Phosphatidylserine (PS) induces PS receptor–mediated macropinocytosis and promotes clearance of apoptotic cells

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Introduction

As cells undergo apoptosis, they are rapidly phagocytosed as intact bodies by cells in the surrounding tissue or by professional phagocytes such as macrophages and immature dendritic cells. This noninflammatory removal process occurs throughout the lifespan of an organism and is the fate of most dying cells. Clearance of apoptotic cells prevents the release of intracellular debris, reducing the likelihood of tissue damage resulting from inflammation or inappropriate autoimmune responses (Wyllie et al., 1980; Savill et al., 1992; Ren and Savill, 1998).

The exposure of phosphatidylserine (PS)* in the outer leaflet of the plasma membrane is one of the most striking changes on the surface of apoptotic cells (Fadok et al., 1992b; Schlegel et al., 1993; Koopman et al., 1994; Homburg et al., 1995; Martin et al., 1995; Vermes et al., 1995; van den Eijnde, 1998; Verhoven et al., 1999). Recent evidence has led to the hypothesis that PS exposure is crucial for the uptake of unopsonized, apoptotic cells (Fadok et al., 2001). Stereo-specific PS recognition has long been included in the list of apoptotic cell removal mechanisms (Fadok et al., 1992a), and we have recently identified and cloned a candidate PS receptor (PSR) that appears to participate in uptake of apoptotic cells and is found on the surface of many cell types (Fadok et al., 2000).

Many receptors have been proposed to mediate the phagocytosis of apoptotic cells (for reviews see Duvall et al., 1985; Savill, 1998; Savill and Fadok, 2000; Savill et al., 1993). These receptors include scavenger receptors (Pearson, 1996; Platt et al., 1996; Shiratsuchi et al., 1999; Zhou et al., 2001), CD14 (Flora and Gregory, 1994; Devitt et al., 1998), CD68 (Ramprasad et al., 1995; Sambrano and Steinberg, 1995; Sambrano et al., 1997), αβ3 (Albert et al., 1998, 2000), and a receptor system involving CD36, a class B scavenger receptor, in conjunction with thrombospondin and an integrin, such as αβ3 (vitronectin receptor [VnR];...
Savill et al., 1992). Additionally, members of the collectin family, such as mannose binding lectin and C1q, are thought to mediate apoptotic cell recognition through opsonization (Balasubramanian et al., 1997; Balasubramanian and Schroit, 1998; Ishimoto et al., 2000; Taylor et al., 2000; Schagat et al., 2001).

On the basis of inhibitor or antibody-blocking studies, phagocytic cells show significant redundancy in apoptotic cell recognition and are apparently able to use many receptors at the same time. Expression of individual receptors in a variety of cell lines has usually led to increased binding of apoptotic cells with minimal effects on uptake (Navazo et al., 1996; Devitt et al., 1998; Hamon et al., 2000). Even genetic deletion of scavenger receptor A (SRA) or C1q has generated only a partial reduction in clearance (Platt et al., 1996; Taylor et al., 2000). These results suggest that some receptors may act as a complex (Pradhan et al., 1997), a concept further supported by the apparent requirement for CD36 in the uptake seen with either the VnR–thrombospondin system (Savill et al., 1992) or that involving the PSR (Fadok et al., 2000).

The individual roles of these receptors in binding, phagocytosis, or transduction of antiinflammatory signals is currently unclear. To address these questions, we have developed a novel system to target individual receptors using erythrocytes conjugated to specific protein ligands or antibodies. Here we demonstrate that ligation of many receptors, including CD14, CD68, CD36, and αβ integrin, resulted in particle binding. However, bound particles were not ingested unless PS was present. The PS-dependent uptake of target particles was stereo specific and inhibited by anti-PSR antibodies. Further, we found that PSR stimulation resulted in membrane ruffling and formation of large, fluid-filled vesicles. We propose that engulfment of apoptotic cells is a two-step process involving an initial tethering event followed by PS-stimulated, PSR-mediated uptake by a process akin to macropinocytosis.

