Appetizing rancidity of apoptotic cells for macrophages: oxidation, externalization, and recognition of phosphatidylserine

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1Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15260; 2Health Effects Laboratory Division, Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; and 3Division of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, 17177 Stockholm, Sweden

Kagan, V. E., G. G. Borisenko, B. F. Serinkan, Y. Y. Tyurina, V. A. Tyurin, J. Jiang, S. X. Liu, A. A. Shvedova, J. P. Fabisiak, W. Uthaisang, and B. Fadeel. Appetizing rancidity of apoptotic cells for macrophages: oxidation, externalization, and recognition of phosphatidylserine. Am J Physiol Lung Cell Mol Physiol 285: L1–L17, 2003; 10.1152/ajplung.00365.2002.—Programmed cell death (apoptosis) functions as a mechanism to eliminate unwanted or irreparably damaged cells ultimately leading to their orderly phagocytosis in the absence of calamitous inflammatory responses. Recent studies have demonstrated that the generation of free radical intermediates and subsequent oxidative stress are implicated as part of the apoptotic execution process. Oxidative stress may simply be an unavoidable yet trivial byproduct of the apoptotic machinery; alternatively, intermediates or products of oxidative stress may act as essential signals for the execution of the apoptotic program. This review is focused on the specific role of oxidative stress in apoptotic signaling, which is realized via phosphatidylserine-dependent pathways leading to recognition of apoptotic cells and their effective clearance. In particular, the mechanisms involved in selective phosphatidylserine oxidation in the plasma membrane during apoptosis and its association with disturbances of phospholipid asymmetry leading to phosphatidylserine externalization and recognition by macrophage receptors are at the center of our discussion. The putative importance of this oxidative phosphatidylserine signaling in lung physiology and disease are also discussed.

phosphatidylserine oxidation and externalization; apoptosis; phagocytosis; cytochrome c; macrophage receptor

APOPTOSIS AND PHAGOCYTOSIS OF CELL CORPSES IN THE LUNG: ROLE OF THE FAS SYSTEM

Apoptosis arises from the active initiation and propagation of a series of highly orchestrated specific biochemical events leading to the demise of the cell (64). Functional consequences of this process are the elimination of specific cells within a population when they are damaged or no longer required for function. For example, apoptosis is an important determinant for tissue morphogenesis during development (32) and also plays a role in the elimination of immune effector cells during lymphocyte selection (20) and the resolution of inflammation and fibrosis (132). Failure of efficient apoptosis can allow the progression of neoplastic disease, permitting the persistence and multiplication of cells suffering significant genotoxic damage. On the other hand, extensive cell loss via apoptosis with concomitant organ dysfunction can arise from a variety of tissue insults including oxidative stress as documented in brain (98), liver (110), and other organs (for review see Refs. 125, 126).

In the lung, as in other tissues, apoptosis is of paramount importance as a regulator of cell homeostasis and also serves as a potential mediator of tissue injury (46). A number of lung diseases, such as idiopathic pulmonary fibrosis (85, 151), acute respiratory distress syndrome (ARDS) (62), chronic obstructive lung disease (137), and bacterial pneumonia (78), are characterized by extensive apoptosis within the alveolar epithelium. In addition, multiple pneumotoxins such as silica (18, 88), bleomycin (52), asbestos (1), and paraquat (13) can induce apoptosis of various cells. Apoptosis plays a fundamental role in the distal airway remodeling during the transition from the canalicular to saccular stages during lung organogenesis (82).
development of an effective alveolar-capillary interface requires extensive cell remodeling and is documented by extensive apoptosis of both epithelial and interstitial cells in both the gestational (133) and postnatal (134) periods. Although much work has focused on the mechanisms for initiation of apoptosis, little information exists regarding the downstream events involved in signaling mechanisms that mark apoptotic cells with “eat-me” signals to govern the fate of these cell corpses or how the lung responds to them.

The Fas/Fas ligand (FasL) system has been implicated in apoptosis within the lung. It appears that many cells within the lung, including the alveolar epithelium, constitutively express Fas (45, 89). Activation of Fas by intranasal administration of an agonistic antibody produced marked alveolar type II epithelial cell apoptosis and pulmonary inflammation in normal but not in Fas-deficient (lpr) mice (99). In addition, soluble FasL was found in a bioactive form in the bronchoalveolar lavage fluid in patients during the development of acute respiratory distress syndrome (ARDS) (100). At least one source of this FasL has been identified as neutrophils (138), whose accumulation within the air spaces represents a hallmark of ARDS. Similarly, FasL was found in bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis but not controls (86). Pulmonary fibrosis can also be mimicked in mice by administration of FasL (53). Importantly, bleomycin-induced lung injury, a well-established model of fibrosis, was also attenuated in lpr and gld mice, which are deficient in Fas and FasL, respectively (84). Lastly, the periods of lung organogenesis characterized by high levels of apoptosis are also marked by increases in tissue expression of FasL (23), an observation that strongly suggests that the Fas/FasL system serves to regulate developmental lung remodeling as well as tissue injury. Thus apoptosis within the lung contributes to both acute and chronic lung injury, as well as lung fetal and neonatal development.

**SELECTIVE PHOSPHOLIPID PEROXIDATION DURING OXIDANT- AND NONOXIDANT-INDUCED APOPTOSIS**

Oxidative stress is one of the most common triggers of apoptosis. In addition, apoptosis is frequently accompanied by the generation of reactive oxygen species (ROS), resulting in part from cytochrome c (cyt c) departure from mitochondria and concomitant disruption of electron transport with enhanced generation of one-electron-reduced species of molecular oxygen within the cell (10, 11, 15, 34, 36, 67, 75, 112, 121, 130). 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. Although effects of oxidative stress on the apoptotic machinery, including the caspases (28, 56), and mitochondrial proteins forming the permeability transition pore (14, 21, 81) have been described, information on peroxidation of phospholipids and in particular on selective oxidation of their specific classes is scarce. This is mainly due to the fact that quantitative assays for oxidation of different classes of phospholipids are not readily available, which, in turn, is due in large part to a highly effective system of remodeling and repair of oxidatively modified phospholipids (106) that interferes with their accurate measurement.

