Chapter 5

Phosphatidylserine Peroxidation During Apoptosis

A signaling pathway for phagocyte clearance

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1. INTRODUCTION

Two distinct pathways, apoptosis and necrosis, may be triggered contemporaneously or independently of each other to cause cell death. Necrotic cell death is generally a consequence of severe stress conditions resulting in increase in the cell volume, disruption of plasma membrane, release of intracellular contents and an inflammatory response. Apoptosis, or programmed cell death, is essential for normal tissue development and organogenesis, immunologic selection, response to tissue injury. It is characterized by chromatin condensation, DNA fragmentation, cytochrome c release, caspase activation and cell shrinkage. Our understanding of apoptosis initiation and execution pathways and mechanisms is far from being complete. In the nematode *Caenorhabditis elegans*, genetic studies resulted in the discovery of 15 genes that are essential for the apoptotic program. Some of these genes have been shown to be conserved across a wide range of species. These 15 genes have been divided into four groups based on temporal order of their action during apoptosis. The products of these genes are involved in: a) decision making (*ces-1* and *ces-2*); b) execution (*ced-3*, *ced-4*, *ced-9* and *egl-1*); c) engulfment of apoptotic cells (*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, *ced-12*) and d) degradation

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of apoptotic cells within phagocytes (Wu and Horvitz, 1988 ab; Liu *et al.*, 1999).

Removal of apoptotic, damaged and other unwanted cells from the bloodstream or tissues, is very important for cell and tissue homeostasis. *In vivo*, apoptotic cells are efficiently removed by professional phagocytes, macrophages or neighboring cells acting as semi-professional phagocytes (Terpstra and Berkel, 2000; Chang *et al.*, 2000; Witting *et al.*, 2000). The mechanisms by which phagocytes recognize apoptotic cells are poorly understood. It is clear that the outer leaflet of plasma membrane in apoptotic cells must be different from that of healthy cells. Promotion of "safe" phagocytic clearance of cells undergoing apoptosis requires the appearance of cell surface "eat-me" signals on the apoptotic cells (Savill, 1998, Ren and Savill, 1998, Savill and Fadok, 2000). Some of the "eat-me" signals, such as changes in surface carbohydrate moieties of membrane proteins and exposure of phosphatidylserine (PS) on the surface of apoptotic cells, are well characterized (Dini *et al.*, 1992; Benner *et al.*, 1995; Martin *et al.*, 1995; Adayev *et al.*, 1998, Savill, 1997, 1998, Savill and Fadok, 2000; Witting *et al.*, 2000). Other signals involved in the removal of apoptotic cells are not well characterized. These particular signals execute removal through adhesive bridging with extracellular matrix proteins that appear on apoptotic target cells.

Execution of the apoptotic program is associated with departure of cytochrome c from mitochondria and disruption of electron transport resulting in generation of reactive oxygen species (ROS), particularly superoxide and hydrogen peroxide. Whether this ROS production during apoptosis is an inevitable but unimportant consequence of dysregulated electron transport, or fulfills important signaling functions during apoptosis, is not fully understood. Recently, formation of various ROS has been implicated as components of the final common pathway leading to the execution of apoptosis following exposure to tumor necrosis factor, growth factor withdrawal, various oxidants, and numerous other insults. It has been established that apoptosis and PS externalization are associated with ROS generation (Wood and Youle, 1994; Jacobson, 1996). Thus, appearance of PS in outer monolayer of the plasma membrane could be related to its oxidation in the plasma membrane of apoptotic cells. Thus, oxidized PS on the cell surface may be recognized as an "eat-me" signal by macrophages and serve to regulate and stimulate phagocytosis of apoptotic cells (Kagan *et al.*, 2000).

