Loss of Phospholipid Asymmetry and Surface Exposure of Phosphatidylserine Is Required for Phagocytosis of Apoptotic Cells by Macrophages and Fibroblasts*

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Removal of apoptotic cells during tissue remodeling or resolution of inflammation is critical to the restoration of normal tissue structure and function. During apoptosis, early surface changes occur, which trigger recognition and removal by macrophages and other phagocytes. Loss of phospholipid asymmetry results in exposure of phosphatidylserine (PS), one of the surface markers recognized by macrophages. However, a number of receptors have been reported to mediate macrophage recognition of apoptotic cells, not all of which bind to phosphatidylserine. We therefore examined the role of membrane phospholipid symmetrization and PS externalization in uptake of apoptotic cells by mouse macrophages and human HT-1080 fibrosarcoma cells by exposing them to cells that had undergone apoptosis without loss of phospholipid asymmetry. Neither mouse macrophages nor HT-1080 cells recognized or engulfed apoptotic targets that failed to express PS, in comparison to PS-expressing apoptotic cells. If, however, their outer leaflets were repleted with the L-, but not the D-, stereoisomer of sn-1,2-PS by lipidosome transfer, engulfment by both phagocytes was restored. These observations directly demonstrate that loss of phospholipid asymmetry and PS expression is required for phagocyte engulfment of apoptotic cells and imply a critical, if not obligatory, role for PS recognition in the uptake process.

In tissues undergoing remodeling or during resolution of inflammation, apoptotic cells are cleared by phagocytes prior to their lysis, supporting the idea that surface changes promoting recognition occur early in the apoptotic process. Removal prior to lysis prevents the release of potentially toxic or immunogenic intracellular contents, thus maintaining normal tissue structure and function in the face of considerable cell death. Little is known about the specific surface markers that trigger recognition by phagocytes. In some cases, exposure of specific carbohydrates on the apoptotic cell surface may trigger binding to phagocyte lectins (1–5). In other cases, molecules such as thrombospondin (6), collectins, or complement products (iC3b) (7) appear to bridge the apoptotic cell to the phagocyte; however, the molecules on the apoptotic cell to which they bind have not been identified. The best studied and most consistent surface change occurring on apoptotic cells is the exposure of phosphatidylserine (PS) associated with loss of phospholipid asymmetry (8–20). We showed several years ago that exposure of PS during apoptosis appeared to trigger recognition of apoptotic cells by subsets of macrophages, because uptake of apoptotic bodies could be inhibited in a dose-dependent and stereospecific manner by liposomes containing PS and by structural analogues of PS but not by liposomes containing other anionic phospholipids (8, 21, 22). Using relatively simple inhibition assays employed by most investigators in the field, we have previously shown that in mouse thioglycollate-elicited peritoneal macrophages, a PS-dependent mechanism appears to be dominant, whereas in unstimulated mouse bone marrow macrophages and human monocyte-derived macrophages, the αvβ3/CD36/thrombospondin mechanism appears to predominate (6, 23). The latter can be stimulated to recognize PS by treating them with digestible particulate stimuli, including β-glucan.

We have recently described a system in which loss of phospholipid asymmetry and PS expression can be divorced from other changes associated with apoptosis (24). HL-60 cells cultured in the presence of the ornithine decarboxylase inhibitor difluoromethylornithine (DFMO) to reduce putrescine and spermidine levels were still able to undergo apoptosis following treatment with UV irradiation. Thus, they showed the classic nuclear morphologic changes and plasma membrane vesiculation associated with apoptosis in these cells. In addition, untreated and DFMO-treated irradiated cells showed identical DNA fragmentation, caspase 3 activity, and depression of aminophospholipid translocase activity. In contrast, apoptotic DFMO-treated HL-60 cells failed to express PS externally, as measured by annexin V binding, and they showed impaired membrane phospholipid flip-flop, as measured by uptake of 1-palmitoyl-2-[6(7-nitro-2-1,3-benzoaxadiazol-4-yl)aminocaproyl]-sn-glycero-3-phosphocholine (24). These membrane changes were restored by repletion of the cells with putrescine prior to induction of apoptosis. Therefore HL-60 cells pretreated with DFMO and induced to undergo apoptosis by UV could provide an apoptotic cell target for phagocytosis that does not undergo loss of phospholipid asymmetry, allowing us to test the published data.

