PS vs a mixture of PC plus PS-OX. As depicted in Fig. 7a, PS-OX-containing liposomes (0–150 nM) were more potent inhibitors of phagocytosis than liposomes containing nonoxidized PS. In a different experimental paradigm, Jurkat cells were pretreated with a low dose of NEM (10 μM) that was determined to inhibit aminophospholipid translocation, as evidenced by a decrease in cellular uptake of fluoresceinyl-labeled PS (1-palmitoyl-2-[6-[[(7-nitro-2,1,3-benzoazadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine), yet failed to induce exposure of endogenous PS (data not shown). These cells were then incubated with liposomes containing PS or a mixture of PS and PS-OX (0–150 nM) and subsequently cocultivated with J774A.1 macrophages. As seen in Fig. 7b, phagocytosis of Jurkat cells in which PS-OX was integrated into the outer membrane was substantially enhanced as compared with cells expressing nonoxidized PS. Moreover, when Raji cells were enriched with exogenous PS by the same method, these cells were also engulfed in a dose-dependent manner by macrophages (Fig. 8a). As in the case of Jurkat cells, phagocytosis was further enhanced when Raji cells were enriched with PS-OX compared with PS alone (Fig. 8b). Importantly, Raji cells treated with PS- or PS-OX-containing liposomes exhibited a similar degree of annexin V binding (Fig. 8c); a similar annexin V staining pattern was also obtained for Jurkat cells (data not shown). Taken together, these results suggest that PS-OX is an important signal that, in conjunction with nonoxidized PS, is recognized by one or more macrophage receptor(s) with subsequent engulfment of the dying cell.

**PS-dependent macrophage recognition of myeloid leukemic HL-60 cells**

To verify that the importance of PS for phagocytosis is not limited to Jurkat and Raji cells, we performed similar experiments in the myeloid leukemic cell line, HL-60. Hence, these cells were preincubated with NEM (10 μM) and then incubated with liposomes containing different mixtures of phospholipids (PC and PS (50%/50%), PC and PS plus PS-OX (50%/50%) or PS-OX (50%/50%)), PC alone, or PC and oxidized PC (PC-OX) and subsequently subjected to annexin V-FITC labeling. Flow cytometric analysis of annexin V staining confirmed specific enrichment with PS and PS-OX in the plasma membrane (Fig. 9a). Moreover, no significant differences in the percentage of annexin V-positive cells were observed in PS- vs PS-OX-enriched cells. As expected, no annexin V staining was detected in the cells with integrated PC or PC-OX. HL-60 cells were then cocultured with J774A.1 macrophages and the number of phagocytes positive for uptake of HL-60 cells was determined. As seen in Fig. 9b, HL-60 cells treated with PS and PS-OX, but not with PC or PC-OX, were readily engulfed. Moreover, PS-OX-treated cells were more efficiently phagocytosed when compared

FIGURE 7. Surface expression of PS-OX is an important stimulus for phagocytosis of Jurkat cells. a, Competitive inhibition of phagocytosis of Fas-triggered Jurkat cells by J774A.1 macrophages in the presence of liposomes containing PS vs a mixture of PS plus PS-OX. b, Phagocytosis by J774A.1 macrophages of nonapoptotic, NEM-pretreated (10 μM) Jurkat cells with liposomes containing PS or PS-OX integrated into the plasma membrane. Data are expressed as mean ± SEM (n = 6). *, p < 0.01; **, p < 0.05 (PS-OX vs PS by Student’s t test).
with PS-treated cells, at all concentrations of liposomes tested (Fig. 9c). HL-60 cells are relatively insensitive to Fas ligation, yet rapidly undergo apoptosis in response to tert-butyl hydroperoxide, with attendant oxidation and externalization of PS (data not shown). We incubated HL-60 cells with tert-butyl hydroperoxide (150 μM) for 3 h and these cells were subsequently cocultured with macrophages in the presence or absence of liposomes containing PS, PS-OX, PC, or PC-OX. As seen in Fig. 9d, PS-OX-containing liposomes were the most effective inhibitors of engulfment of apoptotic HL-60 cells. Hence, these data provide further evidence that PS exposure is important for macrophage recognition and corroborate that both PS-OX and nonoxidized PS act as recognition signals for phagocytes.