2. MEMBRANE PHOSPHOLIPID ASYMMETRY DURING APOPTOSIS

Active maintenance of membrane phospholipid asymmetry is universal in normal cell membranes. Phosphatidylcholine (PC) and sphingomyelin (SPH) are found predominantly in the outer monolayer of plasma membrane, whereas phosphatidylinositol (PI) and phosphatidylethanolamine (PE), are confined predominantly to its inner leaflet. Phosphatidylserine (PS) is, however, the only phospholipid that localizes almost entirely to the inner leaflet of plasma membrane (Zwaal and Schroit, 1999; Bevers *et al.*, 1999). Surface exposure of PS has important physiological and phathological implications for blood coagulation, apoptosis and cell-cell recognition (Bevers *et al.*, 1998). The loss of transmembrane asymmetry of plasma membrane phospholipids and consequent externalization of PS on the surface of apoptotic cells is considered to be the critical triggering event required for their recognition by some macrophages (Martin *et al.*, 1995; Hampton *et al.*, 1996; Fadok *et al.*, 1998, 2001). Several proteins have been identified for the function of maintaining asymmetric transbilayer distribution of phospholipids. Maintenance of PS in the inner leaflet of the plasma membrane is accomplished by ATP-dependent aminophospholipid translocase (APT), P-type ATPase (Tang *et al.*, 1996; Daleke and Lyles, 2000; Ding *et al.*, 2000). APT transports aminophospholipids, PS and PE, from the outer to the inner leaflet of the plasma membrane, with a preference for PS over PE (Daleke and Huestis, 1985; Bitbol and Devaux, 1988). Originally discovered in erythrocytes, the APT activity has been identified in plasma membranes of a number of cells (Daleke and Lyles, 2000). Mechanisms of PS exposure on the surface of apoptotic cells appear to involve down-regulation of APT (Verhoven *et al.*, 1999; Bratton *et al.*, 1997). APT activity has been shown to be sensitive to oxidation and SH-modification and can be regulated in a redox-dependent manner (Fabisiak *et al.*, 2000, Herrmann and Devaux, 1990). In our experiments, direct inhibition of APT, as measured by NBD-PS internalization, occurs at the same time as PS oxidation (Fabisiak *et al.*, 2000). Oxidized PS may fail to be recognized by APT, and thus, escape its surveillance function. Alternatively, reactive oxidation products of PS may covalently modify APT within its active catalytic site and serve to "poison" the enzyme (Kagan *et al.*, 2000).

The steady-state level of PS in the inner leaflet of plasma membrane is the result of two opposite processes, an inward translocation mediated by APT, and an outward translocation mediated by Mg^{2+}, ATP-dependent floppase (Haest *et al.*, 1997). Similar to APT, floppase activity could be abolished by ATP depletion and sulfhydryl oxidation (Bevers *et al.*, 1999).

It has been demonstrated that floppase in red blood cells is identical to the multidrug resistance protein (MDRP) (Kamp and Haest, 1998). MDRP1 has very broad substrate specificity and translocates a number of lipid-soluble compounds, including anti-tumor drugs, from the inner to the outer leaflet. Recently, MDRP1 P-glycoprotein has been shown to transport NBD-labeled phospholipids (Van Helvoort *et al.*, 1996) including NBD-PS (Kamp and Haest, 1998). In mammalian cells, transbilayer arrangement of lipids is also mediated by ATP-binding cassette (ABC), proteins related to MDRP (Luciani and Chimini 1996; Wu and Horvitz, 1998b). ABC-1 transporter (Hamon *et al.*, 2000), a homologue of the *C.elegans* protein CED-7, is likely to function in both apoptotic cells and macrophages (Luciani and Chimini 1996; Wu and Horvitz, 1998b). It has been shown that, in addition to its externalization during apoptosis in target cells, PS is expressed on the surface of macrophages as well and is functionally significant and required for the phagocytosis of PS-expressing target cells (Marguet *et al.*, 1999; Callahan *et al.*, 2000). Furthermore, ABC-1 induced modification of lipid distribution in macrophage membranes, yielding exofacial PS flopping, generates the biophysical microenvironment required for the docking of apoA-1 at the cell surface (Cambenoit *et al.*, 2001).

