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Review

If phosphatidylserine is the death knell, a new phosphatidylserine-specific receptor is the bellringer

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Abstract

Recognition of phosphatidylserine (PtdSer) is essential for engulfment of apoptotic cells by mammalian phagocytes. Engagement of a new phosphatidylserine-specific receptor (PtdSerR) appears to be necessary for uptake of apoptotic cells. Many other mammalian receptors have been described to function in the clearance of apoptotic cells. The emerging picture is that many of these receptors may provide the strong adhesion needed to increase the likelihood of contact between the PtdSerR and its phospholipid ligand, which is required for uptake. Furthermore, stimulation of this receptor on different types of phagocytes by apoptotic cells, PtdSer-containing liposomes or an IgM monoclonal anti-PtdSer antibody initiates release of TGFβ, known to be involved in the anti-inflammatory effects of apoptotic cells. Although highly homologous genes exist in *C. elegans* and *Drosophila melanogaster*, their role in engulfment of apoptotic cells remains to be determined. *Cell Death and Differentiation* (2001) **8,** 582–587.

Keywords: phagocytosis; apoptosis; phosphatidylserine; receptor; cytokines

Abbreviations: β2GPI, beta 2 glycoprotein 1; BMDM, bone marrow-derived macrophages; HMDM, human monocyte-derived macrophages; LDL, low density lipoprotein; PAF, platelet activating factor; PGE2, prostaglandin E2; POP-L-PS, 1-palmitoyl-2-oleoyl-sn-3 glycerophospho-L-serine; POP-D-PS, 1-palmitoyl-2-oleoyl-sn-3 glycerophospho-D-serine; PtdSer, phosphatidylserine; PtdSerR, phosphatidylserine receptor; TGFβ, transforming growth factor-beta

In order for phagocytes, including macrophages, to recognize and engulf them, apoptotic cells must lose phospholipid asymmetry and expose phosphatidylserine on the outer leaflet of the plasma membrane.[1] Learning how macrophages and other phagocytes recognize this phospholipid has been a crusade for our laboratories and those of many other investigators. Many different types of receptors have been implicated in the uptake of apoptotic cells. An emerging pattern is that many of the same recognition receptors responsible for recognizing and engulfing pathogens also mediate recognition and engulfment of apoptotic cells, yet the outcome is very different. These include lectins, Class A and Class B scavenger receptors, CD68 and other receptors for oxidized LDL particles, CD14, selected integrins, C1q and collectin binding proteins, complement binding proteins, and β2GPI binding proteins.[2–22] Uptake of apoptotic cells appears to be anti-inflammatory and possibly even immunosuppressive, at least with regard to self antigens. For example, it was recently shown that only necrotic tumor cells, but not apoptotic cells, could drive dendritic cell maturation and subsequent activation of T lymphocytes.[23] In contrast, uptake of pathogens is proinflammatory and promotes an adaptive immune response. Understanding this conundrum is an important goal for investigators studying the clearance of apoptotic cells by phagocytes.

A brief history of the quest for a phosphatidylserine-specific receptor

We have recently cloned the gene for a novel protein that appears to mediate specific recognition of phosphatidylserine on apoptotic cells by phagocytes and is highly conserved throughout phylogeny.[24] It was first reported as a gene of unknown function cloned from a human brain library; however, the authors made the observation that the gene was highly homologous to an undescribed gene from *Caenorhabditis elegans* contained on cosmid F2929.[25] A highly homologous gene is also found in the genome of *Drosophila melanogaster*; its function in this organism is also unknown. For lack of a better name, since it does not fall into any of the known receptor families, we have called this protein the phosphatidylserine-specific receptor (PtdSerR).[24]

