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Auto-Oxidation and Oligomerization of Protein S on the Apoptotic Cell Surface Is Required for Mer Tyrosine Kinase-Mediated Phagocytosis of Apoptotic Cells

Hiroshi Uehara and Emily Shacter

Prompt phagocytosis of apoptotic cells prevents inflammatory and autoimmune responses to dying cells. We have previously shown that the blood anticoagulant factor protein S stimulates phagocytosis of apoptotic human B lymphoma cells by human monocyte-derived macrophages. In this study, we show that protein S must first undergo oxidative activation to stimulate phagocytosis. Binding of human protein S to apoptotic cells or to phosphatidylserine multilamellar vesicles promotes antioxidation of Cys residues in protein S, resulting in covalent, disulfide-linked dimers and oligomers that preferentially bind to and activate the human Mer tyrosine kinase (MerTK) receptor on the macrophages. The prophagocytic activity of protein S is eliminated when disulfide-mediated oligomerization is prevented, or when MerTK is blocked with neutralizing Abs. Protein S oligomerization is independent of phospholipid oxidation. The data suggest that membranes containing phosphatidylserine serve as a scaffold for protein S-protein S interactions and that the resulting auto-oxidation and oligomerization is required for the prophagocytic activity of protein S. In this way, apoptotic cells facilitate their own uptake by macrophages. The requirement for oxidative modification of protein S can explain why this abundant blood protein does not constitutively activate MerTK in circulating monocytes and tissue macrophages. The Journal of Immunology, 2008, 180: 2522–2530.

Apoptosis is a physiological form of cell death that plays an essential role in tissue and organ development and homeostasis in multicellular organisms. Rapid removal of apoptotic cells by macrophages and certain types of neighboring cells before the loss of plasma membrane integrity prevents the leakage of potentially toxic and immunogenic cellular contents and thereby prevents inflammation and autoimmune responses to cell death (1, 2). This is thought to be one of the main physiological advantages of death through apoptosis instead of necrosis, in which cellular macromolecules may leak out and stimulate an inflammatory response before removal of the cells from the tissue (3, 4). Engulfment of apoptotic cells by macrophages also triggers production of anti-inflammatory and immunosuppressive cytokines, further limiting an immune response (5, 6). Studies using gene knockout mice have shown an association between autoimmune disease and abnormal clearance of apoptotic cells (7, 8).

The removal of apoptotic cells involves apoptotic cell surface molecules, phagocyte receptors, and soluble factors that modulate cell recognition and uptake (1, 9). Upon induction of apoptosis, cells lose the phospholipid asymmetry normally found in the plasma membrane and expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane (10, 11). The PS exposure is thought to be essential for the recognition and uptake of apoptotic cells by phagocytes (12–14).

Several different macrophage receptors have been identified as being involved in the phagocytosis of apoptotic cells (reviewed in Ref. 15). These include a PS receptor, integrins, CD14, CD36, and receptors for oxidized low-density lipoprotein. One key macrophage receptor is Mer tyrosine kinase (MerTK), which is a member of the TAM family of receptor tyrosine kinases (16). It is expressed on epithelial cells and monocyteic cells (17), both of which have phagocytic activity. The essential role of MerTK has been demonstrated for the phagocytosis of photoreceptor outer segment cells by retinal pigment epithelium cells (18, 19).

In addition to these phagocyte surface molecules, a number of soluble molecules have been identified that may control the interaction between apoptotic cells and phagocytes (2). These include protein S, MFG-E8, gas6, C1q, mannose-binding lectin, thrombospondin, pentraxin, β2-glycoprotein I, and surfactant proteins A and D. Opsonization of target cells with bridging molecules may expand the repertoire of potential phagocytic targets by allowing classical phagocyte receptors that are customarily associated with infection and immunity to mediate recognition and uptake of apoptotic cells and thereby limit the undesirable side effects from exposure to dead cell debris (20, 21).

We and others showed that the anticoagulant factor protein S is required for the efficient uptake of apoptotic lymphoma cells by macrophages in vitro (22, 23). Protein S, originally defined as an anticoagulant protein that is a nonenzymatic cofactor for activated protein C, is a vitamin K-dependent, 68-kDa monomeric protein present at a concentration of ~25 µg/ml in the blood (24, 25). It

2-methyl-propaimidaamide; (±)-9-HODE, (±)-9-hydroxy-10E,12Z-octadecadienoic acid; IAA, iodoacetamide; NEM, N-ethylmaleimide; TnCl, taurine chloramine; sMer, soluble Mer; LMV, large multilamellar vesicle; oxPLPS, oxidized PLPS; LDS, lithium dodecyl sulfate.
is known to bind to PS on cell surfaces in a Ca\(^{2+}\)-dependent manner using a Gla domain at the N terminus of the protein. Abruptly low levels of protein S may lead to inefficient uptake of early apoptotic cells and expose immune cells to potentially immunogenic cellular contents and thus trigger an autoimmune response (26). Deficiencies in protein S, either hereditary or acquired through autoantibody formation, lead to excess thrombosis (27) and are associated with autoimmune diseases such as systemic lupus erythematosus (28, 29). The newly discovered role of protein S in stimulating the phagocytosis of apoptotic cells reveals a novel functional link between the coagulation system and autoimmunity (26).