Another protein, phospholipid scramblase, mediates non-specific bi-directional transbilayer movement of membrane phospholipids, including PS, under conditions of elevated calcium and acidification of cytosol (Zhao *et al.*, 1998; Verhoven *et al.*, 1999, Fadok *et al.*, 1998). Activation of phospholipid scramblase is also important for the appearance of PS on the surface of apoptotic cells. It has been reported that up-regulation of ATP-independent phospholipid scramblase during apoptosis is involved in PS externalization (Verhoven *et al.*, 1999; Bratton *et al.*, 1997). A recent study reported that scramblase activation precedes the appearance of PS on the cell surface. This supports the hypothesis that increased scamblase activity is required for PS externalization (Frasch *et al.*, 2000). PS externalization has been demonstrated to occur in a number of cell types undergoing apoptosis, including neutrophils, thymocytes, lymphocytes, actrocytes, as well as various tumor cell lines (Adayev *et al.*, 1998; Chang *et al.*, 2000; Fadok *et al.*, 1992; 2001; Schlegel *et al.*, 2001; Verhoven *et al.*, 1999; Witting *et al.*, 2000). Externalization of PS, however, is not an obligatory component of the apoptotic phenotype. Fadeel *et al.*, (1999) detected PS exposure in Jurkat and CEM cells subjected to different apoptotic treatments, whereas P39, HL-60 and Raji cells failed to externalize PS in response to the same pro-apoptotic stimuli (Fadeel *et al.*, 1999).

3. ROLE OF PS IN RECOGNITION OF APOPTOTIC CELLS BY MACROPHAGES

Recognition and removal of PS-displaying cells has been extensively studied. It has been demonstrated that not all macrophages recognize externalized PS (Fadok *et al.*, 1992; Schwartz *et al.*, 1999). Those macrophages that recognize PS on apoptotic cell use at least two different recognition systems (Schliegel *et al.*, 2000). Binding and uptake of PS-displaying cells appears to involve a multiple receptor-mediated system that recognizes PS either directly or indirectly through intermediate proteins that form a molecular bridge between apoptotic cell and macrophage (Savill and Fadok, 2000). A recent study showed several receptors and molecules to be important in the uptake of PS-displaying apoptotic cells in mammals. CD36 is a member of the family of class B scavenger receptor s like SR-B1 and is the first well-defined receptor shown to bind to apoptotic cells through a PS-related mechanism (Rigotti *et al.*, 1995). CD36 has been implicated as a putative receptor for oxidized LDL (Nicolson *et al.*, 1995; Krieger, 1997; Stainberg, 1997; Febbraio *et al.*, 1999), and appears to be the major PS-binding protein on the surface of retinal pigment epithelial cells (Ryeom *et al.*, 1996), platelets (Miao *et al.*, 2001), endothelial cells (Nicolson *et al.*, 1995; Febbraio *et al.*, 1999), human macrophages (Fadok *et al.*, 1998), and monocytes (Tait and Smith, 1999) and is specific for PS among anionic phospholipids. Several human macrophages use the vitronectin receptor ($\alpha_V\beta_3$-integrin) associated with CD36 to phagocytize apoptotic cells (Fadok *et al.*, 1992, 1998; Savill *et al.*, 1992). Furthermore, CD36 seems to be an important cofactor for recognition mechanisms mediated by PS-specific receptors (Fadok *et al.*, 1998). Existing data suggest that human macrophages might phagocytize PS-displaying apoptotic cells using the surface CD14 receptor, a glycophosphatidylinositol anchored protein (Devitt *et al.*, 1998: Schlegel *et al.*, 1999) that may function separately or jointly with the integrin and phospholipid receptor. Phosphatidylinositol (PI) and PS are more likely than the other phospholipids tested to be natural CD14 ligands (Wang *et al.*, 1998; Akashi *et al.*, 2000). Phagocyte scavenger receptor CD68 is involved in the binding of oxidized LDL (Sambrano *et al.*, 1994; Chang *et al.*, 1999), PS vesicles and apoptotic PS-displaying thymocytes to mouse peritoneal macrophages (Ramprasad *et al.*, 1995, 1996; Fadok *et al.*, 1992). A lectin-like receptor for oxidized LDL, LOX-1, was cloned from endothelial cells (Sawamura *et al.*, 1997) and it was shown to bind and phagocytize aged red blood cells and apoptotic cells through a PS-dependent mechanism (Oka *et al.*, 1998). Recently, a cDNA encoding a novel class of scavenger receptor s, SR-PSOX, that binds PS and oxidized LDL was isolated (Shimaoka *et al.*, 2000). Several studies proposed the