**Results**

**Ligation of many putative phagocytic receptors results in particle adhesion, but not ingestion**

To determine which phagocytic receptors may act to promote apoptotic cell binding and which induce engulfment, we developed a novel system using defined ligands on the surface of erythrocytes. The surface proteins of erythrocytes (E) were biotinylated (E_b) and coated with avidin (E_ba). These erythrocytes were then incubated with human monocyte–derived macrophages (HMDM), murine thioglycolate-elicited peritoneal macrophages (MPM), or other phagocytic cells. The use of FACS®-based uptake assays was explored (Licht et al., 1999; Albert et al., 2000), but was not useful in distinguishing between E_ba–anti-CD32 and E_ba–anti-CD36. The use of erythrocytes as target particles has several advantages. First, erythrocytes without surface alterations do not bind to phagocytes. Second, hypotonic lysis can be used to distinguish between E_ba–anti-CD32 and E_ba–anti-CD36. The use of erythrocytes to bind particles has several advantages. First, erythrocytes without surface alterations do not bind to phagocytes. Second, hypotonic lysis can be used to distinguish between erythrocytes that are bound to the phagocytes and those that have been ingested (Gigli and Nelson, 1968). Third, PS may be deliberately inserted into the erythrocyte membrane, allowing for recreation of one of the hallmark characteristics of apoptotic cells.

The efficient coating of erythrocytes with antibody, protein ligand, or various forms of PS was verified by flow cytometry (Fig. 1 A). The E_ba were then incubated with human monocyte–derived macrophages (HMDM), murine thioglycolate-elicited peritoneal macrophages (MPM), or other phagocytic cells. The use of FACS®-based uptake assays was explored (Licht et al., 1999; Albert et al., 2000), but was not useful in distinguishing between E_ba that had attached to the outside of the HMDM and those that had been engulfed. Therefore, light microscopy was used to accurately quantitate attachment and engulfment of E_ba (Fig.
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1 B). As expected, ligation of the Fcγ receptor IIA (CD32) with Ebab-anti-CD32 induced high levels of both binding and uptake, representing the phagocytic potential of the HMDM under these conditions. The use of antibodies for adequate triggering of uptake was further supported by the observation that Ebab-anti-C3bi receptor (anti-CD18) also demonstrated high levels of binding and uptake (unpublished data). Isotype control IgG or IgM on the erythrocytes induced neither binding nor uptake.

Using this system, it was determined that engagement of putative apoptotic cell receptors including anti-CD36, anti-αβ3 integrin (CD51/61), anti-CD14, and anti-CD68 resulted in binding of the erythrocytes to the macrophages without substantial uptake. Combining antibodies against CD36 with those against the VnR (CD51/61) did not significantly increase uptake (Fig. 2 A). Recently the αβ3 integrin has been described as mediating uptake of apoptotic cells by dendritic cells (Albert et al., 1998, 2000). However, engagement of this receptor using our system also promoted tethering with no uptake by HMDM (Fig. 2 B).

Protein ligands were also used to target specific receptors. Ebab–vitronectin and Ebab–thrombospondin, the natural ligands for αβ3 integrin, and the proposed thrombospondin receptor system, respectively (Pytela et al., 1985), were bound by the phagocytes, but were not substantially ingested (Fig. 2 C). As mentioned above, another candidate receptor for uptake is SRA (Platt et al., 1996). Because antibodies against the human version of this receptor are unavailable, the role of SRA was examined using MPM. Anti-SRA antibodies promoted some uptake of bound Ebab, but were far less efficient than the positive controls (Fig. 2 D).

PS induces uptake of tethered particles in a stereo-specific manner and is mediated by the PSR