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The abbreviations used are: PS, phosphatidylserine; DFMO, difluoromethylornithine; POP-L-S, 1-palmitoyl-2-oleoyl-sn-3-glycerophospho-L-serine; POP-D-S, 1-palmitoyl-2-oleoyl-sn-3-glycerophospho-D-serine; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody.

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the hypothesis that loss of phospholipid asymmetry is required for uptake by macrophages and other phagocytes. Additionally, we found that undifferentiated PLB 985, a human myelomonocytic cell line (25) that undergoes nuclear apoptosis following exposure to UV, fails to express PS externally because of defective phospholipid scrambling. This may be contrasted with PLB 985 cells differentiated toward either neutrophils with dimethyl sulfoxide or retinoic acid or macrophages with phorbol esters or vitamin D3; these cells do express PS and show enhanced membrane phospholipid flip-flop (scrambling) following induction of apoptosis. These cells provided a second system with which to test the hypothesis that exposure of PS was required for recognition and uptake of apoptotic cells by phagocytes.

EXPERIMENTAL PROCEDURES

Cell Culture—The human promyelocytic cell line HL-60 and the human leukemia T cell line Jurkat were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI containing 20% fetal calf serum (Gemini Bioproducts, Calabasas, CA), glutamine, penicillin, and streptomycin. The human myelomonoblastic PLB 985 cells (25) were a gift from Dr. Christina Leslie and were cultured in the same medium, except that 10% fetal calf serum was used. For some experiments, the cells were differentiated into monocytes by treatment for 5 days with 10⁻⁷ M 1,25-dihydroxyvitamin D3. Mouse bone marrow-derived macrophages derived from C3H/Hej mice were cultured in macrophage colony-stimulating factor in 96-well tissue culture plates as described (21, 26) and used for phagocytosis after 7 days of culture.

FIG. 1. Phosphatidylserine exposure is inhibited in cells treated with DFMO but restored when polyamines are repleted with putrescine. A, cyto metric data from one representative experiment. The dot plots illustrate annexin binding on the horizontal axis and propidium iodide binding on the vertical axis. The numbers in each quadrant represent the percentage of the total population. The lower left quadrant represents annexin-low cells, the lower right quadrant represents annexin-high (i.e. PS exposed externally), and the upper right quadrant represents cells that are annexin-high and permeable to propidium iodide. FL1-H, green fluorescence (annexin V); FL2-H, red fluorescence (propidium iodide). B, the percentage of annexin-high (PS-positive) cells in five experiments.

FIG. 2. Phagocytosis of apoptotic HL-60 cells is correlated with loss of phospholipid asymmetry. Unstimulated bone marrow-derived macrophages (BMDM) were those cells derived from the bone marrow after 7 days in macrophage colony-stimulating factor. Stimulated bone marrow-derived macrophages were treated for 48 h with β-glucan to induce the ability to recognize PS on apoptotic cells. Put, putrescine.

To induce the ability to overtly recognize phosphatidylserine on apoptotic cells, bone marrow macrophages were treated with 75 μg/ml β-glucan (Accurate Chemical Co.) on day 5 of culture and were used 48 h later (21). HT-1080 human fibrosarcoma-derived cells (27), ob-
tained from the American Type Culture Collection were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, nonessential amino acids, and sodium pyruvate.

Treatment with DFMO and/or Putrescine—Polyamines were depleted as described (24). Briefly, HL-60 cells were plated at a density of 1.25 × 10^6 cells/ml in culture medium containing 1 mM 2-(difluoromethyl)-DL-ornithine monohydrochloride monohydrate, a generous gift of Dr. Eckhard Bohme, Hoescht Marion Roussel, Inc., Cincinnati, OH. This compound inhibits ornithine decarboxylase and thereby depletes the cells of polyamines, in particular putrescine and spermidine (24). The cells were used after 5 days of culture. For reconstitution of polyamines, 10 μM putrescine was added on day 3 of culture. Control cells were plated at 6.25 × 10^5 cells/ml, because they grew faster than DFMO-treated cells. All cultures achieved a final density of 1–1.25 × 10^6 cells/ml.