existence of a so-called PS receptor (Fadok *et al.*, 1992, 1998; Tait and Smith, 1999). Lately, Fadok and co-workers cloned a gene that appears to recognize PS on apoptotic cells. Transfection of this gene to B and T lymphocytes results in recognition and engulfment of apoptotic cells in a PS-specific manner. It was found that newly-identified stereo-specific PS receptor (PSR) is expressed by professional phagocytes, fibroblasts and epithelial cells (Fadok *et al.*, 2000).

Rapid clearance of the PS-displaying particle from circulation and phagocytosis of apoptotic cells is associated with PS-bridging complexes between macrophage and the PS displaying particle or apoptotic cell (Chonn *et al.*, 1995; Sugihara *et al.*, 1993; Fadok *et al.*, 1998; Balasubramanian, *et al.*, 1997 Balasubramanian and Schroit, 1998, Mevorach, 2000). On the phagocyte, CD36 receptor and vitronectin receptor ($\alpha_v\beta_3$-inegrin) have the ability to bind a bridging molecule thrombospondin, that is present in plasma and found in the basement membrane of endothelial cells (Savill *et al.*, 1992; Jimenez *et al.*, 2000; Bornstein 2001). It was suggested that PS is the partner for thrombospondin bridging on the apoptotic cells (Sugihara *et al.*, 1993; Fadok *et al.*, 1998) and PS-displaying erythrocytes (Manodori *et al.*, 2000). PS induces conformational changes in plasma glycoprotein, β_2-glycoprotein I, that exposes a specific epitope that is recognized by a macrophage receptor distinct from CD36, CD68 and CD14 (Balasubramanian and Schroit, 1998). Recently it has been shown that compliment receptors CR3 and CR4 are also important for rapid elimination of the PS-containing liposomes from blood circulation (Huong *et al.*, 2001) and uptake of apoptotic cells by human macrophages (Mevorach *et al.*, 1998). Activation of the compliment is mediated, at least in part, by PS and results in coating the apoptotic cell surface with C3bi (Mevorach, 2000).

4. ROLE OF LIPID OXIDATION IN MACROPHAGE RECOGNITION

Macrophages are able to recognize and effectively take up from the circulation oxidized LDL particles as well as a wide variety of apoptotic and oxidatively damaged cells when they are no longer functionally required (Sambrano *et al.*, 1994; Eda *et al.*, 1997; Mold and Morris, 2001). Oxidized LDL is taken up and degraded by mouse macrophages significantly faster than native lipoproteins (Babiy and Gebidcki, 1999). It is tempting to assume that both oxidized LDL and apoptotic cells may have common ligands on their surface, consisting of oxidatively modified moieties that are recognized by common macrophage receptors, including CD36, CD68 and LOX-1. Chang and co-workers demonstrated that when cells undergo