The PtdSerR is expressed on macrophages, fibroblasts, endothelial cells, epithelial cells, melanoma cells (Fadok, unpublished data), and human dendritic cells (Fadok, unpublished data); in short, on virtually all the cells described to mediate clearance of apoptotic bodies.[24] It is not expressed on the surface of circulating cells such as lymphocytes, neutrophils, monocytes, or red blood cells. On macrophages, expression is variable. The expression on human monocyte-derived macrophages or mouse bone marrow-derived macrophages is low, unless they are stimulated with digestible particles such as β-glucan, zymosan, or even apoptotic cells themselves.[24] In contrast, thioglycollate-elicited peritoneal macrophages express high surface levels of this receptor, which

correlates with their ability to recognize apoptotic cells in a PtdSer-inhibitable manner. The human cDNA, when transfected into human Jurkat T cells and mouse M12.C3 B lymphocytes (two undisputedly nonphagocytic cell lines) enabled them to bind to and engulf apoptotic cells and PtdSer-expressing red blood cells.[24]

Several years ago we proposed the hypothesis that the ability to recognize PtdSer by macrophages was dependent on the macrophage subpopulation used.[26,27] These observations were based on whether uptake of apoptotic cells could be inhibited by PtdSer-containing liposomes or not. Based on our current data and those of others, we believe it is time to dispel this notion. It has become clear that all macrophages, and in fact, all phagocytes, recognize phosphatidylserine on apoptotic cells. Our earlier misinterpretation arose from the fact that inhibition assays, particularly when using liposomes, are relatively insensitive in determining how a macrophage recognizes apoptotic cells. Insensitive though they are, inhibition assays were all we knew to use in the infancy of the study of clearance. Savill and coworkers had originally reported that human monocyte-derived macrophages (HMDM) could recognize apoptotic cells using a complex of receptors, the $\alpha v\beta 3$ vitronectin receptor and CD36, which were bridged to the apoptotic cells by thrombospondin.[7,8] Uptake by these cells, as well as by mouse bone marrow-derived macrophages (BMDM), was inhibited by the tetrapeptide RGDS and antibodies against $\alpha v\beta 3$ or CD36, but not by PtdSer-containing liposomes.[26,28] In contrast, HMDM or BMDM treated with TGFβ and β-glucan lost their ability to be inhibited by the $\alpha v\beta 3$ antibodies, and acquired the ability to be inhibited by PtdSer-containing liposomes.[26,28] Pradham and coworkers, however, later went on to demonstrate that while uptake of apoptotic cells by BMDM was not inhibited by PtdSer-containing liposomes, it could be inhibited by PtdSer-expressing symmetric red blood cells.[29] They also observed that pretreatment with annexin V, PtdSer binding protein, could inhibit uptake of apoptotic cells by all macrophages studied.[30,31]

Recently, we reported that apoptotic cells which failed to express phosphatidylserine externally were not engulfed by either macrophages (stimulated or not to upregulate the PtdSerR) or fibroblasts.[1] Restoration of PtdSer in the outer leaflet by liposome transfer or by differentiating the cells prior to induction of apoptosis restored recognition and uptake by either type of macrophage or by fibroblasts. Furthermore, only L stereoisomers were effective; 1-palmitoyl-2-oleoyl-sn-3 glycerophospho-L-serine (POP-L-PS) but not POP-D-PS was able to signal the phagocyte for uptake, and inhibition was inhibited by the monoclonal anti-PtdSerR antibody. All the data at this point, therefore, are most consistent with the hypothesis that phosphatidylserine on apoptotic cells must be engaged by the PtdSerR on phagocytes before engulfment can proceed.

Distribution, structure, and function of the mammalian PtdSer receptor

The gene for the PtdSer-specific receptor encodes a type II protein of approximately 48 kd. A simple bar structure of the protein is shown in Figure 1. There are multiple serines in the predicted extracellular domain which appear to be glycoslyated, as deglycosylation reduces the apparent molecular weight in Western blots from approximately 70 to 48 kd.[24] There is at least one potential tyrosine phosphorylation site in the predicted intracellular domain. There are runs of basic residues in the extracellular domain, which could provide a binding site for the negatively charged phosphoserine head group of PtdSer. In addition, there is a weak WW domain as well; this domain has been implicated in the binding of phosphoserine and phosphotyrosine residues in proteins. It, therefore, could provide a potential binding site for phosphoserine in a phospholipid.[32] This protein does not contain the proposed consensus binding sequence for phosphatidylserine (FxFxLKxxxKxR) found in protein kinase C isoforms, phospholipase C, and phosphatidylserine decarboxylase.[33] Nor does it have any sequence similarities to the annexins, coagulation factors, vitronectin, or complement proteins known to bind to PtdSer.[34–38]