To characterize phospholipid oxidation during oxidative stress-induced apoptosis, we have developed a protocol based on metabolic labeling of cellular phospholipids with a natural oxidation-sensitive and highly fluorescent fatty acid, cis-parinaric acid (PnA). This reagent has been extensively used in its free (nonesterified) form for structural measurements in membranes as well as in assays of oxidative stress in simple model systems (63, 87). Metabolic labeling yields cells containing the major phospholipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol, diphasphatidyglycerol, and sphingomyelin (SPH)] fluorescently labeled with PnA and extremely low intracellular concentrations of free PnA (124). Because free PnA is not available for phospholipid repair, resolution of major phospholipid classes by fluorescence HPLC can be used to quantify their oxidative damage (as a decreased content of fluorescent PnA residues in respective phospholipid classes). The level of PnA labeling of endogenous phospholipids (~1–3mol%) is low enough to have minimal effects on cell viability and functions yet sufficient to permit quantitative detection of oxidative stress (124). Importantly, the PnA-based assay can identify the selectivity of phospholipid oxidation on the basis of their polar head groups, and it is obviously independent of the fatty acid composition of phospholipids (30, 124).

Using metabolic labeling of phospholipids with PnA, we were able to establish that different phospholipids undergo nonrandom peroxidation during oxidant-induced apoptosis in a number of different cell lines (Table 1). In particular, preferential oxidation of PS was typical of apoptosis induced by a number of oxidants, such as organic hydroperoxides, paraquat, and azo-initiators of peroxyl radicals. Most notably, there was a strong correlation between PS peroxidation and its externalization during apoptosis (see below). We observed that in all cases when enhanced PS peroxidation occurred, PS externalization took place as well and vice versa; the lack or inhibition of preferential PS peroxidation during apoptosis was accompanied by the lack of PS externalization. The fundamental association of PS oxidation with apoptosis was strengthened by experiments in which we used a vitamin E homolog, 2,2,5,7,8-pentamethyl-6-hydroxy-chromane (PMC). Here, we employed the lipophilic azo-initiator of radicals 2,2'-azobis(2,4-dimethylsovaleronitrile) (AMVN) to generate membrane-confined oxidative stress and induce apoptosis in HL-60 cells (36). As an effective radical scavenger, PMC was able to completely protect all phospholipids against oxidation with the remarkable exception of PS. Furthermore,
The temporal sequence of PS oxidation and externalization on the cell surface is compatible with a causal link between these two events. Indeed, if this is the case, PS oxidation should occur within the plasma membrane where PS translocation events during apoptosis are known to occur. To address this issue, we performed subcellular fractionation experiments in PnA-labeled cells challenged with tert-butyl hydroperoxide (t-BuOOH). We documented that t-BuOOH-induced apoptosis and prominent PS oxidation in cells and different organelles. Most importantly, the highest rate of PS oxidation was detected in the plasma membrane compared with other organelles such as mitochondria, nuclei, lysosomes, and microsomes (Fig. 1). The causative link between PS oxidation and apoptosis was further supported by our experiments with DMSO-differentiated HL-60 cells showing inducible NADPH oxidase activity (4). Activation of the NADPH oxidase by phorbol 12-myristate 13-acetate (PMA) or NADPH oxidase activity (4). Activation of the NADPH oxidase by phorbol 12-myristate 13-acetate (PMA) or tert-BuOOH in HL-60 cells. PnA-labeled HL-60 cells (2 × 10⁶) were incubated in the presence of tert-BuOOH (150 μM) for 20 min at 37°C. Lipid oxidation was terminated by addition of 10 μM butylated hydroxytoluene (BHT), and cells were incubated an additional 40 min. Lipids were extracted and resolved by HPLC. The rate of tert-BuOOH-induced oxidation of PnA-PS was calculated as the difference between the specific PnA content of PS in t-BuOOH-treated cells and that in control cells divided by 20 min. PM, plasma membranes; MS, microsomes; LS, lysosomes; N, nuclei; MT, mitochondria. *P < 0.05 vs. MS, LS, N, or MT, n = 6.

Fig. 1. Rate of oxidation of cis-parinaric acid (PnA)-labeled phosphatidylinerse (PS) during apoptosis induced by tert-butyl hydroperoxide (t-BuOOH) in HL-60 cells. PnA-labeled HL-60 cells (2 × 10⁶) were incubated in the presence of t-BuOOH (150 μM) for 20 min at 37°C. Lipid oxidation was terminated by addition of 10 μM butylated hydroxytoluene (BHT), and cells were incubated an additional 40 min. Lipids were extracted and resolved by HPLC. The rate of t-BuOOH-induced oxidation of PnA-PS was calculated as the difference between the specific PnA content of PS in t-BuOOH treated cells and that in control cells divided by 20 min. PM, plasma membranes; MS, microsomes; LS, lysosomes; N, nuclei; MT, mitochondria. *P < 0.05 vs. MS, LS, N, or MT, n = 6.

Table 1. Oxidation and externalization of PS during apoptosis in various cells

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Nonoxidant-induced apoptosis

