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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.

Apoptosis is a program of selective deletion of unwanted cells whose survival would prejudice that of the organism as a whole (1, 2). A prominent feature of this well-choreographed mode of cellular demise is the recognition and phagocytosis of apoptotic cells by macrophages or nonprofessional phagocytes. Clearance of cell corpses precludes release of noxious cellular contents, thus allowing for extensive deletion of cells without eliciting tissue disruption or inflammation (3). A common recognition signal for phagocytes is the loss of phospholipid asymmetry and attendant externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane of apoptotic cells (4). A number of macrophage receptors have been suggested to be involved in the recognition of apoptotic cells, and several of these molecules, including the scavenger receptors CD36 and CD68 and a recently characterized molecule termed the PS receptor, are known to bind anionic phospholipids such as PS (5, 6). PS exposure is thought to depend on the concomitant activation of a plasma membrane phospholipid scramblase and inhibition of an ATP-dependent aminophospholipid translocase (7, 8). Moreover, apoptotic PS exposure is known to occur downstream of caspase activation in some cell types (9, 10), although examples of caspase-independent externalization of PS have also been documented (11, 12). Nevertheless, the signaling events resulting in the selective egress of PS and its subsequent recognition by macrophages remain poorly characterized.

Apoptosis is frequently accompanied by the generation of reactive oxygen species (ROS), resulting in part from cytochrome c departure from mitochondria and attendant disruption of electron transport with enhanced generation of one-electron-reduced species of molecular oxygen within the cell (13). ROS represent attractive candidates for final common mediators of apoptosis, yet a specific role for ROS in the execution or resolution of the apoptotic program has not been established. We have recently demonstrated that oxidant-induced apoptosis yields rapid oxidation of different classes of phospholipids with substantial oxidation of PS (14). This oxidation of PS precedes its externalization during apoptosis (15).
and was blocked by the antiapoptotic protein Bcl-2 (16), thus im-
plying 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

Agonistic anti-Fas Ab (clone CH-11) was obtained from Medical & Bio-
logical Laboratories (Nagoya, Japan). The fluorogenic peptide substrate
aspartate-glutamate-valine-aspartate (DEVD)-7-amino-4-methyl-coumarin
(AMC) and the broad range caspase inhibitor benzoxycarbonyl-alanine-
asparginyl-aspartyl-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-dihydriodizidol[1,2]-pyrazin-3-one, hydrochloride (MCLA),
diethyriodihydroethidium (DHE), and the Amplex Red hydrogen peroxide assay kit
were from Molecular Probes (Eugene, OR). HPLC grade solvents were
obtained from Nycomed, Oslo, Norway. ATP and the agonistic anti-Fas Ab
(clone CH-11) were obtained from Medical & Biological Laboratories
(Nagoya, Japan). The phospholipid analyses representing the amount of
phosphatidylethanolamine (PE) and phosphatidylserine (PS) in both
phospholipid classes were determined to underivatized aminophospholipid based on spectrophotometrical
integrity and thus were permeable to propidium iodide, were gated out
before analysis.

Fluorescamine labeling of externalized PS and phosphatidylethanolamine (PE)

