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Forum Review

Plasma Membrane Alterations During Apoptosis: Role in Corpse Clearance

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ABSTRACT

Apoptosis is a program of cellular self-destruction culminating in the clearance of cell corpses by neighboring macrophages. Studies in recent years have served to characterize a number of structural and molecular plasma membrane alterations that act in concert to mediate efficient engulfment of cell corpses. Hence, "eat me" signals, including the anionic phospholipid phosphatidylserine (PS) and its oxidized counterpart, PS-OX, as well as the PS-binding protein, annexin I, are exposed on the surface of effete cells and function to mediate engulfment by neighboring phagocytic cells. Plasma membrane blebbing (zeiosis), a common feature of the apoptotic program, provides a structural context for the exposition of recognition signals insofar as PS molecules aggregate on the surface of these membrane protrusions. Apoptotic cells also secrete chemotactic factors ("seek me" signals), such as the phospholipid lysophosphatidylcholine, that recruit phagocytes to the site of the apoptotic lesion. Taken together, these events serve to mediate the disposal of effete cells prior to their necrotic disintegration, thus preventing the inflammation and tissue scarring that would otherwise ensue. *Antioxid. Redox Signal.* 6, 269–275.

INTRODUCTION

APOPTOSIS is a process of cellular suicide that is essential for the sculpting of organs and the deletion of vestigial structures during embryogenesis and for the maintenance of tissue homeostasis in the adult organism (37). Moreover, dysregulation of the apoptotic program has been implicated in the pathogenesis of numerous human diseases (57, 72). Considerable effort has been made in recent years to elucidate the mechanisms underlying the execution phase of this mode of cellular demise; however, the subsequent resolution phase, *i.e.,* the recognition and removal of apoptotic corpses by neighboring phagocytes, has received less attention. Nevertheless, if apoptotic cells were to escape clearance *in vivo*, then secondary necrosis and tissue damage would ensue. In other words, macrophage engulfment of apoptotic cells defines the "meaning" of cell death (63). Indeed, a cell can be considered to be "functionally" dead, as a corpse is placed in a coffin, when it is engulfed by neighboring cells, irrespective of the occurrence of other apoptotic features. The current review aims to discuss the molecular and structural surface alterations that occur during apoptosis, as well as the importance of these alterations in the recognition and engulfment of apoptotic cells by macrophages.

EXPOSITION OF RECOGNITION SIGNALS

Specific alterations in the molecular composition of the plasma membrane occur during apoptosis. Hence, cells undergoing apoptosis express "eat me" signals, including lipids, proteins, and modified sugar moieties, that facilitate recognition and ingestion by macrophages. Duvall and associates (21) demonstrated some 20 years ago that macrophage recognition of apoptotic thymocytes is mediated by a sugar-dependent mechanism, inhibited in this model by *N*-acetylglucosamine, but not by mannose or fucose, and only to a minor extent by other

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monosaccharides, including galactose. These pioneering studies suggested the involvement of lectin-like macrophage receptor(s) in the clearance of apoptotic cells. A similar sugar-dependent binding to macrophages was demonstrated in studies of apoptotic neutrophils (33, 64) and hepatocytes (20), although here mannose and fucose moieties were implicated. Apoptotic cell surfaces lose existing cell membrane due to surface blebbing and budding of apoptotic bodies (discussed below), and gain new membrane through fusion of vesicles of dilated endoplasmic reticulum with the plasma membrane (3, 53). Such membrane loss and replacement could explain the change from the expression of mature surface glycan groups seen in viable cells to the immature glycan groups seen in apoptotic cells, leading to the focal exposure of sugars normally found in the interior of glycan structures, such as *N*-acetylglucosamine (3).

Biological membranes are vectorial structures insofar as lipid constituents are asymmetrically distributed between the outer and the inner leaflets of the plasma membrane (59). Hence, the outer leaflet of plasma membranes is formed predominantly with the cholinephospholipids [sphingomyelin and phosphatidylcholine (PC)], whereas the majority of the aminophospholipids [phosphatidylserine (PS) and phosphatidylethanolamine] are confined to the inner leaflet of the membrane. Phospholipid asymmetry is likely to be of major physiologic importance as cells invest energy to catalyze lipid movement in order to maintain a specific transmembrane phospholipid distribution (6, 85). Importantly, loss of membrane asymmetry and attendant exposure of PS is seen in aging erythrocytes and in activated platelets, and serves to stimulate the coagulation cascade, as well as to mediate cell recognition by the reticuloendothelial system (85). Moreoever, PS exposure is a common feature during apoptosis (23, 24, 47, 56), and is essential for the clearance of apoptotic cells by phagocytes (26, 39); the egress of PS thus constitutes an example of a lipid "eat me" signal that is displayed by cell corpses.