In this report, we investigated the biochemical and molecular mechanisms for stimulation of phagocytosis by protein S. We demonstrate that in order for protein S to serve as a phagocytic molecule, it must bind to membrane PS, either on the surface of apoptotic cells or in artificial phospholipid vesicles. Protein S then undergoes oligomerization through oxidation of cysteine residues and formation of intermolecular disulfide bonds. This oxidative activation of the protein is required for binding to and activation the macrophage MerTK. Thus, under physiological conditions, protein S activates MerTK and stimulates macrophage phagocytic activity only in the presence of apoptotic cells, thereby preventing circulating protein S from randomly activating macrophage activity.

Materials and Methods
Reagents and Abs

The following reagents were purchased from the vendors indicated. FBS, RPMI 1640, DMEM, HBSS, and PBS were from Mediatech. FITC-conjugated annexin V and PE-conjugated annexin V were obtained from BD Biosciences. Human protein S was obtained from Enzyme Research Laboratories. R&D Systems provided recombinant human gas6 protein (C-terminal 559-aa residues linked to an N-terminal hexameric His tag) and recombinant human Mer-Fc, which is a chimeric recombinant protein containing the extracellular domain of MerTK fused to the Fc region of human IgG. Protein assay reagent was obtained from Bio-Rad. Protein S was covalently modified by incubating with 100 mM IAA or 10 mM NaN\(_3\) in PBS at 5 mM and incubated with 2 mM AAPH for 3–4 h at 37°C. Lipids were extracted with chloroform:methanol (2:1, v/v), dried under \(\text{N}_2\) gas, and resuspended in PBS. Lipid oxidation was assessed by quantifying conjugated dienes from the secondary derivative of the UV spectrum around 230 nm using (±)-9-HODE as a standard (31). Approximately 15–18% of PLPS fatty acid chains were estimated to be oxidized by the AAPH treatment, which is similar to the level achieved by others (32) while no conjugated dienes were detected in DOPC or DOPC treated with AAPH.

Preparation of phospholipid vesicles

Large multilamellar vesicles (LMV) were prepared by mixing phosphatidylcholine (PC), PS, and rhodamine-labeled phosphatidylethanolamine in chloroform at a 70:30:0.5 molar ratio, or by mixing PC and rhodamine-labeled phosphatidylethanolamine at a 100:0.5 molar ratio, drying under a stream of \(\text{N}_2\) gas, and resuspending in PBS at a concentration of 5 mM by vigorous vortexing. DOPS and DOPC were used in all phospholipid vesicle experiments unless otherwise indicated.

Oxidation of phospholipids was performed by incubation with 2 mM AAPH. Protein-LPMV prepared as described above were suspended in PBS. Lipids were extracted with chloroform:methanol (2:1, v/v), dried under \(\text{N}_2\) gas, and resuspended in PBS. Lipid oxidation was assessed by quantifying conjugated dienes from the secondary derivative of the UV spectrum around 230 nm using (±)-9-HODE as a standard (31). Approximately 15–18% of PLPS fatty acid chains were estimated to be oxidized by the AAPH treatment, which is similar to the level achieved by others (32) while no conjugated dienes were detected in DOPC or DOPC treated with AAPH.

Phagocytosis assay

Phagocytosis was quantified as described previously (13). Briefly, human mononuclear cells were obtained by elutriation of human peripheral blood and cultured for 4–7 days in RPMI 1640 with 10% heat-inactivated FBS and 100 ng/ml recombinant M-CSF at a cell density of 0.5–0.8 × 10\(^6\) cells/well in 24-well culture plates. For phagocytosis of apoptotic cells, BL-41 cells were labeled with 0.12 \(\mu\)g/ml CFDA in binding buffer for 20 min at 37°C before apoptosis induction. The CFDA-labeled cells were treated with 0.1–0.2 mM LMV at 37°C for 1 h. The macrophages were harvested by centrifugation at 14,000 rpm for 12 min at 4°C. The washed cells were resuspended in serum-free RPMI 1640 supplemented with 100 ng/ml recombinant M-CSF, 10% FBS, and protease inhibitor mixture (Roche Diagnostics) for 20 min on ice. The nuclei were removed by passing the solution through a Sephadex G-25 desalting column.