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HL-60, human leukemia cell line; dHL-60, human leukemia cells differentiated by dimethylsulfoxide; NHEK, normal human epidermal keratinocytes; PC12, rat pheochromocytoma cells; A549, human pulmonary adenocarcinoma cell line; NCI-H226, squamous nonsmall cell lung cancer cell line; 32D, murine hematopoietic progenitor cells; Jurkat, Jurkat T lymphocytes; AMVN, 2,2′-azobis(2,4-dimethylisovaleronitrile); NCS, neocarzinostatin. PS, phosphatidylinerse; PMC, 2,2,5,7,8-pentamethyl-6-hydroxy-chromane; NO·, nitric oxide; zVAD-fmk, Z-Val-Ala-Asp-fluoromethylketone. The above data indicate that PS oxidation may be largely associated with the execution of apoptotic program. This association, however, is obscured during oxidant-induced apoptosis due to high background nonspecfic oxidation. Therefore, we have used several models of nonoxidant-induced apoptosis in which we...
studied phospholipid peroxidation and revealed that PS was again selectively oxidized compared with other more abundant phospholipids (Table 1). In particular, decreased levels of Bcl-2 protein expression achieved via manipulating the level of \( bcl-2 \) mRNA translation in the squamous nonsmall cell lung cancer line NCI-H226 by use of a synthetic antisense-\( bcl-2 \) oligonucleotide resulted in selective oxidation of PS in the subpopulation of cells with externalized PS. No significant differences in oxidation of \( \text{cis-PnA-labeled PE \ and \ PC} \) in cells were found after treatment with nonsense or antisense-\( bcl-2 \) oligonucleotides (80). Similarly, by exposing HL-60 cells to staurosporine, a protein kinase inhibitor without direct prooxidant activity, we were able to induce apoptosis in HL-60 cells without triggering confounding nonspecific oxidation reactions. PS underwent preferential oxidation at an early stage of apoptosis, whereas the most abundant phospholipid, PC, and GSH, the most abundant cytosolic thiol, remained unoxidized (97). Finally, Fas triggering of Jurkat T lymphocytes resulted in oxidative stress with specific PS oxidation and externalization, whereas Raji cells, which are defective for PS exposure, did not undergo PS oxidation (74) (Fig. 2). Expectedly, anti-Fas triggering of PS oxidation/externalization was accompanied by phagocytosis of apoptotic Jurkat cells (but not Raji cells) by J774A.1 macrophages (Fig. 3). Because several recent reports suggest that Fas/FasL are implicated in apoptosis within the lung (discussed above), we further utilized the human pulmonary adenocarcinoma A549 cell line as a convenient model for alveolar epithelial cells to evaluate the effect of Fas-triggered apoptosis on membrane phospholipid oxidation. We were able to demonstrate that induction of apoptosis by Fas triggering was accompanied by phagocytosis of target cells by J774A.1 macrophages.

Fig. 2. Cell type-specific externalization of PS. Jurkat cells (A) and Raji cells (B) were triggered to undergo apoptosis in response to Fas ligation for 4 h and subsequently costained with Hoechst 33258 (blue) and annexin V (green) for visualization of nuclear condensation and PS exposure, respectively.

Fig. 3. Typical fluorescence photomicrographs showing phagocytosis of target cells by J774A.1 macrophages. A, B: phagocytosis of Jurkat cells (A, control; B, apoptotic cells after stimulation with 250 ng/ml of anti-Fas agonistic antibody, 2 h). C, D: nonapoptotic HL-60 cells [C, control; D, after enrichment with nonoxidated PS (PS/oxidated PS (PSOx) liposomes). Target cells were fluorescently labeled with Cell Tracker Orange (red); macrophages were fluorescently labeled with Hoechst 33342 (blue).
of apoptosis in interferon-\(\gamma\)-pretreated A549 cells by anti-Fas monoclonal antibody caused specific PS oxidation along with its externalization (Table 1).

In sum, three important features of PS oxidation during apoptosis have been established during the course of our studies. First, the preferential oxidation of PS is observed only in intact living cells undergoing apoptosis and not in cell-free liposome preparations incubated with oxidants. Second, PS oxidation occurs early during execution of the apoptotic program and precedes the appearance of such hallmarks of apoptosis as DNA fragmentation and, most importantly, PS externalization. Finally, PS peroxidation is blocked in cells overexpressing antiapoptotic gene products such as Bcl-2 and is sensitive to pancaspase inhibitors (4, 33, 34, 74, 77, 92, 142, 158). Overall, our findings clearly indicate that execution of the apoptotic program involves initiation of oxidative stress that targets specific phospholipids in the plasma membrane including PS. These observations raise two important questions: 1) What are the catalytic mechanisms involved in PS oxidation during apoptosis? and 2) What are the consequences of PS oxidation for the execution of apoptosis?

MECHANISM OF PS OXIDATION DURING APOPTOSIS: EVIDENCE FOR A ROLE OF CYT C

Selective PS oxidation suggests that there may be specific catalytic mechanism(s) responsible for the predominant oxidation of this particular class of phospholipids (Fig. 4). One possible mechanism relies on the involvement of cyt c released from mitochondria into the cytosol during apoptosis. Cyt c release, an early and common marker of apoptosis, is involved in the formation of the so-called apoptosome complex and the subsequent activation of caspases downstream of mitochondria (70). This proapoptotic role of cyt c seems to be redox independent and does not require the presence of the heme moiety (59, 79). However, a signifi-
cant part of cyt c is not released as an apoprotein but rather contains heme with its redox-active iron (140, 141). Moreover, as cyt c is a basic protein (pI 10.3) that is positively charged at neutral pH (90), it may be predisposed to interact electrostatically with negatively charged phospholipids such as PS (103, 149). In fact, it has been demonstrated that cyt c effectively binds to negatively charged PS-containing membranes (22). Electrostatic interaction of cyt c with negatively charged sites of membranes induces disruption of the Met80-Fe(heme) coordination bond and partial unfolding of the protein globule, thus facilitating orientation of the heme moiety along the membrane surface (113). Disturbance of Met80-Fe(heme) coordination renders Fe more catalytically redox active, while unfolding of the protein positions its heme catalytic site closer to phospholipid targets. Oxidized cyt c is less stable, and the energy of its unfolding is lower than for the reduced form (109), suggesting that PS in the inner leaflet of the plasma membrane may preferentially interact with the oxidized form of cyt c. Importantly, departure of cyt c from mitochondria is accompanied by a massive production of H2O2, which may promote oxidation of cyt c heme (113). Thus one may speculate that, once released from mitochondria, cyt c becomes oxidized, binds to PS-rich lipid rafts on the inner surface of plasma membrane, and unfolds to expose its redox-active heme-iron moiety to phospholipids, particularly to PS, the electrostatically most attractive phospholipid species. As a result, PS may be more susceptible to cyt c-catalyzed oxidation than other phospholipids during apoptosis (Fig. 5).