Reconstitution of Plasma Membrane Outer Leaflet with Phosphatidylserine—Small unilamellar liposomes containing a 50:50 molar ratio of phosphatidylserine (derived from brain; Avanti Polar Lipids, Alabaster, AL) to phosphatidylcholine (derived from bovine liver; Avanti Polar Lipids) were made. For some experiments, 1-palmitoyl-2-oleoyl-sn-3-glycerylphospho-L-serine (POPL-S) and 1-palmitoyl-2-oleoyl-sn-3-glycerylphospho-D-serine (POPD-S) were used instead of brain-derived phosphatidylserine. POP-L-S was purchased from Avanti Polar Lipids, Alabas-

All populations were labeled with annexin V-FITC and analyzed by flow cytometry. These dot plots represent one experiment of a total of five. The upper left panel shows annexin binding to viable HL-60 cells, the upper middle panel shows binding to UV-treated HL-60 cells, and the upper right panel shows the binding to DFMO-pretreated, UV-treated HL-60 cells. The lower left panel shows annexin binding to viable HL-60 cells treated with liposomes containing PS, the lower middle panel shows viable HL-60 cells treated with liposomes but then washed with bovine serum albumin and cultured for an additional hour, and the lower right panel shows DFMO-pretreated, UV-irradiated HL-60 cells treated with liposomes containing PS. FL1-H, green fluorescence; FL2-H, red fluorescence.

PS was confirmed by determining the ability of the cells to bind FITC-conjugated annexin, determined by flow cytometry. In some cases, the added PS was removed by washing the cells three times in PBS containing 2% bovine serum albumin, and the cells were incubated in tissue culture medium at 37 °C for 1 h to allow translocation of remaining PS to the inner leaflet by the aminophospholipid translocase. Removal of PS was confirmed by assessing the ability to bind annexin-FITC, as determined by flow cytometry.

Induction of Apoptosis and Its Quantitation—Apoptosis was induced in HL-60 cells and PLB 985 cells by exposure to UV irradiation at 254 nm for 5 min. Jurkat T cells were exposed for 10 min. The cells were then cultured for 2 h and harvested. For phagocytosis, the cells were resuspended in Dulbecco’s modified Eagle’s medium at a concentration of 0.5 × 10^6 cells/ml. An aliquot of cells was prepared by cytocentrifugation, and apoptosis was quantitated by evaluation of nuclear morphology at the light microscopic level.

Annexin Binding and Flow Cytometry—Loss of phospholipid asymmetry and exposure of PS were evaluated by analysis of annexin-V-FITC binding. Propidium iodide was used as a control to determine the level of secondary necrosis. The cells were stained exactly as recommended by the manufacturer of the annexin kit (RD Systems, Minneapolis, MN). Stained cells were analyzed using a FACScan cytometer and FCLysys software (Beckton Dickinson, Franklin Lakes, NJ).

Phagocytosis Assay—Targets for uptake (0.5 × 10^6 cells/well in 96-well plates) were added to mouse bone marrow-derived macrophages in serum-free medium for 30 min. For HT-1080 cells, 5 × 10^5 targets were added per well in 24-well plates in growth medium containing serum for 1 h. Unphagocytosed cells were washed away with PBS, and then the wells were stained with a modified Wright-Giemsa stain (Leukostat, Fisher). Phagocytosis was counted as described previously by light microscopy (8, 21, 22). The only modification was that a phagocytic index was not calculated; all data are reported as percent phagocytes positive for uptake.
RESULTS

HL-60 cells were treated with DFMO to deplete intracellular polyamines; to some cells, putrescine was added to replete the polyamine stores (24). The cells were then exposed to UV light to induce apoptosis. The percentage of apoptotic cells, as determined by nuclear morphology, was the same (48.7 ± 3.9% for apoptotic HL-60 cells and 51.7 ± 5.2% for apoptotic DFMO-treated HL-60 cells). Exposure of phosphatidylserine was assessed by flow cytometry, using FITC-labeled annexin V, and induction of apoptosis was confirmed by cellular morphology. The population of HL-60 cells previously cultured in DFMO showed a dramatic reduction in PS-positive cells (Fig. 1). In contrast, if DFMO-pretreated cells had been repleted with putrescine for 2 days prior to induction of apoptosis, PS exposure was restored (Fig. 1).

These populations of HL-60 cells were subsequently fed to mouse bone marrow-derived macrophages, half of which had been stimulated to recognize PS overtly, as suggested by PS inhibition of uptake. As expected, very little phagocytosis was seen when macrophages of either type were fed viable HL-60 cells (Fig. 2). In contrast, in HL-60 cells that had been UV-irradiated to induce apoptosis, uptake was significantly increased. If the cells had been pretreated with DFMO prior to induction of apoptosis to inhibit loss of phospholipid asymmetry, uptake was significantly reduced. Importantly, uptake into nonstimulated macrophages, which are thought to use primarily the avb3/CD36/thrombospondin mechanism, was also greatly reduced. Those HL-60 cells that had been repleted by putrescine prior to induction of apoptosis and that therefore lost phospholipid asymmetry and exposed PS externally were recognized by macrophages of either type (Fig. 2).