Phospholipid scramblase (PLS), a Ca^{2+}-activated enzyme that catalyzes the bidirectional movement of phospholipids in the plasma membrane, has been shown to mediate Ca^{2+}-stimulated PS externalization (80, 82). Moreover, previous work suggested that activation of a lipid scramblase and suppression of ATP-dependent aminophospholipid translocation are responsible for PS exposure during apoptosis (10, 75). In contrast, recent studies show that PS exposure is modulated by the level of intracellular ATP and transpires downstream of Bcl-2-regulated mitochondrial events, irrespective of the expression of PLS (31, 73). Scott syndrome is a severe congenital bleeding disorder associated with an impairment in the exposure of PS on the surface of platelets and other blood cells following intracellular Ca^{2+} elevation (85); absence of activation of PLS was suggested to account for this defect (70, 83). Of note, recent studies show that apoptotic PS exposure is normal in Scott syndrome cells (48, 76), thus supporting the notion that different mechanisms account for the egress of PS in cells undergoing Ca^{2+} stimulation and apoptosis, respectively.

Of note, PS externalization is also seen under certain circumstances in viable, nonapoptotic cells (19, 74). How such cells manage to escape engulfment is a conundrum, as PS externalization is considered to be an important "eat me" signal. However, one possible mechanism through which macrophages distinguish apoptotic from viable cells is the existence of a threshold of sensitivity on behalf of the macrophage; in other words, surface exposure of PS will not induce a phagocytic response unless the "eat me" signal is sufficiently strong. Indeed, recent studies have documented that the level of externalized PS is markedly below the engulfment threshold in nonapoptotic cells, whereas apoptotic cells were found to display above-threshold amounts of PS sufficient to trigger phagocytosis (8). Another possible explanation is that the context of PS exposure during apoptosis, *i.e.,* the spatial organization of PS molecules on the surface of dying cells (discussed below), serves to alert macrophages.

Apoptotic cells have been shown to expose oxidized lipid moieties. Chang and colleagues (13) have shown that monoclonal antibodies against oxidized low-density lipoprotein (LDL) bind to the surface of apoptotic cells and inhibit their uptake by macrophages. Recent studies show that C-reactive protein, an acute-phase protein that opsonizes apoptotic cells, thus rendering these cells appetizing to macrophages (30), binds to oxidized PC on the surface of "late" apoptotic cells (*i.e.,* apoptotic cells that have undergone secondary necrosis due to prolonged *in vitro* culture) (14). Moreover, selective oxidation of PS and subsequent externalization have been demonstrated in several instances of oxidative stress-induced apoptosis, as well as in models of non-oxidant-induced apoptosis (40). Furthermore, the exposition of oxidized PS (PS-OX), in conjunction with its nonoxidized counterpart, serves as an "eat me" signal, and is required for efficient engulfment of apoptotic cells (4, 39). Of note, preincubation of target cells with antioxidant enzymes, catalase and superoxide dismutase, prevents death receptor-triggered oxidation of PS and the subsequent ingestion of these cells by macrophages, despite the fact that PS molecules are present on the cell surface (39). The latter findings indicate that PS oxidation is dispensable for the exposure of PS molecules; however, clearance of cells appears to be critically dependent on the concomitant oxidation and externalization of PS, at least in this model.

Apoptotic cells also exhibit protein recognition signals. For instance, ICAM-3 (intercellular adhesion molecule-3) has been shown to act as a cell corpse ligand for macrophage receptors, including CD14 (52). Furthermore, an increased expression of the tetraspanins CD53 and CD63 was observed on the surface of apoptotic neutrophils (7). Tetraspanins have been implicated in cell activation and signal transduction, as well as in cell adhesion (46), and could serve to mediate recognition and clearance of aging neutrophils, although this remains to be tested. Of note, recent proteomics studies have identified annexin I as a recognition signal (5). Thus, annexin I was shown to be recruited from the cytosol to the plasma membrane during apoptosis, and was found to be required for efficient uptake of apoptotic cells by human umbilical vein endothelial cells, *i.e.,* nonprofessional phagocytes. Interestingly, annexin I (also known as lipocortin I) is a PS-binding protein (67) and, consequently, was found to colocalize with PS on the surface of apoptotic cells, leading to the clustering of PS receptors on the phagocytic cell surface (5). Taken together, these data suggest that annexin I and PS (and/or its oxidized counterpart) act in concert to promote tethering and ingestion of dying cells.

Finally, detachment signals transmitted through the cell surface molecules, CD47 (54) and CD31 (11), have been shown

to prevent macrophage ingestion of viable erythrocytes and neutrophils, respectively. Interestingly, studies of the interaction of neutrophils and macrophages demonstrated that apoptosis converts the repulsive signals that are conferred by CD31 to adhesive signals, thus promoting ingestion (11). The molecular mechanism underlying the switch from detachment to adhesion remains to be elucidated; nevertheless, one can conclude that corpse clearance is a complex process that involves both exposition of recognition signals (including PS and PS-OX) and disabling of detachment signals on the surface of the apoptotic cell.