**Discussion**

In this study we have demonstrated the oxidation of PS during Fas-induced apoptosis in Jurkat cells, and we provide evidence that oxidation and externalization of PS, but not of PE, is critical for phagocytic recognition and uptake of apoptotic cells. These studies thus indicate that oxidation of PS is not restricted to oxidative stress-induced apoptosis (14) and suggest that PS oxidation is an intrinsic feature of the apoptotic program, at least in some cell types. Our studies in Jurkat cells disclosed a correlation between the induction of oxidative stress (i.e., generation of ROS and dissipation of intracellular GSH) and oxidation of PS upon Fas ligation. It has previously been proposed that cytochrome c may act as an endogenous catalyst for PS oxidation during apoptosis, because PS was markedly oxidized when intact HL-60 cells were loaded with exogenous cytochrome c (14). However, further studies are warranted to elucidate the putative role of GSH depletion, ROS generation, and release of mitochondrial constituents, including cytochrome c, in the process of Fas-induced oxidation of PS. Nevertheless, the current observations suggest that oxidative stress upon apoptosis induction should be viewed not as a trivial side effect of the disruption of mitochondrial electron transport but rather as an important signal involved in the resolution of this mode of cellular demise.

The ability of macrophages to discriminate between self and non-self or “altered self” in the defense against microbial infections and in the process of tissue turnover is of paramount importance (26). The present study emphasizes the role of PS exposure in this recognition process insofar as apoptotic Jurkat and HL-60 cells are readily ingested by various classes of macrophages, whereas the PS-defective Raji cells remain unengulfed. However,
we found that incubation of Raji cells with high doses (5 mM) of the thiol-reactive agent, NEM, resulted in the externalization of PS and ingestion of these cells by phagocytes. While the concomitant induction of other hitherto unknown recognition signals is difficult to exclude, these data nevertheless provide strong evidence for a role of PS in this process. These findings thus concur with previous work by Fadok et al. (21) and Pradhan et al. (27), who proposed that certain macrophage populations use PS as a signal for phagocytic uptake. Moreover, recent studies have indicated that PS recognition is used by both professional and nonprofessional phagocytes (28). Interestingly, we found that oxidation of PS was less pronounced in the PS exposure-defective cell line Raji. However, while SOD and catalase effectively prevented Fas-induced oxidation of PS in Jurkat cells, the percentage of annexin V-positive cells was unchanged. Thus, while it is tempting to speculate that preferential oxidation of PS may dictate the specificity of phospholipids present on the surface of apoptotic cells (Ref. 15 and the present study), exposure of PS does not appear to depend solely on its oxidation. These findings are in agreement with previous studies in which NO was found to dissociate lipid peroxidation from PS externalization during oxidant-induced apoptosis (29).

**FIGURE 9.** Involvement of PS and PS-OX in macrophage clearance of HL-60 cells. a, HL-60 cells were preincubated with NEM (10 μM for 5 min), then incubated with liposomes containing different mixtures of phospholipids at 500 nM (PC and PS (50%/50%), PC and PS plus PS-OX (50%/50%), PC alone, or PC and PC-OX) and subsequently subjected to annexin V-FITC labeling. Propidium iodide-positive cells were gated out before determination of the percentage of annexin V-positive cells. Data shown are mean ± SEM (n = 3). b, HL-60 cells treated as above were cocultivated with J774A.1 macrophages and the number of phagocytes positive for uptake was determined. Data are expressed as mean ± SEM (n = 3). *, p ≤ 0.01; PS vs control; **, p ≤ 0.01; PS-OX vs PS. c, Phagocytosis of HL-60 cells treated as above with different amounts of PS- or PS-OX-containing liposomes (0–500 nM), respectively. Data shown are mean ± SEM (n = 3). *, p ≤ 0.01; PS-OX vs PS. d, HL-60 cells triggered to undergo apoptosis by tert-butyl hydroperoxide (150 μM) for 3 h were subjected to phagocytosis by J774A.1 macrophages preincubated in the presence or absence of liposomes (0–150 nM) as indicated. Data are shown as mean ± SEM (n = 3). *, p ≤ 0.05 PS or PS-OX vs control.
that scavenger receptors act as receptors for apoptotic cells (5). For instance, the class B scavenger receptor, CD36, is known to be the major PS-binding protein on THP-1 and J774A.1 cells (31) and is required for phagocytosis of apoptotic cells by various classes of human macrophages (23). Indeed, in preliminary experiments we observed that Abs specific for CD36 blocked phagocytosis of Fas-stimulated Jurkat cells, and similar results were obtained when PS-OX-enriched HL-60 cells, but not cells enriched for PS alone, were cocultivated with macrophages (our unpublished data). Chang et al. (32) have previously shown that mAbs against oxidized LDL bind to the surface of apoptotic cells and inhibit their uptake by macrophages. This suggests that apoptotic cells express oxidation-specific epitopes, including oxidized phospholipids, on their cell surface, and that these serve as ligands for recognition and phagocytosis by macrophages. Indeed, our observation that protection against PS oxidation abrogates macrophage recognition of Fas-triggered cells suggests that oxidation of PS itself is involved in the clearance of cell corpses. We cannot rule out other non-PS-related effects of SOD plus catalase, such as the inhibition of expression of other yet-to-be-identified recognition signals. Nevertheless, the current data raise the possibility that scavenger receptors are involved in the recognition of PS-OX on the surface of apoptotic cells. Furthermore, our data indicate that SOD plus catalase are more effective in inhibiting phagocytosis of Fas-triggered Jurkat cells after β-glucan stimulation of HMDMs. Future studies should address whether HMDMs switch from a PS-dependent to a PS-OX-dependent mode of cellular uptake upon β-glucan stimulation, e.g., by up-regulation of one or more receptors for PS-OX.