As mentioned before, we have observed the PtdSerR on many different types of cells which engulf apoptotic bodies, including macrophages, fibroblasts, epithelial cells, and endothelial cells.[24] Staining with the monoclonal anti-PtdSerR antibody on unfixed and nonpermeabilized cells has shown that the distribution of the PtdSer-specific receptor on the surface of fibroblasts, macrophages, and epithelial cells appears to be punctate rather than diffusely distributed around the membrane; other cell types remain to be tested. There are two possible explanations for this distribution which are currently being explored. First, the PtdSerR is associated with specific membrane lipid domains, such as caveolae or rafts; this remains to be explored but preliminary evidence obtained from mammary epithelial cells suggests that caveolin-1, a marker for caveolae, does not colocalize with the PtdSerR. Second, it is possible that PtdSer-containing vesicular debris from ongoing cell death in the cell cultures is continually cross-linking this receptor on the phagocyte surface. Figure 2 shows a fibroblast which has engulfed fluorescently-labeled apoptotic cells. In this case, the phagocytes were fixed prior to staining, to show that the PtdSerR clusters around the engulfed apoptotic bodies. With regard to tissue distribution, using RT–PCR, we have preliminary evidence that the gene is expressed in multiple tissues and organs, and as early as day 7 of embryogenesis in the mouse (unpublished data). This tissue expression pattern is consistent with our observation that the receptor is present on many different cell types.

Using a novel biotinylated red cell system, we have created particles for uptake which will trigger single receptors using specific antibodies or natural ligands (Hoffman P, Fadok VA, Henson PM, unpublished data). The red blood cells are biotinylated; biotinylated antibodies are then attached via a streptavidin bridge. Antibodies against human CD36, $\alpha v\beta 3$, CD68, CD14, or mouse SRA promoted binding but not uptake of these particles; anti-CD36, in particular, appeared to promote very strong binding. The red cells were only engulfed, however, if their outer plasma membranes had been reconstituted with phosphatidyl-L-serine; phosphatidyl-D-serine was not effec-

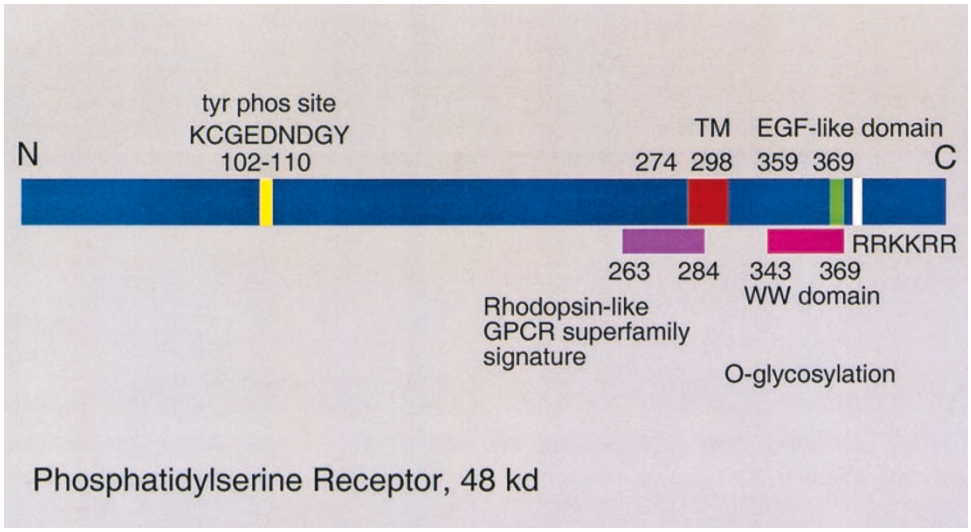

Figure 1 Bar diagram of the phosphatidylserine receptor, which is predicted to be a type II protein. The extracellular domain contains a weak motif for a WW domain which could represent a possible binding site for phosphoserine (see text). Alternatively, the run of basic residues (RRKKRR) could bind to the anionic head group of PtdSer. There is a potential tyrosine phosphorylation and multiple potential PKC phosphorylation site (not shown) in the predicted intracellular domain, which could provide signaling capabilities. TM=transmembrane domain. The significance of the rhodopsin GPCR-like motif and the EGF-like motif is not known at this time