STRUCTURAL SURFACE ALTERATIONS

Structural cell-surface alterations, the most characteristic being the vigorous pulsation and blebbing (zeiosis) of the plasma membrane, occur during apoptosis (41, 78). Plasma membrane blebbing is thought to be caspase-dependent (38, 43, 81), although stimulus-specific differences may exist (18, 49). Furthermore, blebbing is dependent on dynamic rearrangements of the actin cytoskeleton, as evidenced by the abrogation of this plasma membrane alteration by the cytoskeleton-disrupting agent, cytochalasin B (17). In addition, recent studies have disclosed that inhibition of p21-activated kinase-2 interferes with blebbing during apoptosis (60). Expression of death-associated protein (DAP) kinase, an actin filament-associated protein, and DAP kinase-related protein kinase-1 triggers membrane blebbing and the formation of so-called autophagic vesicles (36). Of note, myosin light chain (MLC), the phosphorylation of which mediates membrane blebbing (51), has been identified as a substrate of DAP kinase (15). Moreover, caspase-mediated cleavage of the Rho effector protein, ROCK I (Rho-associated coiled coil-containing protein kinase I), was recently found to induce MLC phosphorylation and membrane blebbing in apoptotic cells (16, 68). Further studies are required to delineate the precise role of each of the above kinases in the dynamic process of cytoskeletal rearrangement and attendant zeiosis of apoptotic cells.

The breakdown of dying cells into membrane-bound vesicles (so-called apoptotic bodies), some containing nuclear fragments and others without (78), likely facilitates subsequent ingestion of cell corpses by macrophages. However, the putative role of blebbing in the clearance of apoptotic cells has not been addressed to date. Nevertheless, recent studies suggest that blebbing is an important feature of apoptosis insofar as macrophage engulfment of cytochalasin B-treated (*i.e.,* nonblebbing) cells was diminished, despite the occurrence of caspase activation and PS externalization in these cells (W. Uthaisang, S. Orrenius, and B. Fadeel, unpublished observations). Of note, PS appears to aggregate on the surface of apoptotic membrane blebs (12; W. Uthaisang, S. Orrenius, and B. Fadeel, unpublished observations). These findings indicate that plasma membrane blebbing may, in fact, serve to facilitate the recognition process, perhaps through the correct presentation (*i.e.,* aggregation) of "eat me" signals, including PS, on the cell surface. Interestingly, nuclear self antigens are present on the surface of membrane blebs (58), and could thus elicit an autoimmune response if cells are not rapidly removed. Indeed, mice that are defective for phagocytosis of apoptotic cells have been shown to develop systemic lupus erythematosus-like disease, with high titers of autoantibodies against nuclear antigens (9).

Shedding of plasma membrane vesicles (surface blebs) from apoptotic cells is well documented (79), and Segundo and associates (69) have shown that surface blebs derived from germinal center B cells are chemotactic for monocytes. Thus, in addition to the presentation of recognition ligands ("eat me" signals) on the cell surface, apoptotic cells appear to emit chemotactic factors ("seek me" signals) that induce the recruitment of phagocytes to the site of cell attrition. Of note, lysophosphatidylcholine (LPC), a phospholipid known to be a chemoattractant for monocytes and T cells (35, 50), was recently identified as a candidate macrophage attraction signal that is released from dying cells; apoptotic vesicles could be excluded in this model because neither filtration nor ultracentrifugation could abrogate the chemotactic activity of apoptotic cell supernatants (44). Interestingly, the release of LPC during apoptosis was linked to caspase-mediated activation of the Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) (44). Kim and colleagues (42) have previously reported that $iPLA_2$ activation during apoptosis can promote cell-surface exposure of LPC, leading to the binding of natural IgM antibodies and subsequent complement opsonization of the dying cell. Future studies should aim to ascertain whether LPC externalization and/or release is a general phenomenon during apoptosis; nevertheless, the current evidence suggests that LPC may play a dual role in the recruitment of phagocytes and the subsequent recognition of cell corpses.