Construction of Ebab–anti-PSR was not possible because the biotinylation of our monoclonal antibody (mAb 217) destroyed its ability to bind the receptor (unpublished data). Therefore, we used erythrocytes coated with PS to investigate the role of the PSR in binding versus uptake of particles. PS alone was not sufficient to mediate adhesion or engulfment of the erythrocytes. However, when membrane PS was combined with any of the ligands capable of tethering the Ebab to macrophages, adhesion was converted into engulfment (Fig. 3 A). PS on the surface of Ebab was sufficient to drive uptake even when Ebab were tethered to CD59, a surface molecule not thought to be involved in phagocytosis. This effect was also seen when PS-coated Ebab were tethered to NIH 3T3 fibroblasts (Ebab–anti-major histocompatibility complex [MHC]-I) or MPM (or Ebab–anti-F4/80-antigen; unpublished data). These results suggest that even though PS on the surface of a particle alone is not sufficient to confer particle binding, its presence is sufficient to trigger the engulfment of a tethered particle regardless of the initial adhesive ligand.
To determine whether the presence of PS on the membrane of E_{bab} promoted uptake in a specific manner, we used synthetic, stereo-isomeric PS in combination with tethering antibodies. The ability of PS to induce uptake of particles was found to be stereo specific in that the L isomer of PS, 1-palmitoyl-2-oleoyl-sn-3-glycerphospho-1-serine (POP-L-S; see Materials and methods), promoted uptake similar to the natural form (PS isolated from bovine brain), whereas the D isomer, 1-palmitoyl-2-oleoyl-sn-3-glycerphospho-2-serine (POP-D-S), had no effect on ingestion of antibody-tethered erythrocytes (Fig. 3 B).

Essential role for the PSR in PS-stimulated engulfment

The stereo-specific, PS-induced engulfment of various tethered E_{bab} suggested the involvement of a PS-specific receptor. In addition to the recently identified PSR (Fadok et al., 2000), other receptors for apoptotic cell engulfment have been reported to recognize PS (Rigotti et al., 1995; Sambrano and Steinberg, 1995; Devitt et al., 1998; Oka et al., 1998). To determine whether the PSR was involved in recognition of PS on erythrocytes, anti-PSR antibody blocking studies were performed. Preincubation of HMDM with anti-PSR antibodies minimally affected uptake through the Fcγ receptor, indicating that the antibody did not inhibit phagocytosis in general. However, preincubation of HMDM with anti-PSR antibodies before the addition of PS-coated E_{bab}–anti-CD36 inhibited the uptake of those particles (Fig. 3 C). This effect was not seen when HMDM were preincubated with an irrelevant isotype control antibody or an IgM that binds to CD11b.

To directly address the requirement for the PSR in the uptake of apoptotic cells, NIH 3T3 fibroblasts, which express the PSR, were transfected with a PSR antisense DNA construct, a sense construct, or an empty vector control. Apoptotic Jurkat cells were fed to transfected fibroblasts and their uptake was determined by light microscopy (see Materials and methods for induction and characterization of Jurkat apoptosis). When expression of the PSR was reduced with antisense transfection to below 25% of control levels (Fig. 4 A), the uptake of apoptotic cells was diminished by a nearly equivalent amount (Fig. 4 B). Transfection did not affect the phagocyte membrane or phagocytosis in a general manner, as both expression of the α chain of the VnR and uptake of latex beads were unaffected.

Finally, an important issue regarding the uptake of apoptotic cells versus other opsonized or nonself particles is the consequential release of antiinflammatory or inflammatory mediators, respectively. TGF-β is released from phagocytes after the uptake of apoptotic cells (Fadok et al., 1998) and is thought to play a key role in downregulating inflammatory responses (Shull et al., 1992; Kulkarni et al., 1993). Transfection of PSR antisense DNA inhibited the TGF-β response in 3T3 cells, after the uptake of apoptotic Jurkat cells (Fig. 4 C).

Stimulation of phagocytes with PS or anti-PSR antibody triggers “bystander uptake” of adherent particles

The above data suggests that the phagocytosis of apoptotic cells involves independent and separable events: particle tethering and engulfment. To investigate this further, we attempted to induce phagocytosis of adherent erythrocytes by secondary stimulation of the PSR. Erythrocytes were created that bound to the surface of NIH 3T3 fibroblasts by coating E_{bab} with biotinylated anti-D9/L^a MHC class I (E_{bab}–anti-MHC-I). These E_{bab} were not internalized in the absence of additional stimulation. Many growth factors, including EGF, PDGF, and macrophage colony-stimulating factor (M-CSF), stimulate the process of macropinocytosis (Brunk et al., 1976; Racoosin and Swanson, 1989; Hewlett et al., 1994), resulting in the ingestion of fluid, solutes, and macromolecules, as well as particles adhered to the cell surface (Galan et al., 1992). We observed that treatment of NIH
3T3 fibroblasts with EGF (or PDGF; unpublished data) stimulated the uptake of adherent Ebab–anti-MHC-I (Fig. 5A). Similarly, treatment of HMDM with M-CSF triggered the uptake of Ebab–anti-CD36 (unpublished data).