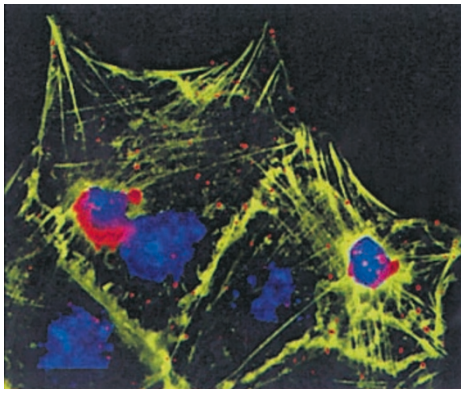

Figure 2 Phosphatidylserine receptor clusters around apoptotic bodies during engulfment. NIH3T3 cells were exposed to apoptotic Jurkat T cells, then fixed and permeabilized briefly with 4% paraformaldehyde in sucrose. The cells were stained with Alexa 488-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) to show actin, with DAPI to identify the nuclei of the phagocytes and the apoptotic bodies, and with mAb 217G8E9 followed by Cy3-labeled anti-mouse IgM (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Apoptotic Jurkat T cells have very little cytoplasm, accounting for the apparent antibody staining around their nuclei

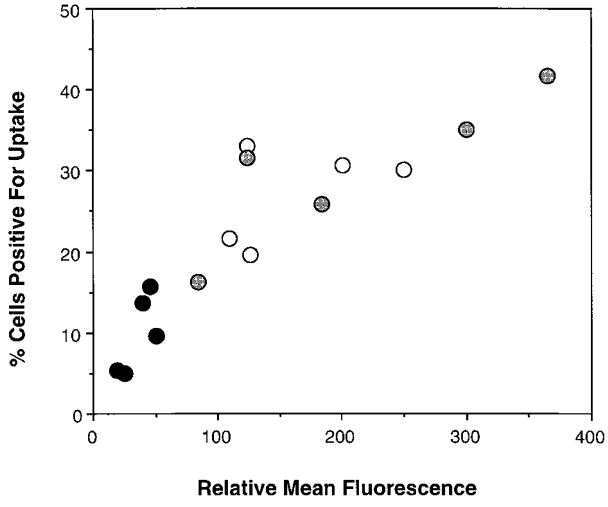

Figure 3 Expression levels of the PtdSerR is correlated with ability to take up apoptotic cells (n=5; r^2=0.78; $P<0.0001$). Expression of the PtdSerR was reduced by transfecting NIH3T3 cells transiently with pcDNA3.1 containing the reverse sequence of the entire PtdSerR, and assessing uptake of apoptotic Jurkat T cells 18 h later. Expression of the PtdSerR was assessed by flow cytometry, and levels are shown as the relative mean fluorescence (*x*-axis). Uptake is expressed as per cent cells positive for uptake (*y*-axis). The black circles represent those cells transfected with the antisense construct; the grey circles represent those cells that were mock transfected; the open circles represent those cells transfected with empty vector. Note that loss of PtdSerR expression is associated with reduced ability to take up apoptotic cells

tive. Uptake was then inhibited by anti-PtdSerR antibody. As an alternative, NIH3T3 cells were transfected with vector containing the sense or antisense sequence of the PtdSer receptor. The antisense construct significantly reduced expression of the PtdSer receptor and significantly inhibited uptake of apoptotic cells (see Figure 3). Taken together, these data suggest that many of the receptors involved in apoptotic cell recognition promote strong adhesion, but that the PtdSer receptor must be engaged for engulfment to occur.