MACROPHAGE RECEPTORS AND BRIDGING MOLECULES

Macrophages are endowed with an array of phagocytosis receptors. These include the so-called PS receptor, the class A scavenger receptor, CD36 (a class B scavenger receptor), CD68 (macrosialin; a class D scavenger receptor), the integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$, and the bacterial lipopolysaccharide receptor CD14 (66). Tissue- and cell type-specific differences in receptor usage provide a tentative explanation for this broad repertoire of phagocytosis receptors. For example, dendritic cells and monocyte-derived macrophages utilize the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin receptor, respectively, for the uptake of cell corpses (1, 65). It is also possible that the engulfment process requires the serial engagement of distinct receptors, some of which are involved in the initial tethering of apoptotic cells and others in the cytoskeletal rearrangement that is required for ingestion of cells (25). Moreover, the recent observation that apoptotic cells display both PS and PS-OX on their surface (39) suggests that the serial or concomitant engagement of distinct, PS-binding receptors may be required for clearance of apoptotic cells. In support of this contention, it was previously reported that disruption of phospholipid asymmetry is sufficient for tethering of erythrocytes to macrophages, whereas additional oxidative changes are required for engulfment to take place (61).

Molecular "bridging" occurs during platelet aggregation in which fibrinogen links the platelet membrane glycoprotein

IIb/IIIa on adjacent platelet surfaces (55). Similarly, soluble factors such as thrombospondin, protein S, β2-glycoprotein I, and milk-fat globule epidermal growth factor 8 (MFG-E8) act as molecular liaisons that link phagocytes to their apoptotic prey (66). For instance, MFG-E8 was shown to bind to the $\alpha_V\beta_3$ integrin on macrophages and to externalized PS on the surface of apoptotic cells, thus enhancing the efficiency whereby macrophages engulfed apoptotic target cells (34). Indeed, MFG-E8 binds both to nonoxidized PS and to PS-OX (S. Iverson, S. Ahlberg, and B. Fadeel, unpublished observations), and could therefore serve to promote phagocytosis of cells in which oxidation of PS has been abrogated (39).

CONSERVATION OF ENGULFMENT PATHWAYS

Studies in the nematode *Caenorhabditis elegans*, a strong candidate for the "most valuable player" in apoptosis research (62), have led to the identification of seven *ced* (cell death abnormal) genes that are involved in engulfment (22, 32). Gene complementation studies have suggested two partially redundant pathways, one comprised of *ced-1, ced-6*, and *ced-7*, and the other of *ced-2, ced-5, ced-10*, and *ced-12*. CED-1 is homologous to mammalian scavenger receptors as well as to the mammalian LDL receptor-related protein (71, 84), and appears to be involved in the binding of cell corpses to neighboring, phagocytic cells (84). CED-6 is an adaptor molecule that interacts with CED-1 (45), and CED-7 (a homologue of the mammalian ATP-binding cassette transporter, ABC1) has been suggested to promote corpse recognition by CED-1, through the externalization of a phospholipid ligand (77, 84) (biochemical evidence of PS externalization, however, has yet to be documented in the nematode). The CED-2/CED-5/CED-10/CED-12 pathway controls both the migration of cells to the distal tip of the gonad and the cytoskeletal reorganization that takes place within the engulfing cell during phagocytosis (32). Importantly, recent studies have provided evidence that the CrkII-DOCK180-Rac1 molecular complex in mammalian cells is functionally analogous to the CED-2/CED-5/CED-10 complex in *C. elegans* (2). Corpse clearance in *Drosophila melanogaster* involves professional macrophages as well as amateur phagocytes (epithelial and glial cells) (27), and the fruitfly thus constitutes a suitable model system in which to dissect the mechanism underlying cell clearance by professional phagocytes. Indeed, Franc and colleagues have identified a CD36-like receptor, croquemort ("catcher of death"), that is expressed on *Drosophila* macrophages during embryogenesis (28). Croquemort was shown to be essential for the removal of apoptotic cells (29), thus emphasizing the importance of scavenger receptors in corpse removal. Genetic analyses of phagocytosis of cell corpses in the nematode and the fruitfly, respectively, have thus disclosed a remarkable degree of conservation of cell clearance through evolution.

CONCLUDING REMARKS

Phagocytosis of cell corpses is an integral part of the apoptotic process. Clearance of apoptotic cells contributes to the resolution of inflammation through the removal of cells prior to the release of noxious cellular constituents. Moreover, apoptotic cells are a potential source of self antigens and clearance of cell corpses is believed to preclude the induction of autoimmune responses. Phagocytosis of effete cells is a complex process, involving the release of "seek me" (attraction) signals, the exposition of "eat me" (recognition) signals, the spatial reorganization of membrane components, and the tethering and ingestion of cell corpses by macrophages through the engagement of specific receptors. Future studies, not only in transformed cell lines, but also in relevant *in vivo* models of cell clearance, should aim to clarify how these processes are orchestrated at the molecular level, and to determine the consequences of such processes in health and disease.

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ABBREVIATIONS

CED, cell death abnormal; DAP, death-associated protein; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; MFG-E8, milk-fat globule epidermal growth factor-8; MLC, myosin light chain; PC, phosphatidylcholine; PLS, phospholipid scramblase; PS, phosphatidylserine; PS-OX, oxidized phosphatidylserine.

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