Preparation of whole cell lysates and membrane fractions and Western blot immunosassay

Whole cell lysates were prepared by lysing cells in a buffer containing 10 mM HEPES (pH 7.4), 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1 M NaCl, 5 mM KCl, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\) (pH 7.4)) followed by addition of PI as described previously (13).

Modification of protein S with iodoacetamide (IAA), N-ethylmaleimide (NEM), or taurine chloramine (TnCl)

Protein S was covalently modified by incubating with 100 mM IAA or 10 mM NEM in PBS for 30 min at room temperature. The reaction was stopped by passing the solution through a Sephadex G-25 desalting column. TnCl was prepared by mixing 10 mM taurine with 2.5 mM HOCl, which results in immediate and quantitative consumption of all of the HOCl into TnCl (30). This solution was diluted to 0.5 mM TnCl in PBS containing 1.5 mM CaCl\(_2\) and incubated with protein S alone (0.25 mg/ml) or protein S bound to PS vesicles at 25°C for 1 h. The sulfhydryl-specific cross-linking of protein S was performed in PBS containing 0.5 mM BMEP00, under the same condition as described for oxidation with TnCl. The reactions were stopped by desalting with Sephadex G-25.

Preparation of plasma membranes

To measure uptake of rhodamine-labeled LMV, the macrophages were labeled with PE-conjugated anti-CD11b and -CD14 in binding buffer for 30 min at 4°C. Macrophages were recovered by incubating with 1% lipocidene in PBS containing 0.5% FBS, fixed with 2.5% formaldehyde in PBS, and quantified by two-color FACS analysis. The cells positive for both PE and CFDA are scored as macrophages that had ingested target apoptotic cells. The phagocytic index (percent) was calculated as the percentage of PE-positive macrophages that are also CFDA positive. For most experiments, the data were expressed as fold increase in the phagocytic index compared with the control. To measure uptake of rhodamine-labeled LMV, the macrophages were labeled with 0.12 ng/ml CFDA in PBS for 20 min at 37°C before incubating with 0.1–0.2 mM LMV at 37°C for 90 min. The macrophages were collected as described above. CFDA-positive and rhodamine-positive cells were scored as macrophages that had ingested LMV and calculated as a percent of the total number of macrophages.

Preparation of whole cell lysates and membrane fractions and Western blot immunosassay

Whole cell lysates were prepared by lysing cells in a buffer containing 10 mM HEPES (pH 7.4), 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1 M NaCl, 5 mM KCl, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), and protease inhibitor mixture (Roche Diagnostics) for 20 min on ice. The nuclei were removed by centrifugation at 14,000 × g for 12 min at 4°C. To isolate membrane...
fractions, the cells were first treated with digitonin buffer (10 mM HEPES (pH 7.4), 0.01% digitonin, 0.14 M NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, and protease inhibitor mixture for 5 min on ice followed by centrifugation at 14,000 × g for 2 min. The supernatant (cytosolic fraction) was removed and the pellet was solubilized with lysis buffer followed by centrifugation to remove nuclei (14,000 × g, 12 min). The supernatant of this fraction was used as a crude membrane fraction. Typically, ~20% of total protein was recovered in the membrane fraction. Protein concentrations were determined using either the Bradford assay or the DC protein assay from Bio-Rad using BSA as a standard. SDS-PAGE was performed using precast 4–12% Bis-Tris gradient polyacrylamide gels and MOPS buffer (Invitrogen Life Technologies). Western blotting to nitrocellulose or polyvinylidene difluoride membranes was performed according to standard protocols.

**Protein S and Mer-Fc coprecipitation**

Protein S (50 nM), recombinant Mer-Fc (10 nM) and PS-LMV (0.5 nM) were incubated in 0.2 ml of binding buffer (see above) containing 0.1% BSA. After 1 h on ice, 1% β-octylglucopyranoside was added and the mixture was incubated an additional 20 min on ice. The mixture was centrifuged at 13,000 × g for 12 min and then 10 μl of protein G-Sepharose beads were added to the supernatants. The suspension was kept at 4°C overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation. The beads were rinsed four times with binding buffer with 0.1% Triton X-100 and 0.1% Nonidet P-40. Proteins bound to the beads were eluted by lithium dodecyl sulfate (LDS) loading buffer overnight with gentle rotation.

**Data analysis**

The FACScan results were analyzed using CellQuest Pro software (BD Biosciences). Most experiments and Western blots were performed at least three times. Data are shown as the mean ± SD. Statistical significance was assessed by the Student’s t test.