To stimulate the PSR, liposomes containing PS or a control phospholipid, phosphatidylcholine (PC), were added to the culture media after Ebab–anti-MHC-I were allowed to adhere to the phagocytes. The addition of POP-L-S liposomes, but not liposomes containing POP-D-S or PC, resulted in internalization of the bound erythrocytes. The same result was obtained when HMDM were stimulated with PS after binding of Ebab–anti-CD36; addition of antibodies to the Fc receptor on these macrophages did not stimulate the ingestion of tethered erythrocytes (unpublished data). We hypothesized that the anti-PSR antibody, an IgM, may aggregate the PSR and result in its activation. In support of this hypothesis, we found that stimulation of the phagocytes with anti-PSR antibodies, but not an IgM isotype control (unpublished data), also triggered the uptake of tethered erythrocytes (Fig. 5A). Importantly, the stimulatory effects of the anti-PSR antibody cease after 30 min, allowing for its dual use as a blocking antibody.

These results suggest that ligation of the PSR with exogenous PS or anti-PSR antibody is capable of triggering the engulfment of any particle tethered to the surface of the phagocyte. The stimulated ingestion of tethered particles is characteristic of a macropinocytosis-mediated engulfment mechanism.

Figure 4. TGF-β secretion and PS-dependent engulfment of targeted erythrocytes and apoptotic cells is mediated by PSR. (A) Transfection of NIH 3T3 fibroblasts with vectors containing PSR antisense DNA resulted in a decrease of PSR expression to 25% of control levels, as measured by flow cytometry. Antisense transfection did not alter the levels of other phagocytic receptors, shown here by flow cytometric analysis of CD51 (α1, integrin) expression. Transfection with sense PSR (unpublished data) or empty vector controls did not alter levels of PSR expression. □, PSR expression; ■, α1 expression. (B) The decreased expression of PSR induced by antisense transfection (Fig. 3A) resulted in a decrease of apoptotic cell engulfment compared with control levels. However, antisense PSR DNA transfection did not alter the ability of these cells to engulf latex beads. Data were normalized to control (unmanipulated) cells. All results represent mean ± SEM from at least three experiments. □, Apoptotic cell uptake; ■, latex bead uptake. (C) PSR is essential for TGF-β release by phagocytes during uptake of apoptotic cells. NIH 3T3 fibroblasts showed decreased production of TGF-β after an overnight feeding of apoptotic cells, as compared with unmanipulated fibroblasts and fibroblasts transfected with empty vector controls. All results represent mean ± SEM from at least two experiments.

Figure 5. PS- and PSR-mediated uptake of apoptotic cells occurs by a process akin to macropinocytosis. (A) To separately examine the tethering and engulfment events, Ebab were first allowed to tether to MHC-I on the phagocyte surface for 15 min, followed by stimulation with various forms of PS, anti-PSR antibody, or EGF (positive control) for 45 min. Ebab–anti-MHC-I were engulfed by fibroblasts when stimulated with EGF, anti-PSR, or POP-L-S, but not when stimulated with POP-D-S stereoisomer or PC. Results represent mean ± SEM from three experiments. (B) Amiloride, a specific inhibitor of macropinocytosis, inhibited the engulfment of apoptotic Jurkat T cells by NIH 3T3 fibroblasts at concentrations as low as 0.3 mM. Results represent mean ± SEM from three experiments.
Amiloride, an inhibitor of the Na⁺/H⁺ antiporter, is known to selectively inhibit the process of macropinocytosis (Swanson, 1989; West et al., 1989; Dowrick et al., 1993). To assess whether the engulfment of apoptotic cells was related to the process of macropinocytosis, we attempted to inhibit the uptake of apoptotic cells with amiloride. When NIH 3T3 fibroblasts were incubated in amiloride for 15 min before the addition of apoptotic Jurkat cells, uptake was inhibited at concentrations as low as 0.3 mM (Fig. 5 B). This result suggests that the phagocytosis of apoptotic cells, regardless of the receptors involved, is an amiloride-sensitive endocytic process.