There are several experimental findings that are compatible with the model outlined above. In one series of experiments, we gently sonicated HL-60 cells in the presence of excess amounts of cyt c (200 μM), resulting in the integration of cyt c into the cells. We found that incorporation of cyt c into PnA-labeled HL-60 cells (in the presence of exogenously added t-BuOOH) resulted in preferential oxidation of PS compared with other phospholipids (Fig. 6) (77). Furthermore, in cell-free model systems, PS proved to be se-

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Fig. 5. Cyt c as a catalyst for selective PS oxidation during apoptosis: release of cyt c from mitochondria into the cytosol is one of the early and common events in apoptosis. In the cytosol, cyt c participates in the activation of the caspase cascade. As a basic protein (pI 10.3), it is positively charged at neutral pH and can electrostatically interact with negatively charged phospholipids such as PS. As a result, redox (prooxidant) catalytic activity of heme-containing cyt c may be directed toward selective PS oxidation. Production of superoxide and H2O2 by disrupted mitochondrial electron transport facilitates formation of reactive oxidants such as oxo-ferryl species of heme-containing cyt c in the immediate vicinity of PS thus providing for PS oxidation. PSox can be externalized and enhance externalization of PS. Molecular model of cyt c (1A7K) was obtained using Cn3D software from the National Center for Biotechnology and Information.
Phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), or phosphatidylcholine (PC) or phosphatidylylethanolamine (PE).

Selective oxidation by cyt c/ascorbate/H$_2$O$_2$-catalytic system compared with PC (73). In another series of experiments, we used a different approach to disrupt mitochondria and release cyt c into cytosol, utilizing the DP-1 peptide. DP-1 is composed of two functional domains: a protein transduction domain, PTD-5 (a 12-mer peptide sequence RRQRRSTKLMKR from M13 phage library), and an antibiotic peptide, KLA ([KLAKLAK]$_2$). PTD-5 is used to guide KLA to target cells and allow for its internalization. PTD-5 is positively charged due to a high content of arginine and lysine residues (~70%) and causes efficient and rapid internalization of conjugated proteins into cells in vitro and in vivo (102). KLA, an antimicrobial peptide, is designed to target and disrupt bacterial cells, as well as mitochondria in eukaryotic cells (31, 69). Therefore, the PTD-5/KLA conjugate (DP-1) is able to preferentially disrupt mitochondria and induce release of cyt c but spare damage to the plasma membrane and other organelles in cells. Incubation of Jurkat cells with DP-1 peptide caused a rapid (within 5 min) release of cyt c from mitochondria into cytosol. Importantly, cyt c released from mitochondria could be completely recovered in the cytosolic fraction. Furthermore, we found that DP-1-induced cyt c release was accompanied by a more than fourfold increase in H$_2$O$_2$ production by Jurkat cells. Next, using the PNA-based assay, we showed that PS was the only phospholipid that was significantly oxidized after incubation of Jurkat cells with 10 µM DP-1 (J. Jiang, B. F. Serinkan, Z. Mi, P. D. Robbins, and V. E. Kagan, unpublished observations). DP-1-induced PS oxidation was accompanied by significant externalization of PS in Jurkat cells and enhanced recognition and phagocytosis of these cells by J774A.1 macrophages. Together, these data suggest that release of cyt c may be involved in PS oxidation. The possibility that this site-specific oxidative stress and selective PS oxidation can play a signaling role in the resolution of apoptosis, through PS-dependent pathways such as PS externalization, recognition of apoptotic cells by specialized macrophage receptors, and subsequent phagocytosis, is discussed below.

**PS ASYMMETRY AND EXTERNALIZATION (RAFTING) DURING APOPTOSIS: THRESHOLD PHENOMENA AND ROLE IN MACROPHAGE CLEARANCE**

The plasma membrane, the barrier between intracellular and extracellular milieus, plays a pivotal role in the communication of cells with their environment. The majority of aminophospholipids (PE and PS) are predominantly confined to the inner leaflet of the plasma membrane, whereas choline-containing phospholipids (PC and SPH) are localized mainly in the outer leaflet (5, 25, 93, 120, 167). This phospholipid asymmetry is maintained by an ATP-dependent aminophospholipid translocase that specifically transports PS and PE from the outer to the inner leaflet (for review see Ref. 6). Aminophospholipid translocation is Ca$^{2+}$-inhibitable and sensitive to the sulfhydryl-reactive agent N-ethylmaleimide (NEM), as well as to vanadate, an inhibitor of P-type ATPases. The molecular identity of this “flippase” is not firmly established, although a candidate P-type ATPase termed ATPase II that possesses phospholipid transporting properties has been identified (29, 54, 105, 108, 143, 146).