apoptosis, they express oxidation-specific epitopes on their cell surface, including oxidized phospholipids and/or oxidized phospholipid-protein adducts, and that these serve as ligands for macrophage recognition and phagocytosis (Chang *et al.*, 1999). Cellular plasma membrane and LDL particles are both composed mainly of phospholipids containing unsaturated fatty acids (Schnurr *et al.*, 1996). Polyunsaturated phospholipids are mostly prone to oxidative attack, thus the phospholipid oxidation products should contain hydroperoxy-fatty acid residues in the *sn*-2 position at the initial stage of free radical oxidation (Kagan, 1988). It has been shown that the lipid moiety of oxidized LDL, derived from oxidation of arachidonoyl phospholipids (Watson *et al.*, 1995), is responsible for its interaction with the scavenger receptor (Terstra *et al.*, 1998, Boullier *et al.*, 2000; Gillotte *et al.*, 2000). Furthermore, minimally oxidized LDL and the oxidation product of 1-palmitoyl-2-arachidonoyl-*sn*-glysero-3-phosphatidyl-choline have been shown to stimulate endothelial cells to increase monocyte-endothelial interaction (Berliner *et al.*, 1990; Lee *et al.*, 2000), which is inhibited by oxidized but not non-oxidized polyunsaturated fatty acids (Scthi *et al.*, 1996). Subbanagounder *et al.* identified 6 new bioactive, oxidized phospholipids that have specific effects on the monocyte-endothelium interaction and demonstrated that the specificity is determined by the *sn*-2 groups of oxidized phospholipids (Subbanagounder *et al.*, 2000).

5. PS OXIDATION IN APOPTOSIS

Oxidative stress in general and lipid peroxidation in particular have been implicated in the final common pathway of apoptosis (Chandra *et al.*, 2000). Cytochrome c released from mitochondria may be involved in specific interaction with PS located in the cytosolic leaflet of plasma membrane and be responsible for selective PS oxidation during apoptosis (Kagan *et al.*, 2000; Tyurina *et al.*, 2000). Recently, we demonstrated that cytochrome c can effectively induce oxidation of PS in both liposomes and intact living HL-60 cells (Kagan *et al.*, 2000). Moreover, PS oxidation during apoptosis may, in some way, participate in its externalization within the membrane. We have recently reported that apoptosis following exposure to paraquat was associated with enhanced sensitivity of PS to oxidation that was attenuated by Bcl-2 (Fabisiak *et al.*, 1997).

To characterize phospholipid oxidation during apoptosis, cellular phospholipids were metabolically labeled at *sn*-2 position with a natural unsaturated florescent fatty acid containing four conjugated double bonds, *cis*-parinaric acid (*cis*-PnA) (Kagan *et al.*, 1998; Tyurina *et al.*, 2001). Oxidative destruction of any part of the conjugated double bond system of

cis-PnA results in irreversible loss of its characteristic fluorescence (Kuypers *et al.*, 1987). The amounts of PnA-labeled phospholipid in each class usually do not exceed 2%. That is low enough to have minimal effects on cell viability and function yet sufficient to permit quantitative detection of oxidative stress (Kagan *et al.*, 1998).

Using this protocol we found that apoptosis induced by a number of different oxidants is associated with selective oxidation of specific phospholipid classes, most notably PS (Kagan *et al.*, 2000, Tyurina *et al.*, 2000). Several cell lines were used to investigate the role of PS oxidation in apoptosis. Preferential oxidation of PS was observed in human leukemia HL-60 cells (Fabisiak *et al.*, 1998, 2000; Kawai *et al.*, 2000), and normal human keratinocytes (Shvedova *et al.*, 2001). Similarly, in pheochromocytoma PC12 cells exposed to a radical-generating antineoplastic drug, neocarzinostatin, externalization of PS was potentiated by its selective oxidation in whole cells (Schor *et al.*, 1999). In contrast, this selective PS oxidation did not occur in liposomes prepared from mixtures of PnA-labeled phospholipids extracted from the cells and exposed to oxidants under the same conditions (Fabisiak *et al.*, 1998; Kagan *et al.*, 2000; Shvedova *et al.*, 2001). Recently, we also documented that PS oxidation during oxidant-induced apoptosis occurred within the plasma membrane, the largest source of oxidized PS compared to other cellular organells (Kawai *et al.*, 2000).