**Apoptotic cells, PS, and anti-PSR antibody stimulate membrane ruffling**

The process of macropinocytosis is subsequent to the induction of membrane ruffling (Francis et al., 1993). To further examine the ability of PS and the PSR to stimulate the uptake of apoptotic cells by the process of macropinocytosis, we tested their ability to induce membrane ruffling in Swiss 3T3 fibroblasts. Cultured fibroblasts were briefly serum starved, and then cultured with viable or apoptotic Jurkat cells or PDGF as a positive control. When apoptotic cells were incubated with Swiss 3T3 fibroblasts, membrane ruffling was induced within 15 min, even when apoptotic cells were not obviously in contact with the phagocytes. Consistent with this observation, we also found that liposomes containing PS, but not PC, induced membrane ruffling (unpublished data). Membrane ruffling was rarely observed in unstimulated cultures or when viable Jurkat cells were added to fibroblast cultures (unpublished data). Anti-PSR antibody, but not an IgM isotype control antibody, induced ruffles in the plasma membrane of quiescent Swiss 3T3 fibroblasts at time points as short as 2 min, with increasing formation of stress fibers over a 20-min period (Fig. 6 A).

**PSR-mediated membrane ruffling requires Cdc42 and Rac1, but not RhoA activity**

Microinjection techniques were used to investigate the role of the low molecular weight GTPases Cdc42, Rac1, and RhoA in PSR-mediated membrane ruffling. Although inhib-
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iting RhoA with the microinjection of C3 transferase did not affect anti-PSR antibody-stimulated membrane ruffling, the microinjection of dominant negative recombinant protein for Cdc42 and Rac1 (N17Cdc42 and N17Rac1) resulted in the inhibition of membrane ruffling. Representative pictures of injected cells are shown, with neighboring uninjected cells shown for comparison (Fig. 6 B). Injected cells were visually inspected for the presence or absence of membrane ruffles. Inhibition of membrane ruffling by microinjection of N17Cdc42 or N17Rac1 was estimated at 20–40% inhibition and 40–90% inhibition, respectively, in four separate experiments. These results indicate that PS and PSR are capable of signaling changes in the actin cytoskeleton and these changes require both Cdc42 and Rac1. These findings are consistent with the requirement for Cdc42 and Rac1 in the uptake of apoptotic cells (Leverrier and Ridley, 2001) and macropinocytosis (Ridley et al., 1992; Swanson et al., 1995; Garrett et al., 2000).

Antis-PSR antibodies and PS stimulate macropinocytosis

Even though membrane ruffling is closely associated with the process of macropinocytosis, stimulation of membrane ruffling does not always result in vesicle formation (Li et al., 1997; Bretscher and Aguado-Velasco, 1998). HMDM were stimulated with either the anti-PSR antibody or PS-containing liposomes and examined for uptake of a water-soluble, membrane-impermeable fluorescent dye, Lucifer yellow (LY). Whereas untreated macrophages showed little or no uptake of LY, treatment of HMDM with M-CSF, which has been shown to induce macropinocytosis in macrophages (Racoosin and Swanson, 1989, 1992), resulted in uptake of LY into large vesicles (Fig. 7 A). Large vesicles containing the dye were also detectable in PS liposome-treated cells. However, little uptake of dye was seen when cells were stimulated with liposomes containing only PC. We also attempted to induce macropinocytosis with an anti-CD36 IgM antibody and saw little or no LY uptake. As observed for M-CSF and PS, anti-PSR antibody also stimulated the uptake of LY into large vesicles.

Macropinocytosis was assessed quantitatively using MPM due to donor-to-donor variability inherent with the use of HMDM. Cells were serum starved for 2 h and stimulated in the presence of dye. Cells were then washed free of extracellular dye, lysed, and dye content was measured by fluorimetry (Racoosin and Swanson, 1989, 1992; Swanson, 1989). Anti-PSR antibodies stimulated dye uptake to levels similar to M-CSF, whereas IgM isotype control antibodies had no effect on dye uptake (Fig. 7 B), similar to the results shown above.