Loss of membrane phospholipid asymmetry with subsequent externalization of PS is known to be an important signaling mechanism, e.g., to stimulate the coagulation cascade during platelet activation and to mediate cell recognition by macrophages (131). This process is believed to be mediated by a Ca$^{2+}$-activated phospholipid scramblase that facilitates the bidirectional movement of all classes of phospholipids across the the lipid bilayer (6). Zhou and coworkers (161, 163) identified a 37-kDa protein in platelets with scramblase activity and have demonstrated a correlation between the expression of this scramblase and the Ca$^{2+}$-ionophore-induced externalization of PS. However, this protein is normal in Scott syndrome patients whose blood cells are defective for scramblase activity (38, 47). PS externalization is considered a general phenomenon in cells undergoing apoptosis (43, 95), although a few instances of apoptosis in the absence of PS exposure have also been described (38, 47). PS exposure is dependent on extracellular Ca$^{2+}$ (7, 57, 96) and occurs downstream of the activation of caspases (37, 94, 155). In addition, previous studies have indicated that PS exposure during apoptosis is dependent on the concomitant inhibition of the
Interestingly, clustering of ced-1, a putative PSR in Caenorhabditis elegans, is seen in response to cell corpse recognition in the nematode system (165). Although it is not known whether PS oxidation is involved in PS aggregation and formation of rafts in the plasma membrane during apoptosis, it is noteworthy that aggregation of phospholipid hydroperoxides resulting in the formation of their clusters has been documented (72). Dillon et al. (27) have shown that annexin V colocalizes with markers of lipid rafts in the outer membrane of activated, nonapoptotic B cells, suggesting that PS exposure can occur in specific membrane microdomains in the absence of cell death. Moreover, mature B cells expose PS on their surface, where it colocalizes with antigen receptors and forms caps that are required for receptor-mediated signaling events that trigger Ig production (27). Similarly, transient, nonapoptotic exposure of PS is seen during development of skeletal and heart muscle and has been suggested to be essential for myotube formation in mice (153). Finally, normal macrophages themselves were reported to display PS on their surface, and this was suggested to be required for phagocytosis of PS-expressing target cells (12). These observations beg the question of how cells that express the common PS-dependent eat-me signal can escape macrophage recognition. One may speculate that the functional outcome of PS externalization may ultimately depend on the density of PS on the cell surface. A low or intermediate level of PS or transient exposure of PS may not suffice to trigger clearance, whereas more extensive externalization of PS during apoptosis may reach a threshold necessary for phagocytosis to occur (see below). Alternatively, viable nonapoptotic PS-positive cells may fail to express additional necessary cofactors, such as chemotactic mediators or accessory surface ligands (such as oxidized PS), required for the stimulation of macrophages.

Evaluations of amounts of externalized PS in nonapoptotic and apoptotic cells. Because apoptosis is accompanied by PS oxidation in the plasma membrane, two different populations of PS, nonoxidized PS (PS) and oxidized PS (PSox), are likely to be present in the outer membrane leaflet of apoptotic cells. These two PS species may behave differently with regard to their topography in the membrane as well as their effects on enzymatic and nonenzymatic pathways involved in the maintenance of PS asymmetry across the plasma membrane. PSox may act as a poison or “suicide substrate” for the aminophospholipid translocase, thus inhibiting translocation of both PSox and PS. This would result in the appearance of both PS and PSox on the surface of the plasma membrane. Alternatively, PSox could be preferentially externalized during apoptosis as a result of poor substrate recognition by the translocase and/or a high rate of spontaneous (nonenzymatic) transbilayer flip-flop. Methods for the quantitative assessment of PS content on the surface of apoptotic cells are clearly needed to determine the levels of native PS, PSox, and potentially other molecular species of PS and the mechanism of their redistribution within the plasma membrane during apoptosis. Although annexin...
V-based assays for PS externalization have been extensively used in numerous studies, the results are usually interpreted in terms of distribution of cell populations with an arbitrary (above threshold) amount of externalized PS rather than the absolute amount (concentrations) of PS expressed on the cell surface. Unfortunately, quantification of PS on apoptotic cells has not received significant attention. Indeed, since the pioneering work by Fadok and colleagues (43), who discovered PS externalization in apoptotic murine thymocytes using chemical derivatization with fluorescamine, a nonpermeable reagent for primary amines, there are only few examples of PS quantitative measurements (e.g., in the human leukemia T cell line Jurkat and the human leukemia HL-60 cell line) (Table 2). However, neither the fluorescamine-based assay nor the annexin V-based flow cytometric assay permits the quantitative estimation of amounts of PS on the surface of nonapoptotic cells. Using a modification of the annexin V method, the annexin V iron-beads/electron paramagnetic resonance assay, we were recently able to quantify PS on the surface of Jurkat cells and HL-60 cells (Fig. 7, Table 2). We found that apoptotic HL-60 cells and Jurkat cells externalized up to 25–250-fold more PS than nonapoptotic controls. This suggests that PS is a prominent signal for clearance of apoptotic cells by macrophages (G. G. Borisenko, T. Matsura, S.-X. Liu, V. A. Tyurin, J. Jiang, B. F. Serinkan, and V. E. Kagan, unpublished observations).

The importance of PS on the cell surface for recognition and phagocytosis by macrophages was first demonstrated by Schroit and colleagues (136, 145) in the early 1980s in experiments using red blood cells with artificially manipulated levels of exogenous PS. These investigators hypothesized that recognition of PS-exposing cells by macrophages involves specific ligand-receptor interactions (136). Further studies demonstrated that activated monocytes are able to bind different tumor cell lines with elevated levels of PS, but not normal human keratinocytes cells with relatively low PS content (152). These results imply that a threshold of PS expression may exist for macrophage recognition of PS-externalizing cells (152). To further explore this hypothesis, we enriched the external leaflet of the plasma membrane of nonapoptotic Jurkat cells and HL-60 cells with known amounts of exogenous PS and determined the sensitivity of macrophages for PS on the surface of the target cells (G. G. Borisenko, T. Matsura, S.-X. Liu, V. A. Tyurin, J.

### Table 2. PS expression on the surface of naïve and apoptotic cells

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>HL-60 Cells</th>
<th>Jurkat Cells</th>
<th>Annexin V-iron bead EPR assay</th>
<th>Fluorescamine-based assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-FAS antibody</td>
<td>N/A</td>
<td>239 ± 18</td>
<td>0.5 μg/ml, 4 h</td>
<td></td>
</tr>
<tr>
<td>Staurosporine</td>
<td>27 ± 13</td>
<td>N/A</td>
<td>1 μM, 4 h</td>
<td></td>
</tr>
<tr>
<td>Camptothecin</td>
<td>22 ± 18</td>
<td>237 ± 62</td>
<td>10 μM, 4 h for HL-60 cell, 50 μM, 4 h for Jurkat cell</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.1 ± 2.4</td>
<td>2.2 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMVN</td>
<td>129.1 ± 21.5</td>
<td>243 ± 27</td>
<td>500 μM, 2 h</td>
<td></td>
</tr>
<tr>
<td>Anti-FAS antibody</td>
<td></td>
<td></td>
<td>0.25 μg/ml, 2 h</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. PS externalization was calculated per total number of cells in control samples or per number of apoptotic cells after different treatments. N/A, not available.
Jiang, B. F. Serinkan, and V. E. Kagan, unpublished observations). The dependence of the phagocytic capacity of macrophages on the amounts of PS integrated into the outer leaflet of plasma membrane in Jurkat cells or HL-60 cells unequivocally demonstrated a nonlinear response with a sensitivity threshold required to initiate macrophage recognition of externalized PS. Hence, at least a 5–10-fold increase of the PS content in the outer leaflet of plasma membrane was required to trigger macrophage recognition and uptake. During apoptosis, e.g., induced by the chemotherapeutic agent camptothecin, both cell lines expressed externalized PS in amounts far in excess of the recognition threshold and were thus effectively phagocytosed.