Labeling of cells with fluorescamine was performed essentially as previ-
ously described (15). Briefly, cells (4 × 10^7) were suspended in 2 ml of
fluorescamine labeling buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl_2,
1 mM CaCl_2, 5 mM NaHCO_3, 5 mM glucose, and 20 mM HEPES (pH 8)).
Fluorescamine was dissolved in DMSO and added to cells (200 μM final
concentration) and the mixture was gently shaken for 15 s at room tem-
perature. The final DMSO concentration was 0.05%. A total of 5 ml of 40
mM Tris-HCl (pH 7.4) was then added. Cells were incubated for 10 min
and resuspended in 40 mM Tris-HCl (pH 7.4) followed by
lipid extraction in the dark. Lipids were analyzed by two-dimensional
high performance thin-layer chromatography (HPTLC) using a solvent
system of chloroform:methanol:28% ammonium hydroxide (65:35:5, v/v/v)
in the first direction and chloroform:acetone:methanol:glacial acetic acid:wa-
ter (50:20:10:5:3, v/v/v/v/v) in the second. The location of each of the
phospholipids was confirmed by comparison to authentic standards (Avanti
Polar Lipids, Alabaster, AL). Results are expressed as the ratio of derivat-
ized to undertivarized 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 × 10^6 cells/ml.
cis-PnA-labeled cells were washed and resuspended in a buffer containing
115 mM NaCl, 1 mM MgCl_2, 5 mM NaHPO_4, 10 mM glucose, and 25
mM HEPES (pH 7.4) at 1 × 10^6 cells/ml. Aliquots were taken for phos-
pholipid analyses representing the amount of cis-PnA incorporated imme-
diately after labeling. Cells were then treated with anti-Fas Abs (250 ng/ml)
or NECA (5 mM) for 2 h at 37°C, centrifuged, and lysed in 0.5 ml of ice-cold methanol containing butylated hydroxytoluene (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 indi-
vidual phospholipid class, as determined from parallel HPTLC.

Caspase-3-like enzyme activity

Cleavage of the caspase-3-like enzyme substrate DEVD-AMC was esti-

mated in a fluorometric assay as described previously (18). Briefly, cells
were pelleted and frozen on microtiter plates at 1 × 10^6 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)dim-
ethylammonio]-1-propanesulfonate (pH 7.25)) and 50
mmol/L HEPES, 10% sucrose, 5 mM DTT, and 0.1% 3-
(3-cholamidopropyl)dimeth

Assessment of nuclear apoptotic morphology

Cells (1 × 10^5) were harvested after apoptosis induction, washed, resus-
pended in paraformaldehyde (4% in PBS), and spun down onto glass slides.
Cytospin preparations were air dried, then rehydrated in D_H_2O and
stained with 10 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The
Netherlands) in the dark for 10 min. Slides were rinsed in D_H_2O, dried in
dark, and mounted with coverslips using glyceropreserved (50–50)
water. Apoptotic nuclei were scored under an 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) op-

ting with CellQuest software (BD Biosciences). Cellular debris and necrotic cells were excluded based on forward and side scatter character-
istics. Alternatively, superoxide was measured by MCLA ECL. Jurkat cells

Annexin V staining of externalized PS

PS exposure was determined by flow cytometric detection of annexin V
staining using the protocol outlined in the annexin V-FITC apoptosis de-
tection kit (Oncogene Research Products, Cambridge, MA). Cells (0.5 ×
tenfold) were stained with propidium iodide (100 μg/ml) before analysis
with a FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped
with a 488-nm argon laser. Ten thousand events were collected and ana-
yzed using the Cellquest software (BD Biosciences). Low-fluorescence
derbris and necrotic cells, defined as cells that had lost their membrane

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(1 × 10⁶ 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 CaCl₂, 1 mM MgCl₂, and 30 mM glucose. Anti-Fas Abs were added to cells, and the chemiluminescence signal was continuously recorded for 15 min in the Luminescent Analyser 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 × 10⁷/100 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 (λex/λem) using a Cytofluor Model 2350 fluorescence microplate reader (Millipore, Bedford, MA).

**GSH levels**

Intracellular glutathione (GSH) was measured as previously described (20). Briefly, 0.5 × 10⁶ 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 in 50 mM N-ethylmorpholine (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-r-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-amidinopropane) 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 × 10⁶) were labeled with 50 μM of the fluorescent dye 5(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 10⁶ 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 10⁶ viable or apoptotic cells were added to macrophages in 24-well tissue culture plates. After incubation at 37°C for 1 h (for J774.A.1