Discussion

Apoptosis occurs throughout the lifetime of an organism, from embryogenesis to death. Recent evidence lends further support to the concept that efficient removal of apoptotic cells and cell bodies prevents exposure of surrounding tissue to potentially cytotoxic, immunogenic, or inflammatory cellular contents (Albert et al., 1998; Sauter et al., 2000). The removal process is completed by a wide variety of cell types and appears to involve multiple receptors and apoptotic cell ligands, yet the phagocytic mechanism has not been clearly defined. The data presented here suggest that uptake of apoptotic cells involves two separate steps: an initial tethering event followed by PS-stimulated macropinocytosis.

Many putative phagocytic receptors appear to mediate tethering of the apoptotic cell to the phagocyte. In contrast, PS by itself appears to promote poor binding of particles to
phagocytes, but plays a critical role in stimulating the actual uptake process through the PSR. The ability of PS to drive internalization of any tethered particle may, in part, explain the recent reports that apoptotic and necrotic cells appear to be recognized and engulfed in a similar manner (Chung et al., 2000; Cocco and Ucker, 2001). In this proposed scenario, binding and ingestion are separable events. However, the requirement for both a tethering receptor and an engulfment receptor is indispensable. It is plausible that the ability of macrophages, or professional phagocytes, to rapidly and efficiently clear apoptotic cells compared with their nonprofessional counterparts may be due to the wide repertoire of adhesion receptors, not to differences in engulfment mechanisms (Parnaık et al., 2000). In addition, we have recently suggested that the collectin family of innate immune system molecules can mediate uptake of apoptotic cells (Ogden et al., 2001), perhaps because they can enhance adhesion to phagocyte surfaces. However, surface calreticulin, a receptor for the collagenous domains common to the collectin family, also appears to be capable of stimulating macropinocytosis, perhaps through CD91 (A2 macroglubulin receptor/LRP), and may provide another example of apoptotic cell removal by this mechanism (Ogden et al., 2001).

The requirement for Rac1 during anti-PSR antibody-mediated membrane ruffling suggests that the PSR utilizes signaling pathways for apoptotic cell engulfment similar to those defined in the Caenorhabditis elegans system (Gumienny et al., 1999; Liu and Hengartner, 1999; Chung et al., 2000; Zhou et al., 2001). In addition, the PSR appears to require the activity of Cdc42 for the induction of membrane ruffling. How the PSR transmits signals to the low molecular weight GTPases is currently unclear. Although our results suggest that receptors such as SRA, α5 integrins, and CD36 do not transmit engulfment signals, these studies do not rule out the possible formation of a complex, where tethering receptors and the PSR act in concert to transmit engulfment signals. The integrin, αvβ3, has been shown to recruit Rac1 and Dock180 via the adaptor protein CrkII in HEK293 cells in the presence of apoptotic lymphocytes (Albert et al., 2000). It is interesting to speculate that the integrins implicated in apoptotic cell engulfment may be activated by the binding of target ligands on the apoptotic cell, resulting in the recruitment of Rac1 to the engulfment site and the induction or amplification of actin cytoskeleton rearrangement.

Surprisingly, PS alone on the surface of erythrocytes resulted in limited adhesion to the phagocyte surface. The lack of particle binding by PS–PSR interactions could reasonably be explained by either low surface expression of the PSR on the phagocytes, i.e., low avidity, or by low affinity between PS and the PSR. HMDM from many donors have been shown to express the PSR at low levels unless stimulated with TGF-β and β-glucan (Fadok et al., 2000). However, preliminary studies using HMDM, stimulated to upregulate PSR expression, have not resulted in increased erythrocyte binding, suggesting that low avidity is not responsible for the lack of PS-coated erythrocyte binding. Affinity studies are currently being conducted to determine the strength and specificity of the PS–PSR interactions. It is imagined that relatively low affinity PSR interactions with PS are markedly enhanced by localized expression of PS on the apoptotic cell and, in particular, by the binding of the apoptotic cell by recognition receptors.