Since in all these cases we used oxidants to induce apoptosis, a relatively massive background oxidation of all PnA-labeled phospholipid classes was observed and masked apoptosis-specific PS oxidation. Therefore, we next determined whether a non-oxidant-induced apoptosis model could be used to reveal more explicitly the selective mode of PS oxidation inherent to the apoptosis execution program. To this end, we used agonistic anti-Fas antibody to induce apoptosis in Jurkat B cells according to a commonly used protocol (Fadeel *et al.*, 1999). Importantly, we found that anti-Fas-triggered apoptosis in Jurkat cells was characterized by early and selective oxidaiton of *cis*-PnA-PS (Kagan *et al.*, 2001) without any significant involvement of other classes of phospholipids.

We further used a quantitative procedure to determine the amounts of externalized PS on the surface of apoptotic cells based on its availability to react with fluorescamine, a non-permeating reagent readily interacting with primary amino-groups. Subsequent separation (by high performance thin-layer chromatography) and quantitation of fluorescamine-modified PS permits determination of absolute amounts of externalized PS on the outer leaflet of plasma membrane. We were also able to determine the amounts of PnA-phospholipids oxidized during apoptosis based on their disappearance in the course of apoptosis. Thus both PS oxidation and externalization could be assessed.

Table 1. Externalization and oxidation of PS during apoptosis

Cell line	Stimuli	PS externalised*	PnA-PS oxidized*
HL-60	AMVN	51.2	12.2
HL-60	tert-BuOOH	41.6	2.2
HL-60	H_2O_2	27.8	2.9
NHEK	Cumene OOH	84.6	5.7
Jurkat	α-Fas antibody	52.8	0.3

* 10^6 molecules/cell

Our data on PS oxidation and externalization summarized in Table 1 show that relatively high amounts of PS were externalized as a result of apoptosis induced by both oxidants and non-oxidants (anti-Fas antibody). Interestingly, between 5-20% of externalized PS was represented by oxidized PnA-PS in the cells when apoptosis was induced by oxidants. In contrast, only <1% of esterified PS was accounted for as oxidized PnA-PS in Jurkat cells triggered with anti-Fas-antibody. This may be indicative of a specific role that oxidized PS may play in its externalization. Given that PnA-PS represents only ≈2 mol% of total PS in cells, realistic amounts of the fraction of oxidized PS in the total pool of externalized PS may be significantly higher. Thus, oxidation of PS may be a significant contributor to its externalization during apoptosis and may consequently play a very important role in recognition of apoptotic cells by macrophages.

6. OXIDATION OF PS AND RECOGNITION OF APOPTOTIC CELLS BY MACROPHAGES

The absolute amounts of externalized PS may be essential for clearance of apoptotic cells. Surprisingly, it has been recently found that viable developing and mature B cells can bind significant amounts of annexin V (Dillon *et al.*, 2000, 2001). These normal PS-displaying cells do not exert any of the biochemical or morphological characteristics associated with apoptosis. One may suggest that the relatively low amounts of externalized PS in normal non-apoptotric cells may be insufficient for recognition by macrophage receptors. PS exposed on the surface of viable cells and may be sequestered in membrane microdomains the characteristics of which are essential for recognition by macrophages. Liposome binding experiments indicate that increasing the amounts of liposomal PS from 10 to 30 mol% leads to a faster disappearance of the liposomes from circulation (Kamps *et*

al., 1999). Moreover, oxidation of PS may be an additional factor contributing to the enhanced recognition by macrophages. In line with this, no uncontrolled uptake of slightly oxidized LDL (fewer than 150 hydroperoxide groups per particle) by macrophages was observed (Babiy and Gebidcki, 1999). Thus, both the amount of PS exposed on the cell surface and its oxidative modification may be critical for recognition and engulfment of apoptotic cells.