An intriguing question is why macrophages express so many putative PS-binding receptors. One explanation for this apparent redundancy is that multiple receptors are required to ensure that dying cells do not persist in vivo, such that the “meaning” of cell death is not precluded (6). Alternatively, cooperation between different receptors may be required for the adherence to macrophages and the subsequent internalization of effecte cells. Importantly, the latter event also requires coordinated reorganization of the cytoskeleton of the engulfing cell and is likely to depend on signals that are transduced via the intracellular domains of the phagocyte receptors. In support of the receptor cooperativity model, Sambrano et al. (33) reported that disruption of phospholipid asymmetry is sufficient for tethering of erythrocytes to macrophages but noted that additional oxidative changes are required for engulfment to take place. Indeed, the current observation that PS-OX-containing liposomes are potent inhibitors of phagocytosis of Fas-triggered Jurkat cells and tert-butyl hydroperoxide-treated HL-60 cells, and the fact that cells (Jurkat, Raji, or HL-60) in which PS-OX is inserted into the plasma membrane are effectively engulfed indicates that PS-OX, in conjunction with nonoxidized PS, serves as an “eat me” signal for macrophages. Shacter et al. (34) recently demonstrated that chemotaxy (etoposide, doxorubicin, cisplatin, and AraC)-induced apoptosis of human Burkitt lymphoma cells is augmented by antioxidant agents; conversely, phagocytosis of etoposide-treated cells was inhibited by hydrogen peroxide. However, the apparent discrepancies between our findings and those of Shacter et al. (34) are most likely related to the different stimuli used to trigger apoptosis. Indeed, while phagocytosis of etoposide-treated cells was inhibited by hydrogen peroxide as a result of its inhibition of the apoptotic process itself (34), in our model of Fas-triggered apoptosis SOD plus catalase blocked ROS production yet failed to prevent cell death. In contrast, these antioxidant enzymes were able to diminish phagocytosis of Fas-triggered cells. Hence, in the current model, the execution of cell death is independent of ROS, while PS oxidation and resolution of the death process by phagocytosis are clearly linked to ROS production.

To conclude, our findings underscore the critical involvement of PS externalization in the recognition and removal of apoptotic cells. Moreover, we provide evidence that oxidation of PS occurs upon Fas ligation and show that this oxidative modification of PS serves as an important stimulus for neighboring phagocytes. The observation that SOD and catalase can block macrophage recognition of apoptotic cells is of particular interest and it will be important to establish to what extent this effect may be a common feature of other natural and synthetic antioxidants. Furthermore, recent studies have demonstrated that PS-dependent phagocytosis of apoptotic cells not only serves to remove dying cells from the tissues but also stimulates macrophage production of anti-inflammatory cytokines such as TGF-β1 and inhibits the production of TNF-α and other proinflammatory mediators (35, 36). Therefore, it will be important to determine whether the exposure on the surface of apoptotic cells of PS-OX vs nonoxidized PS differentially modulates these functional responses in macrophages. Finally, failure to clear apoptotic debris may be linked to the production of autoantibodies (37, 38). Thus, manipulation of PS exposure and of the macrophage receptor(s) involved in the recognition of PS-OX and nonoxidized PS on the surface of cell corpses may ultimately yield new strategies for the treatment of autoimmune diseases.

References