As macropinocytosis represents a nearly universal mechanism for ingestion by eukaryotic cells, it follows that PS may be capable of initiating this mechanism in a wide variety of cell types. It is tempting to speculate that the presence of PS is a powerful and ubiquitous engulfment signal, capable of stimulating the ingestion of apoptotic cells by many different types of phagocytes, regardless of the initial tethering ligand. These data suggest that PS stimulation of macropinocytosis and apoptotic cell engulfment is mediated in majority by the PSR. The PSR appears to be expressed by nearly all tissue types, and PSR homologues have been identified not only in man and mouse but also in Drosophila melanogaster and C. elegans (Fadok et al., 2000). Although playing a minor role in the binding of apoptotic cells, the PSR is critical for their ingestion, as indicated by antisense transfection data. In addition to conducting a signal that contributes to apoptotic cell uptake, the PSR appears to be responsible for inducing the release of a key antiinflammatory, antimimunogenic molecule, TGF-β. The structure, localization, and characterization of domains on the PSR that mediate signaling for phagocytosis and cytokine production are currently under investigation.

**Materials and methods**

**Cells and cell lines**

Human monocytes were obtained from normal donors and cultured in X-Vivo 10 (BioWhittaker) containing 10% human serum. The differentiated macrophages (HMDM) were used on days 6 and 7 of culture. MPM were elicited with thioglycolate, harvested, and plated as previously described (Fadok et al., 1992a). NIH 3T3 fibroblasts were obtained from the American Type Culture Collection and cultured in DME (Cellgro®; Media- tech, Inc.), containing 10% fetal calf serum, sodium pyruvate, glutamine, penicillin, and streptomycin. Swiss 3T fibroblasts were obtained and cultured as previously described (Ridley and Hall, 1992; Ridley et al., 1992).

The Jurkat human leukemia T cell line was obtained from the American Type Culture Collection and cultured in RPMI 1640 (Cellgro®), containing 20% fetal calf serum (Gemini Bioproducts), glutamine, penicillin, and streptomycin. Apoptosis was induced by exposure to UV irradiation at 254 nm for 10 min, followed by 3 h of culture as previously described (Fadok et al., 2001). 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, according to the manufacturer’s instructions for using the annexin kit (R&D Systems).

Under these conditions, cells are <5% propidium iodide positive and 50–80% annexin V positive. Cells were also quantified by electron microscopy at the light microscopic level. By this method, these cells are typically 70–90% apoptotic.

**Antibodies and protein reagents**

Antibodies against human macrophage receptors included anti-CD32 (FL18.26; Pharmingen), anti-CD36 (CB38; Pharmingen), anti-CD11a/1b (23C6; Pharmingen), anti-CD68 (KP1; Dako), anti-CD59 (p282; Pharmingen), anti-α5β1 (P1F6; Chemicon), and anti-CD14 (MEM-18; Monosan®).

Anti–human PSR antibody (mAb 217; Fadok et al., 2000) was used for all experiments. Anti–H-2Dq/H-2Lq (anti-MHCI, D2.4G2; Pharmingen) and anti-SRA (2F8; Serotec) isotype control antibodies used included murine IgG (28–14–8, anti-H-2Dδ, provided by T. Potter, National Jewish Medical and Research Center, Denver, CO) or IgM (G155–228, anti-TNP; Pharmingen [or mouse myeloma; CalBiochem]) for HMDM experiments and rat IgG (A95–1, anti-TNP; Pharmingen) for murine macrophage experiments. Anti–H-2Dδ/H-2Lδ (anti-MHCI, KH17; Pharmingen) was used in experiments with NIH 3T3 cells. Antibodies used to block Fcγ receptors in phagocytosis assays included anti–human CD16, anti–human CD32, anti–human CD64, and anti–murine CD16/32 (3G8, FL18.26, 10.1, and 2.4G2, respectively; Pharmingen). Pro-
teins used as ligands for phagocytic receptors included human vitronectin (Life Technologies) and thrombospondin (Calbiochem). All antibodies and ligands were biotinylated (Boehringer) and resuspended in PBS before coating of erythrocytes.