These data suggested that loss of phospholipid asymmetry and exposure of PS on apoptotic cells were required for macrophage phagocytosis, regardless of which recognition phenotype was involved. It was then important to determine whether introduction of PS into the outer leaflet by liposome transfer might restore phagocytosis of DFMO-pretreated HL-60 cells. Brain-derived PS was introduced into the outer leaflet of DFMO-pretreated HL-60 cells after they had undergone apoptosis. Because aminophospholipid translocase activity is reduced during apoptosis (24, 31, 32), outer leaflet PS would not be expected to be transported back into the inner leaflet. As is shown in Fig. 3, these cells did express PS externally (assessed by annexin V binding), and they were also recognized and engulfed by macrophages (Fig. 2).

Nonapoptotic HL-60 cells were also treated with liposomes to introduce brain-derived PS into the outer leaflet. As is shown in Fig. 3, these cells acquired the ability to bind annexin V. If the liposomes were washed away and the cells were cultured for 1 h, they no longer bound annexin V, presumably because they translocated any remaining outer leaflet PS into the inner leaflet (Fig. 3). Viable HL-60 cells expressing PS in the outer leaflet were recognized and engulfed by bone marrow-derived macrophages (Fig. 4); if, however, they had been cultured to allow for PS translocation into the inner leaflet, they were not recognized and therefore were not engulfed (Fig. 4).

We have recently found a cell line, PLB 985 (25), that fails to lose phospholipid asymmetry during apoptosis. Although these cells undergo morphologic changes of apoptosis, they fail to express PS externally, as is shown in Fig. 5. Uptake of apoptotic PLB cells, compared with apoptotic Jurkat T cells and apoptotic HL-60 cells, by unstimulated mouse bone marrow-derived macrophages was significantly reduced (Fig. 6). Thus, in a second system in which drug manipulation was not required to prevent loss of phospholipid asymmetry, PS exposure was confirmed as a requirement for efficient phagocytosis by macrophages.

We next tested the hypothesis that loss of phospholipid asymmetry was required for uptake by phagocytes other than macrophages. HT-1080 cells are an adherent cell derived from a human fibrosarcoma (27). These cells were exposed to fresh and apoptotic Jurkat T cells or PLB 985 cells, and uptake was evaluated. Fig. 7 shows that HT-1080 cells were able to phago-

![Fig. 4. Mouse bone marrow macrophages phagocytose viable HL-60 cells that express PS externally via liposome transfer. Uptake is compared with that for viable cells, apoptotic HL-60 cells, and viable HL-60 cells treated with liposomes containing PS derived from brain but then washed and cultured to allow translocation of PS. Data shown are the means ± S.E. for three experiments.](http://www.jbc.org/)

![Fig. 5. Phosphatidylserine expression, as indicated by annexin V binding, for undifferentiated and vitamin-D3 differentiated apoptotic PLB 985 cells compared with apoptotic Jurkat T cells and apoptotic HL-60 cells. For all target cells, apoptosis was induced by irradiation with UV light. These histograms are from one experiment representative of four. FL1-H, green fluorescence; FL2-H, red fluorescence.](http://www.jbc.org/)
cytose apoptotic Jurkat T cells but not apoptotic PLB 985 cells, unless the latter were treated to replete the outer leaflet with brain-derived PS. Furthermore, viable Jurkat cells were phagocytosed if their outer leaflets were loaded with PS by liposome transfer. PLB 985 cells, like HL-60 cells, can be differentiated into monocytic cells with 1,25-dihydroxyvitamin D3 (33). Apoptosis was induced in the differentiated PLB 985 cells by UV irradiation, and the cells were assessed by annexin V binding for PS exposure. Differentiated PLB 985 cells were found to express PS on their outer leaflets (Fig. 5) and were recognized and engulfed by HT-1080 cells (Fig. 8).