MACROPHAGE RECOGNITION OF PS VS. PSox: OXIDIZED PHOSPHOLIPIDS ENHANCE PHAGOCYTOSIS OF APOPTOTIC CELLS

Many receptors have been implicated in the removal of dying, apoptotic cells by macrophages. Not only do different populations of phagocytes use discrete receptors, but a single phagocyte may express a number of receptors that cooperate in the ingestion of their prey (119). These different receptors include the class A scavenger receptor (114, 116), $\alpha_v\beta_3$-integrin (vitronectin receptor) (42, 44), CD68 (16), CD14 (26), lectin-like oxidized low-density lipoprotein receptor, and CD36 (44, 123). An interesting alternative point of view is that CD31 acts as a cell surface molecule that normally prevents phagocyte ingestion of viable cells by transmitting “detachment” signals (9). CD31-mediated detachment is disabled in apoptotic cells by an unknown mechanism, resulting in the promotion of tethering of cell corpses to phagocytes. In addition, serum proteins such as $\beta_2$-glycoprotein I (5, 19) and C1q, the first component of complement (101), bind to apoptotic cells and enhance their uptake. Many of the above receptors can bind PS, but not all of them are specific for this phospholipid (26, 115). Furthermore, activated macrophages were recently reported to secrete a glycoprotein, milk fat globule epidermal growth factor 8, that specifically binds to PS on apoptotic cells and to $\alpha_v\beta_3$-integrin on the macrophage surface, thus serving as a “molecular bridge” between the two cells (60). Regardless of the receptors engaged or disengaged in phagocytosis, ingestion does not occur in the absence of PS exposure (39, 74). Specifically, recognition of surface PS was reported to be dependent on the presence of the so-called PSR, a recently cloned receptor that is expressed by phagocytes and mediates pinocytosis and initiates uptake of apoptotic cells (40, 68, 156). Indeed, the requirement for PS expression and the ensuing ligation of PSR on phagocytes by PS on the apoptotic cell surface may be essential to signal uptake of cells that are tethered to phagocytes via other receptors (144). However, additional signals, such as oxidative changes, may also be required for the engulfment of target cells (129). In fact, many of the receptors implicated in phagocytosis of apoptotic cells can strongly bind oxidized phospholipids (48, 129), which arise during apoptosis and provide additional ligands for recognition receptors (74, 150). Oxidized epitopes on the surface of apoptotic cells may thus act as important signals for the recognition of target cells by macrophages (16, 128). In particular, C-reactive protein, a component of the innate immune response, was shown to bind to oxidized PC species on the surface of “late” apoptotic (propidium iodide-positive) cells (17). Moreover, Podrez et al. (117, 118) recently described a novel family of oxidized PC homologs that were able to act as a ligands for the scavenger receptor CD36 and promote macrophage foam cell formation.

Because plasma membrane PS may be a specific target for oxidation, it is tempting to speculate that a combination of PS and PSox may be essential for recognition and uptake of apoptotic cells. In support of this notion, inhibition of PS oxidation in cells during apoptosis has been demonstrated to block phagocytosis of Jurkat cells and HL-60 cells by macrophages (4, 74). Moreover, we found that integration of PSox along with PS into plasma membrane of nonapoptotic cells significantly stimulated their phagocytosis compared with the incorporation of similar amounts of native PS alone (Fig. 3) (74). In addition, liposomes containing PSox acted as potent inhibitors of phagocytosis of apoptotic cells (anti-Fas-triggered Jurkat cells and t-BuOOH-treated HL-60 cells) (74). Together, these findings indicate that PSox, indeed, may act as an important signal on the cell surface that can act alone or in combination with native PS to facilitate recognition of apoptotic cells. Nevertheless, many questions remain regarding the role of PSox in the signaling for engulfment. For example, what is the fraction of PSox vs. PS on the cell surface during apoptosis? Can other oxidized phospholipids such as PCox, PEOx, etc. synergistically interact with PS to facilitate recognition of apoptotic cells? What are the actual concentrations of PCox, PEOx, and other oxidized phospholipids compared with PSox on the cell surface during apoptosis? Interestingly, our preliminary experiments indicate that PSox is capable of markedly reducing the threshold for recognition of PS-containing cells by macrophages. Another important question relates to the type of receptors involved in recognition of PS and PSox. It seems likely that recognition of PS and PSox involves different receptor(s). Indeed, we found that anti-PSR antibody, but not anti-CD-36 antibody, was able to inhibit phagocytosis of Jurkat cells with PS integrated into their plasma membrane. In contrast, both anti-PSR antibody and anti-CD36 antibody were effective in suppressing phagocytosis of Jurkat cells enriched with both PS and PSox (Fig. 8); anti-CD36 antibody was also efficient in suppressing the engulfment of Pas-triggered Jurkat cells, which display both PSox and its nonoxidized counterpart on the cell surface (C. Elenström-Magnusson and B. Fadeel, unpublished observations). These data imply that CD36 and PSR might cooperate to recognize oxidized PS. In conclusion, selective oxidation and externalization of PS in the plasma membrane of apoptotic cells likely creates conditions whereby oxidized PS on the external surface of...
the cell could act as a preferred ligand (or eat-me signal) for certain macrophage receptors, including scavenger receptors such as CD36, resulting in the recognition and disposal of cell corpses.