The anti-inflammatory properties of the phosphatidylserine receptor

Exposure to apoptotic cells is found to be not only non-inflammatory,[49–51] but actively anti-inflammatory.[52–54] By

inducing the release of TGFβ and PGE2, apoptotic cells caused downregulation of chemokines, TNFα, IL1β, and IL10 from endotoxin-stimulated macrophages, as anti-TGFβ antibodies, indomethacin, and a platelet-activating factor receptor antagonist restored production of these cytokines.[52] Exposure of mouse macrophages (J774 cells) to apoptotic cells also resulted in the release of TGFβ and reduction in proinflammatory cytokines.[53] These effects could be mimicked by treatment with PtdSer-containing liposomes, as well as by mAb 217, suggesting that the interaction of phosphatidylserine on the apoptotic cells with this receptor on the phagocyte caused the release of anti-inflammatory mediators.[24] This interpretation receives further support from two of our preliminary observations: first, that the fibroblasts transfected with anti-sense constructs for PtdSerR not only fail to engulf apoptotic cells but also fail to produce TGFβ, and second, that apoptotic cells which fail to express phosphatidylserine externally fail to induce TGFβ secretion.

Does the PtdSerR play a role in engulfment of apoptotic cells in invertebrates?

The role of this gene product in engulfment of apoptotic cells by phagocytes from other organisms remains to be determined. It is intriguing to note that there is high homology between the mammalian receptor and the genes of unknown function in *Drosophila melanogaster* and *C. elegans*. To date, the role of this gene is being actively studied in both these organisms. The notion that this gene would function in engulfment of apoptotic cells in nonmammalian organisms is appealing, as phosphatidylserine, at least with regard to its polar head group, is not polymorphic. This phospholipid is found in the plasma membranes of all eukaryotic cells. Exposure of phosphatidylserine in the membranes of *Drosophila* cells undergoing apoptosis has been convincingly demonstrated by van den Eijnde and colleagues,[55] and it seems likely that worm cells would also undergo loss of phospholipid asymmetry, although this remains to be proven.

The study of cell corpse engulfment in *C. elegans* has revealed a wealth of information on the genes used by viable cells to recognize and engulf their dying neighbors, and it is encouraging to note that many similar genes are involved in mammalian recognition and engulfment.[56–60] Pairing the genetic and molecular studies in *C. elegans* and *Drosophila melanogaster* with the mammalian functional studies has proved to be a powerful tool, as illustrated by the characterization of *ced-7* and one of its human homologs the ABC1 transporter,[58,61–63] *ced-6* and its human homolog,[56,57,64,65] and *ced-2*, ced-5, and *ced-10* with CRKII, DOCK 180, and Rac-1[60,66–69] and fly croquemort and its human homolog CD36.[8,70,71] Furthermore, the identity of *ced-1* has recently been reported and the protein appears to be a surface receptor for recognition of apoptotic cells with homology to mammalian scavenger receptors.[72]

The future of the PtdSer-specific receptor

The PtdSer receptor we have identified will provide years of interesting work for us and hopefully others. There are many questions to be asked and answered. Our preliminary observations that this protein is expressed in intracellular membranes suggests a role in addition to recognition of apoptotic cells. It is possible that the PtdSerR could be important in intracellular phosphatidylserine transport, although it has no homologs in yeast as far as we have been able to determine and this is only speculation. How the protein recognizes phosphatidylserine and what accounts for the stereospecificity remains to be determined. It is unknown whether glycosylation is important for this function, or for some other function. The presence of charged residues within the predicted transmembrane domain suggests the potential for interaction with other proteins within the membrane, and the presence of cysteines in the extracellular domain suggest this protein may function as an oligomer, rather than a monomer.

What is clear at this point is that the PtdSer receptor binding to phosphatidylserine on the apoptotic cell is necessary for uptake of apoptotic cells and release of anti-inflammatory mediators. This PtdSer receptor is widespread, with regard to phylogeny, embryogenesis, tissue distribution, and cell type and is therefore likely to play a central role in clearance of apoptotic cells.

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