To test whether oxidation of PS in plasma membrane may be functionally linked not only with PS externalization but with subsequent phagocytosis as well,we performed experiments to compare phagocytosis of HL-60 cells in which we integrated PS or oxidized PS into plasma membrane. We found that recognition of the cells with externalized PS was strongly facilitated when oxidized PS was also present on the surface of target cells. Integration of PS containing-liposomes into HL-60 cells pretreated with N-ethylmaleimide to inhibit APT (and prevent PS internalization) stimulated their phagocytosis by J774A.1 macrophages (Figure 1, A, B). This effect was markedly enhanced when oxidized PS (15 mol%) was integrated into HL-60 target cells along with PS (Figure 1, C). Neither PC, nor oxidized PC were able to stimulate phagocytosis of HL-60 cells by J774A.1 macrophages.

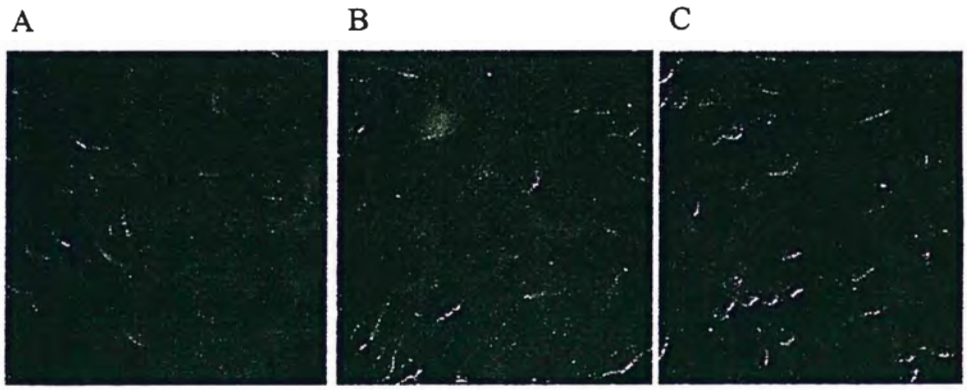

Figure 1. Phagocytosis of HL-60 cells by macrophages. Photographs depicting representative examples of phagocytosis of HL-60 cells by macrophage cell line. (A) naive (untreated); (B) HL-60 cells were pretreated with APT inhibitor, NEM, after which, PS was integrated into outer leaflet of plasma membrane; (C) HL-60 cells were pretreated with APT inhibitor, NEM, after which oxidized PS was integrated into outer leaflet of plasma membrane. HL-60 cells were labeled with CellTracker Orange (Molecular Probes, Eugene, OR) (2 mM in serum free medium at 37 °C for 15 min). Labeled cells were pre-treated with N-ethylmaleimide (10 μM, 5 min) and then incubated with liposomes containing either PS or oxidized PS at 37 °C for 30 min. HL-60 cells containing either externalized PS or oxidized PS were added to J774A.1 and phagocytosis was observed and photographed using Nikon fluorescence imaging system.