ANTIOXIDANTS AS REGULATORS OF APOPTOSIS AND PHAGOCYTOSIS OF APOPTOTIC CELLS

The potentially important signaling function of PS oxidation in the plasma membrane and the subsequent externalization of PSox on the cell surface suggest that antioxidants theoretically could play an unusual role in the regulation of apoptosis and phagocytosis of apoptotic cells. Thus if PS oxidation is essential for its externalization and, furthermore, if PSox acts as an additional stimulatory signal facilitating recognition and engulfment of apoptotic cells, then inhibition of PS oxidation by antioxidant enzymes and/or water- and lipid-soluble antioxidants might interfere with the execution of these critical functions and, hence, with the resolution of the apoptotic process. Obviously, total nonspecific prevention of oxidative stress during oxidant-induced apoptosis by antioxidants via blocking the initiation of apoptotic program is a trivial and highly predictable effect that has been documented in a number of reports (50, 111). Therefore, models of nonoxidative apoptosis, where oxidative stress functions only as a specific component of the execution of apoptotic program, are preferable for studies of antioxidant effects. In particular, observations related to PS-specific effects of antioxidants may be of considerable interest. We found that antioxidant enzymes can indeed modulate PS oxidation-dependent signaling pathways in apoptosis. This was demonstrated in experiments with Fas-triggered apoptosis in Jurkatt cells (74). Anti-Fas antibody triggered selective oxidation of PS accompanied by PS externalization, caspase activation, and recognition and phagocytosis of apoptotic cells by several classes of macrophages. Remarkably, high doses of the antioxidant enzymes superoxide dismutase (SOD)/catalase (50 U/ml of each) were able to inhibit oxidative stress (e.g., production of superoxide and H$_2$O$_2$) and block PS oxidation and recognition/phagocytosis of apoptotic target cell by J774A.1 macrophages without affecting other biomarkers of apoptosis, such as PS externalization, caspase-3 activation, and nuclear condensation (74). Hildeman et al. (66) showed that an SOD mimetic, Mn(III) tetrakis (5,10,15,20-benzoic acid) porphyrin, protected T cells after activation through Fas/TNF-α (i.e., death receptor)-independent pathways. This protection, however, was due to decreased mitochondrial damage and subsequent caspase-dependent DNA fragmentation. It seems, therefore, that ROS may be differentially involved in signaling and execution pathways during apoptosis depending on the initial triggering mechanisms.

Not only antioxidant enzymes but also low-molecular-weight chain-breaking antioxidants can affect apoptotic pathways by inhibiting PS oxidation. For example, an effective lipid antioxidant, etoposide (VP-16), at pharmacologically relevant concentrations (50 μM) was able to completely block PS oxidation in HL-60 cells during H$_2$O$_2$-induced apoptosis (Y. Y. Tyurina, B. F. Serinkan, V. A. Tyrin, V. Kini, J. C. Yalowich, B. Padeel, and V. E. Kagan, unpublished observations). Under these conditions, etoposide inhibited PS externalization in HL-60 cells as well as their phagocytosis by J774A.1 macrophages. It is important that effects of antioxidants on PS oxidation and subsequent PS-dependent pathways are studied and determined specifically during apoptosis rather than when both apoptotic and necrotic mechanisms are realized concomitantly. In this regard, Shaecter and colleagues (3, 139) reported that high concentrations of H$_2$O$_2$ inhibited phagocytosis of apoptotic cells upon etoposide treatment, largely due to the shifting of cell death from apoptosis to necrosis. Vitamin E (α-tocopherol) is one of the major natural lipid-soluble chain-breaking antioxidants of membranes. We experimentally determined the effects of vitamin E on Fas-triggered oxidation of PS, apoptosis in Jurkat cells, and their phagocytosis by J774A.1 macrophages. We found that substantial inhibition of Fas-induced phospholipid oxidation could be achieved only at relatively high pharmacological concentrations of vitamin E. Complete inhibition of Fas-induced PS oxidation was observed only when vitamin E levels in cells were >20-fold in excess of those in nonsupplemented cells. At these high doses, however, vitamin E did not affect the outcome of apoptosis as shown by PS externalization and nuclear morphological alterations (B. F. Serinkan, Y. Y. Tyurina, M. Djukic, H. Babu, A. Schroit, and V. E. Kagan, unpublished observations). No changes in the effectiveness of phagocytosis of apoptotic cells occurred in the presence of vitamin E. Notably, physiologically relevant levels of vitamin E were not able to completely block PS oxidation. It should be mentioned, however, that the effects of vitamin E on phagocytosis may be realized through its antioxidant-independent mechanisms of macrophage activation, such as induction of the expression of cell-cell adhesion molecules (24, 127).

Fig. 8. PS- and PSox-induced phagocytosis elicits different sensitivity toward anti-PSR and anti-CD35 antibodies. J774A.1 macrophages were pretreated for 30 min at 37°C with MAb against PSR (11 μg/ml) or MAb against CD36 receptor (100 μg/ml) before addition of target cells. *P < 0.05 vs. control.
Thus physiological levels of antioxidants are not likely to be sufficient to completely block PS oxidation during apoptosis and hence to interfere with PS-dependent pathways of phagocytosis. High pharmacological doses of antioxidants, however, are able to cause inhibition of PS oxidation sufficient to affect PS externalization (etoposide) or phagocytosis (SOD/catalase). Antioxidants are commonly believed to be effective anti-inflammatory agents (122). However, it is important that their use at high doses be considered carefully, as their specific mechanism of action predicts a potential effect as inhibitors of PS-dependent pathways in apoptosis and phagocytosis of apoptotic cells, thus precluding the noninflammatory removal of dying cells (discussed below).