**FIGURE 1.** Caspase-dependent PS exposure upon Fas ligation of Jurkat, but not Raji, cells. a, Caspase-3-like enzyme activity, expressed as the number of picomoles of AMC released per minute, in cells treated for 4 h with anti-Fas Abs (250 ng/ml). Data shown are mean ± SEM (n = 4). b, Percentage of apoptotic cells, as determined by assessment of nuclear condensation of Hoechst 33342-labeled cells. Data are expressed as mean ± SEM (n = 3). c, Flow cytometric detection of PS exposure in Jurkat and Raji cells treated as above. The percentage of propidium iodide-negative, annexin V-positive cells in each sample is shown. Data are representative of at least three independent experiments.
macrophages) or 3 h (for THP-1 macrophages or HMDMs), nonphagocy-
tosed cells were washed off with several washes in cold PBS and the re-
ming cells were fixed in 4% paraformaldehyde. Phagocytosis was eval-
uated by counting macrophages in visual light and thereafter counting
macrophage-engulfed cells under UV illumination using an inverted fluo-
rescence 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 incu-
bated for another 30 min at 37 °C together with different amounts of lipo-
somes 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. Phagocy-
tosis 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 signi-
cant 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 ago-
nistic 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 nu-
clear condensation (Fig. 1, a and b). However, when these cells
were subjected to flow cytometric analysis using fluorescently la-
beled annexin V, PS exposure was detected only in Jurkat cells
(Fig. 1c), in line with our previous observations (22). These apo-
ptotic 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 pro-
vide a precise quantitative measurement of the amount of PS on
the cell surface. Furthermore, use of annexin V provides no infor-
mation 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 fluores-
camine; 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-di-
imensional HPTLC as described in Materials and Methods. Data are expressed as mean ± SEM (n = 3). n.d., Not detectable.
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.

![Diagram](https://example.com/diagram.png)

**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).
FAS-INDUCED OXIDATION OF PS

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

<table>
<thead>
<tr>
<th>Additions</th>
<th>n</th>
<th>Phosphatidylinositol</th>
<th>PE</th>
<th>PS</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>100.0 ± 11.0</td>
<td>100.0 ± 14.0</td>
<td>100.0 ± 9.5</td>
<td>100.0 ± 13.0</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>9</td>
<td>92.2 ± 11.0</td>
<td>85.5 ± 6.3</td>
<td>66.3 ± 5.8</td>
<td>75.0 ± 5.8</td>
</tr>
<tr>
<td>zVAD + anti-Fas</td>
<td>6</td>
<td>94.1 ± 4.2</td>
<td>93.9 ± 17.0</td>
<td>116.8 ± 6.6**</td>
<td>102.4 ± 9.3</td>
</tr>
<tr>
<td>SOD + Cat + anti-Fas</td>
<td>5</td>
<td>102.6 ± 15.0</td>
<td>109.8 ± 18.0</td>
<td>102.0 ± 8.3</td>
<td>94.1 ± 15.0</td>
</tr>
</tbody>
</table>

*a cis-PnA-loaded cells (10^6 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.

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

<table>
<thead>
<tr>
<th>Additions</th>
<th>n</th>
<th>Phosphatidylinositol</th>
<th>PE</th>
<th>PS</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>100.0 ± 7.2</td>
<td>100.0 ± 8.6</td>
<td>100.0 ± 9.8</td>
<td>100.0 ± 6.9</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>6</td>
<td>86.0 ± 3.0</td>
<td>106.0 ± 9.4</td>
<td>82.6 ± 5.2</td>
<td>122.6 ± 11.0</td>
</tr>
<tr>
<td>zVAD + anti-Fas</td>
<td>6</td>
<td>88.5 ± 17.0</td>
<td>109.8 ± 9.4</td>
<td>111.5 ± 9.2</td>
<td>128.4 ± 14.0</td>
</tr>
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</table>

*a cis-PnA-loaded cells (10^6 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) 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 a 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).

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 fluorescamine-based assay. The amount of PS accessible to fluorescamine 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).
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 fluorescence 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 fluorescently 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 in 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%), 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).

Macrophage scavenger receptors were originally identified based on their ability to bind chemically modified structures, such as acetylated or oxidized low-density lipoprotein (LDL), but not their unmodified counterparts (30). A common feature of these proteins is their ability to recognize a wide range of structurally unrelated ligands, including oxidized LDL and the anionic phospholipid PS, and this lack of specificity is consistent with the idea

**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 receptor 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 HMDDMs. Future studies should address whether HMDDMs 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 effete 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 phagocytic 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-butyldihydroperoxide-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 chemotherapy (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