Lastly, it became important to determine whether phosphatidylserine inserted into the plasma membrane was recognized in a stereospecific manner, as we have previously reported (8, 9). We used two different 1-palmitoyl-2-oleoyl-sn-3-glycerophosphoserine compounds, containing either L- (POP-L-S) or D-serine (POP-D-S), to load apoptotic PLB 985 cells and viable Jurkat T cells; loading of each lipid was confirmed by annexin V staining and flow cytometry. These targets were exposed to mouse bone marrow-derived macrophages that were unstimulated or stimulated with transforming growth factor-β and β-glucan in the presence or absence of mAb 217G8E9 (directed against a newly described receptor for phosphatidylserine; Ref. 9) or its isotype control. Fig. 9 shows that only PLB cells loaded with POP-L-S were engulfed and that uptake was inhibited by Mab 217G8E9, implicating the PS receptor in recognition of these cells. Furthermore, this antibody inhibited uptake of POP-L-S-loaded targets even by unstimulated macrophages. The isotype control had no effect on the engulfment of either macrophage population (data not shown). We also observed that viable Jurkat T cells loaded with POP-L-S but not POP-D-S were taken up by both sets of macrophages; 30 ± 5% unstimulated and 42 ± 6% glucan-stimulated macrophages engulfed POP-L-S-loaded Jurkat T cells, whereas 4.2 ± 0.7% unstimulated and 5.1 ± 1.2% stimulated macrophages engulfed POP-D-S-loaded cells. These data suggest that phosphatidylserine is recognized stereospecifically by the receptor for PS.

**DISCUSSION**

Apoptotic cells that failed to lose phospholipid asymmetry and that did not express PS externally were not phagocytosed by macrophages or by fibrosarcoma cells, despite the fact that they showed the morphological changes of apoptosis and displayed the characteristic biochemical markers, including DNA fragmentation and caspase 3 activity (24). These data suggest that loss of phospholipid asymmetry and external expression of PS are required for recognition of apoptotic cells by macrophages and other phagocytes.

Phosphatidylserine appears to be a critical ligand required for uptake of apoptotic cells. It is clear that some, if not all, macrophages recognize this phospholipid specifically, because their uptake of apoptotic cells and lipid-symmetric red cells can be inhibited stereospecifically by PS and its structural analogues glycerophosphorylserine and phosphoserine (8, 34–40). The data...
presented herein suggest that PS exposure is required for uptake by all macrophages, including those that predominantly use the αβ3/CD36/thrombospondin mechanism. The obvious question that then arises is why uptake by the latter is not inhibited by PS liposomes. One likely explanation is that the inhibition assays traditionally used in analysis of apoptotic cell clearance are relatively insensitive, even when specific antibodies are used. This hypothesis receives some support from the observations by us and many other investigators that complete inhibition is never seen, even when inhibitors of multiple receptors are combined (for example, see Ref. 41). We have recently described a new receptor that appears to mediate phosphatidylserine-specific recognition of apoptotic cells and phospholipid-symmetric red cell ghosts, as well as the release of transforming growth factor-β following uptake of apoptotic cells (9). This receptor is expressed at low levels on macrophages typically reported to use the αβ3/CD36/thrombospondin mechanism for uptake but is up-regulated on macrophages that have been stimulated to recognize PS by β-glucan. In fact, the antibody against this new PS receptor (Mab 217G8E9) is a poor inhibitor of apoptotic cell uptake by human monocyte-derived macrophages or mouse bone marrow-derived macrophages that have not been stimulated to up-regulate the receptor by a particulate stimulus such as glucan (9). By contrast, we found that uptake of apoptotic PLB 985 cells and viable Jurkat T cells loaded with POP-L-S could be inhibited by Mab 217G8E9, suggesting involvement of the PS receptor on these cells also. We hypothesize that the disparities between these two systems (apoptotic cells versus lipid-loaded cells) results from the likelihood that the lipid loading yields a target cell with more recognizable PS on its surface than occurs during apoptosis. Additionally, given that MAb 217G8E9 is an IgM, we believe it to be of relatively low affinity for both determining expression by flow cytometry and for inhibition of uptake; we are currently developing new reagents to better explore this disparity. However, that this receptor is functional on unstimulated macrophages is supported by our additional observation that the anti-PS receptor monoclonal antibody stimulates transforming growth factor-β production and inhibits tumor necrosis factor-α production, as does uptake of apoptotic cells, as we have reported previously (42). Other macrophage receptors incriminated in uptake of apoptotic cells (6, 20, 43) have been suggested to bind PS (as well as other phospholipids) offered in liposomes; these receptors include CD36, CD68, and CD14 (44–46). Whether these receptors specifically recognize PS exposed on an apoptotic cell and/or how they cooperate with the receptor we have described remain to be determined.