ROLE OF APOPTOTIC CELL CLEARANCE IN LIMITING INFLAMMATORY RESPONSES IN THE LUNG

Neutrophils are an important line of host defense against invading microorganisms, and the production of ROS in these cells is an essential step in the killing of ingested bacteria. The apoptotic death of neutrophils and their subsequent engulfment by macrophages is believed to be a critical component in the resolution of inflammation, as this serves to remove these cells from the inflammatory site with minimal damage to surrounding tissues (132, 159). Conversely, a mismatch between the rate of apoptosis and the rate of phagocytic clearance of apoptotic cells may underlie detrimental inflammatory responses, as shown in, e.g., mice receiving agonistic anti-Fas antibodies (104). These animals die from hepatic failure as a result of massive apoptosis of hepatocytes and fulminant inflammation in the liver, due most likely to “secondary” necrosis of unengulfed cells. The harnessing of apoptotic mechanisms involved in the resolution of inflammation and restitution of tissue homeostasis may thus yield novel therapeutic strategies in conditions of excessive inflammation.

Cystic fibrosis (CF) is an inflammatory disease of the lung characterized by a sustained influx of inflammatory cells into the airways and release of proteases from these cells (8). The fact that inflammation is persistent and that necrotic (or postapoptotic) cells accumulate in the airways of CF patients suggests that the normal mechanism for removal of effete cells is impaired. Indeed, Vandivier et al. (156) recently demonstrated an abundance of unengulfed apoptotic cells in the airways of CF as well as non-CF bronchiectasis patients. These investigators also provided evidence that neutrophil elastase, an intracellular protease that is released by inflammatory cells into the airways during inflammation, cleaves the PSR on the surface of phagocytes, thus contributing to the disruption of apoptotic cell clearance. Consequently, therapies that augment macrophage engulfment of apoptotic cells, for instance by targeting the PS-dependent pathway of cell clearance, may be envisaged for conditions of pulmonary decline due to excessive inflammation.

Another example of chronic inflammation is the rare hereditary disease known as chronic granulomatous disease (CGD), which is characterized by severe and sometimes fatal infections, including fungal or bacterial pneumonia (71). The basic defect arises from an inability of neutrophils to generate superoxide and H$_2$O$_2$ due to mutations in the membrane-bound NADPH oxidase. We have previously shown that neutrophils possess both caspase-dependent and oxidant-dependent modes of PS exposure, which are employed during constitutive apoptosis and activation-induced (nonapoptotic) death, respectively (37, 55). We have yet to determine whether PS is also oxidized in neutrophils undergoing apoptotic vs. nonapoptotic cell death. Nevertheless, our studies of CGD neutrophils demonstrated that although these cells exhibit a normal apoptotic response, with caspase activation and plasma membrane exposure of PS, they fail to externalize PS when incubated with the potent neutrophil activator PMA (37). In addition, our recent studies using DMOS-differentiated, neutrophil-like HL-60 cells as well as neutrophils from healthy donors confirm that both PMA and opsonized zymosan beads, a more physiological stimulator of the NADPH oxidase, are capable of triggering ROS-dependent, DPI-inhibitable PS externalization (4). Concomitant oxidation of PS was also observed in the HL-60 model. These data thus provide evidence for the involvement of NADPH-derived ROS in the oxidation and externalization of PS. Our findings were corroborated in a recent report, in which H$_2$O$_2$-dependent PS externalization and macrophage uptake of neutrophils ingesting Staphylococcus aureus were documented (58). It appears reasonable to speculate that the failure to activate this mode of PS exposure in vivo could result in defective clearance of cells by macrophages and hence contribute to the formation of the characteristic granulomatous lesions and subsequent tissue destruction evidenced in CGD patients. We surmise that the augmentation of PS- and/or PSox-dependent cell clearance may serve as a therapeutic approach in CGD and related granulomatous disorders.

Molecules other than PS and PSox may also contribute to the removal of effete cells during inflammation. For instance, ligation of the cell surface adhesion molecule CD44 is known to promote the uptake of apoptotic neutrophils but not of apoptotic lymphocytes (61). Teder et al. (147) recently reported that mice deficient for CD44 exhibit a >10-fold increase in unengulfed apoptotic cells in the lung tissue after bleomycin treatment compared with wild-type animals. Importantly, lack of CD44 resulted in increased mortality from lung injury due to unremitting inflammation, thus serving to underscore the importance of clearance of apoptotic neutrophils in the resolution of lung inflammation.

In view of the above observations concerning the importance of PS oxidation in the plasma membrane during apoptosis, it seems prudent to reconsider the clinical utilization of antioxidants, believed to act as promising therapeutics in the regulation of inflammatory responses. Thus it will be important to establish regimens and conditions for antioxidant treatments...
that do not affect important PS oxidation signaling events in apoptotic cells and hence interfere with macrophage recognition of such cells. Conversely, one can envision that directed and targeted delivery of PSox to the surface of damaged cells can be used to enhance their safe clearance and may be useful for limiting the inflammatory response in the lung and in other tissues.

CONCLUDING REMARKS

In summary, generation of ROS is an integral component of the apoptotic program and results in the preferential oxidation of PS in the plasma membrane of the dying cell. Oxidized PS, in turn, serves as an important signal through which macrophages recognize and eliminate apoptotic cells. Oxidative stress within the apoptotic cell may promote this clearance process either via enhanced externalization of oxidized PS on the surface of apoptotic cells and/or through more effective recognition of apoptotic cells exhibiting PSox (along with its nonoxidized counterpart) on their surface. Furthermore, we speculate that the mitochondrial release of cyt c during apoptosis is critically involved in the selective oxidation of PS and its subsequent externalization. Regardless of the specific mechanism(s) involved, the final outcome of macrophage recognition of PS and Psox is the nonphlogistic disposal of potentially harmful cells, e.g., at sites of inflammation. In this context, the possibility that antioxidants capable of inhibiting PS oxidation might interfere with PS externalization and/or its recognition by macrophages needs to be carefully considered. Nevertheless, as outlined in the present review, apoptosis-dependent, mitochondrially derived oxidative stress is mechanistically linked with the oxidation of PS and the stimulation of PS-dependent signaling pathways culminating in the disposal of cells by macrophages.

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REFERENCES

23. De Paepe ME, Rubin LP, Jude C, Lesieur-Brooks AM, Mills DR, and Lukas FI. Fas ligand expression coincides with...