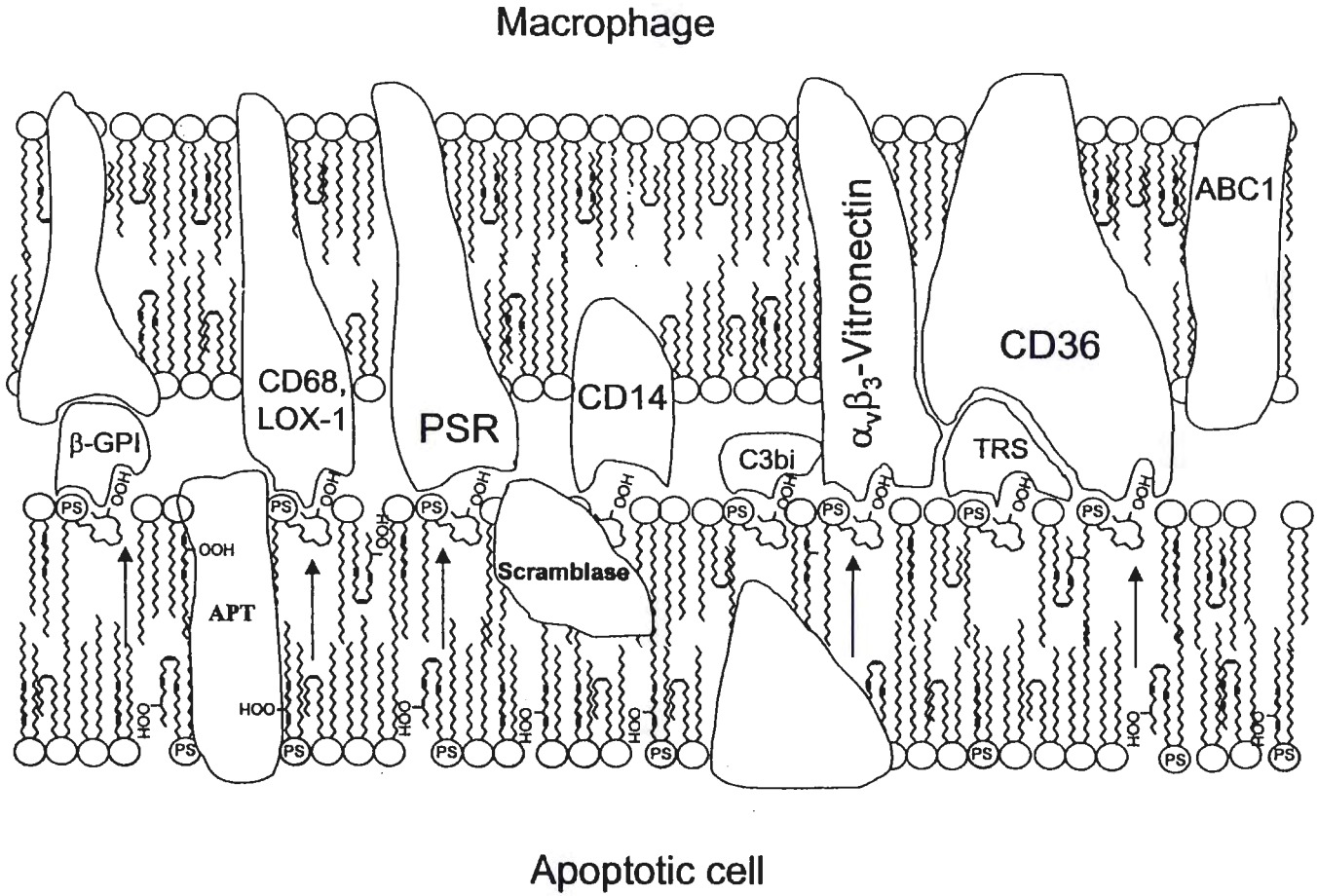

Figure 2. A model illustrating participation of oxidized PS in its externalization and recognition and engulfment of apoptotic cells.

Thus, selective oxidation and externalization of PS in plasma membrane are likely to create conditions where oxidized PS on the external surface of the plasma membrane may act as a preferred ligand for macrophage receptor.

Oxidized phospholipids have been shown to have a number of biological effects on cultured cells including activation of neutrophils (Smiley *et al.*, 1991) and induction of endothelial cell adhesion molecules (Watson *et al.*, 1995). We suggest that oxidized phospholipid, essentially oxidized PS, plays a major role in recognition of apoptotic cells by the macrophage receptor. Our proposed model for participation of oxidized PS in the engulfment of apoptotic cells is shown in Figure 2. Activation of free radical reactions during apoptosis results in oxidation of phospholipids, most notably PS (plasma membrane bilayer shown in the lower part of the panel). Oxidized PS fails to be internalized by APT thus resulting in its appearance in the outer leaflet of plasma membrane (e.g., by up-regulated activity of phospholipid scramblase). Polar fatty acid degradation products resulting from PS oxidation reorient themselves in plasma membrane with polar moieties presenting themselves at the water-lipid interface. Consequently, oxidatively modified PS may adopt configurations recognizable by one of macrophage scavenger receptors thus facilitating adhesion, recognition and PS-dependent engulfment of apoptotic cells by phagocytes (as shown in the upper part of the panel, Figure 2).

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