**Construction of EBAB**

Human erythrocytes were obtained from normal donors and washed in PBS (pH 8.0). 3 × 107 erythrocytes were resuspended in 800 μl PBS and incubated with EZ-Link-Sulfo-NHS-LC-Biotin (Pierce Chemical Co.) according to the manufacturer’s instructions. The biotinylated erythrocytes (EB) were washed with PBS, resuspended in PBS, pH 8.0, and incubated with 120 μg streptavidin (Sigma-Aldrich) at room temperature for 30 min. Avidin-coated, biotinylated erythrocytes (EBa) were washed, resuspended in PBS, pH 7.4, and incubated with 5–10 μg of biotinylated antibody or protein. EBa were then washed and resuspended in DME before addition to phagocytes. Efficient coating of EBa was confirmed by flow cytometry using the appropriate fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

**Coating of erythrocytes with phospholipids**

Bovine brain PS, bovine liver PC, 1-palmitoyl-2-oleoyl-sn-glycerophosphorylcholine (POPC), and POP-L-S were obtained from Avanti Polar Lipids Inc. POP-D-S was synthesized from POPC by phospholipase D–catalyzed headgroup exchange in the presence of D-serine (Comfurius and Zwaal, 1977; Juneja et al., 1989). Unilamellar liposomes containing brain PS, liver PC, POPC, POP-L-S, and POP-D-S were prepared as previously described (Fadok et al., 2001). Association of liposomes containing the various forms of PS with erythrocytes was confirmed by flow cytometry after staining with phycoerythrin-annexin V (PharMingen).

**Phagocytosis assays**

For phagocytosis assays involving EBa, macrophages were plated in 24-well plates at 3 × 105 cells/well. Culture media was washed from macrophages and replaced with serum-free DME. Fc receptors on macrophages were blocked by preincubating the cells with a cocktail of monoclonal antibodies directed against Fcγ receptors for 10 min at room temperature. In some experiments, monoclonal antibodies against HMDM surface molecules were added at 100 μg/ml and incubated at 4°C for 30 min before addition of target EBa. Target EBa were added to macrophages at a ratio of 15:1 and allowed to incubate at 37°C for 30 min. Unbound EBa were washed away with HBSS. Lysis of unengested EBa was performed by adding deionized H2O for 10 s, followed by immediate replacement with DME. Cells were fixed with 0.75% glutaraldehyde, stained with dianisidine and H2O2 (Sigma-Aldrich), and counterstained with eosin. Binding and engulfment of EBa were quantified by light microscopy before and after hypotonic lysis, respectively. 200 cells were scored per well and experiments were repeated at least three times. Assays using apoptotic Jurkat cells as targets and NIH 3T3 fibroblasts as phagocytes were performed as previously described (Fadok et al., 2001). For some experiments, amiloride (Sigma-Aldrich) was added at varying concentrations in serum-free DME for 15 min before the addition of apoptotic target cells.

For stimulated phagocytosis assays, NIH 3T3 fibroblasts were plated in 24-well plates at 104 cells/well. EBa were allowed to adhere to fibroblasts for 15 min before stimulation with either 10 ng/ml EGF, 100 μM liposomes containing PC or a 50:50 molar ratio of PS/PC (prepared as described above). Cells were washed with PBS and stimulated with M-CSF (3,000 U/ml), mAb 217 (100 μg/ml), or IgM isotype control (10 μg/ml, PharMingen) in PBS containing 0.2% FCS. Before the experiments were initiated, equivalent cell numbers between individual wells were confirmed by the addition 5% WST-1 reagent (Roche Molecular Biochemicals) in macrophage culture media for 1 h during serum starvation. Supernatants were read at 450 nm in an ELISA plate reader. Using this method, relative cell number between untreated and stimulated cells was found to be consistent throughout the 3-h time course. Cells were washed in PBS and stimulated with M-CSF (3,000 U/ml), mAb 217 (100 μg/ml), or IgM isotype control (10 μg/ml, PharMingen) in PBS containing 0.2% FCS in the presence of 0.5 mg/ml Cascade blue. To terminate dye uptake, the media was aspirated and plates were washed by immersion in 1.0 liter ice cold PBS (three times, 5 min per wash). Cells were subsequently lysed with 300 μl of 0.1% Triton X-100 (Bio-Rad Laboratories) in distilled H2O. 100 μl of lysis was diluted into 2.9 ml ultrapure distilled H2O and read in a SLM 8000 fluorimeter (Spectronic Analytical Instruments) at 400 nm excitation, 440 nm emission.

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