This newly described PS receptor is also expressed on HT-1080 cells as well as other fibroblasts and epithelial cells, and anti-PS receptor antibody inhibits their uptake of apoptotic cells (9).

Other phagocytes, including Sertoli cells, endothelial cells, and smooth muscle vascular cells have been shown to recognize PS on apoptotic cells (47–49); whether they express and use the new PS receptor remains to be determined, although preliminary assessment of human umbilical vein endothelial cells by flow cytometry was positive for PS receptor expression. These cells have been shown to utilize scavenger receptors for uptake of apoptotic cells, some of which may recognize PS. The HT-1080 cells used herein engulf apoptotic cells poorly when compared with macrophages; similar results were obtained when primary fibroblasts were used (50). The engulfment assay used herein determines the percentage of phagocytes containing apoptotic bodies recognizable by light microscopy at any one point in time. This type of assay does not take into account the rate of uptake or the rate of digestion. In our hands, a higher percentage of macrophages can be scored positively at any time point when compared with fibroblasts or epithelial cells, suggesting to us that macrophages are more efficient.

Loss of phospholipid asymmetry could also contribute to recognition of apoptotic cells by facilitating exposure of other ligands that are cryptic when cells are viable. The loss of phospholipid asymmetry associated with apoptosis is characterized by changes in membrane fluidity relative to that seen in cells maintaining normal asymmetric phospholipid distribution. Alterations in fluidity could contribute to changes in protein conformation, distribution in the membrane, and/or function. For example, it was shown that the ligand specificity for the vitronectin receptor αβ3 (CD51/CD61) was altered depending on the phospholipid milieu in which the receptor was placed (51). Alternations in lipid packing have also been implicated in the function of lymphocyte function antigen-1 (LFA-1) (CD11a/CD18) (52). It is intriguing to note that intercellular adhesion molecule-3 (ICAM-3) appears to be a recognition ligand for macrophage uptake of apoptotic cells but does not facilitate function of viable cells, suggesting that it undergoes some qualitative change during apoptosis (53). It is possible that loss of phospholipid asymmetry contributes to exposure of an otherwise cryptic epitope on this adhesion molecule. However, introduction of PS into the outer leaflet of viable cells induced their uptake, as shown in Fig. 4, suggesting that PS alone is a sufficient signal for uptake by phagocytes, although we cannot rule out the possibility that introduction of PS into the outer leaflet changes the membrane in a way that exposes otherwise cryptic recognition ligands.

There is other experimental support that loss of phospholipid asymmetry is required for macrophage recognition of apoptotic cells. It was recently reported that preincubation of apoptotic cells and lipid-symmetric erythrocytes with annexin V inhibited their uptake by all macrophages tested, whereas it did not inhibit uptake of opsonized red cells via the Fe receptor (54). In addition, Verhoven et al. (55) correlated the appearance of PS...
on the outer leaflet with recognizability by thio-hydril-reactive agents that can inhibit aminophospholipid translocase activity (57–60). Even though N-ethy-lmaleimide-treated cells did not undergo apoptosis, they were recognized and engulfed by macrophages, albeit at very low levels compared with uptake of apoptotic cells. This uptake appeared to be PS-dependent, because it was inhibited by PS liposomes, although the possible effects of N-ethylmaleimide on other membrane proteins could also have contributed to uptake.

Uptake of apoptotic cells by human macrophages was found to be enhanced in the presence of serum, and it was suggested that complement opsonization was responsible for the enhancement (7). Uptake into macrophages in the experiments reported herein were performed in the absence of serum; however, the total levels of uptake by macrophages and fibroblasts but does not abrogate the requirement for PS recognition by either population. It is possible therefore that the serum either provides additional molecules to promote adhesion prior to uptake or that factors in serum stimulate uptake by phagocytes in a ligand-independent manner.

In summary, the data presented herein suggest that loss of phospholipid asymmetry and exposure of PS are required for recognition and removal of apoptotic cells by macrophages and other phagocytes. Based on our observations that only L stereoisomers induce ingestion of lipid-loaded cells, we suspect that this requirement implicates the newly reported PS receptor as a key molecule for signaling ingestion, regardless of which adhesion ligands are initially involved in binding the target to the phagocyte. We speculate that the ability to induce uptake by PS liposomes or the anti-PS receptor antibody (an IgM believed to be of relatively low affinity) may require a threshold level of this receptor on the phagocyte, providing one explanation for the disparity between the requirement for PS exposure described herein and the inability to inhibit uptake with PS liposomes in unstimulated macrophages.

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