**Results**

**Protein S stimulates uptake of PS-containing LMV**

As described previously, phagocytosis of apoptotic BL-41 cells by human monocyte-derived macrophages is enhanced significantly by the presence of FBS in the phagocytosis medium (22). Biochemical fractionation of the FBS and immunodepletion studies revealed that protein S is the only serum component required for the FBS-dependent stimulation of phagocytosis. As shown in Fig. 1, A and B, purified human protein S fully replaces the phagocytosis stimulatory activity of the serum. Here, we further investigated the role of PS in protein S-stimulated phagocytosis by measuring the ability of protein S to stimulate macrophage uptake of rhodamine-labeled LMV composed of PS and PC at a 30:70 ratio (PS-LMV), or PC alone (PC-LMV) (Fig. 1C). The uptake of LMV occurs primarily through phagocytosis and not through lipid membrane fusion (33). Consistent with the results with apoptotic cells expressing PS, the addition of protein S increased the uptake of PS-LMV by 3-fold, resulting in ~22% rhodamine-positive macrophages. Only ~7% of macrophages became rhodamine positive in the absence of protein S. No uptake of PC-LMV was observed, either in the presence or absence of protein S. These results further confirm the essential role of PS in the protein S-dependent stimulation of phagocytosis of apoptotic cells and phospholipid vesicles.

**Protein S stimulates phagocytosis through activation of MerTK**

For protein S to stimulate phagocytosis, it must not only bind to apoptotic cells but must also bind to and activate the phagocytic machinery of macrophages. Previous reports have suggested that protein S, like the homologous protein gas6, is a ligand for the TAM receptor tyrosine kinases such as MerTK and Tyro3 that are expressed on monocytes and macrophages (34). gas6 shares 44% sequence identity with protein S (35). To investigate whether a TAM tyrosine kinase plays a role in protein S-mediated phagocytosis, we examined the level of expression of MerTK, Axl, and Tyro3 in the macrophages by immunoblot analysis of macrophage lysates. A ~160-kDa band corresponding to MerTK was detected when neither Axl nor Tyro3 proteins were detected under the conditions used (data not shown). Based on these observations and recent reports implicating the role of protein S in MerTK-mediated clearance of outer segment by retinal pigment epithelium (18, 19), we focused our investigation on MerTK in macrophages. First, the role of MerTK in protein S-mediated phagocytosis was assessed using a neutralizing Ab to MerTK. As shown in Fig. 2, addition of
an anti-MerTK Ab completely inhibited the protein S-induced phagocytosis of apoptotic cells, indicating that MerTK is required for the protein S-dependent recognition and uptake of apoptotic BL-41 cells by macrophages. Because MerTK is known to be activated by tyrosine autophosphorylation, we investigated the effect of protein S on the activation of MerTK in macrophages co- incubated with apoptotic cells by measuring the level of tyrosine phosphorylation in MerTK protein immunoprecipitated from whole cell lysates. As shown in Fig. 3A, MerTK was rapidly tyrosine-phosphorylated after coincubation with apoptotic cells and protein S; phosphorylation was induced in <7 min and gradually decreased thereafter. Importantly, maximal phosphorylation of MerTK required the presence of both apoptotic cells and protein S; coinoculation of macrophages with apoptotic cells alone or protein S alone resulted in no MerTK phosphorylation above the basal level (see blots in Fig. 3B and densitometric quantification in Fig. 3C). The results with protein S contrast to those seen with the homologous serum protein gas6, which activated MerTK even in the absence of apoptotic cells. The time course of MerTK phosphorylation in response to protein S is consistent with the view that MerTK phosphorylation/activation precedes ingestion of the apoptotic cells (which takes ~20–30 min; Refs. 4 and 13).

**Protein S bound to apoptotic cells and to PS vesicles undergoes oxidative oligomerization**

The weak ability of protein S to stimulate MerTK activity in the absence of apoptotic cells suggested that protein S might be modified and activated in some way by apoptotic cells. To address this possibility, we analyzed the SDS-PAGE/Western blot profile of membrane-bound protein S following a 30-min incubation with apoptotic cells. Proteins in the membrane fractions were analyzed by Western blot immunoassay using a polyclonal Ab against protein S (Fig. 4A). Under nonreducing conditions, protein S bands at ~130 and 210–220 kDa were detected in addition to the 68-kDa monomer band. When the disulfide bonds were reduced with DTT, these high molecular mass bands disappeared and two bands at 68 and 72 kDa were seen. These match the bands seen with the protein S starting material. Based on the molecular masses and the elimination of the high molecular mass bands by a disulfide bond reducing agents, we conclude that the 130- and 210- to 220-kDa bands are disulfide-linked dimers and trimers of protein S, respectively. Disulfide-linked protein S dimers and trimers were also formed in protein S that had been incubated with PS-LMV, although with the vesicles the dimer band was predominant and the trimer band was only weakly formed (Fig. 4B). Because protein S did not bind significantly to PC, very little protein was recovered following incubation and centrifugation with PC-LMV; only a faint monomer band was detected under these conditions.

The role of free cysteine residues in the formation of protein S dimers/oligomers on PS surfaces was confirmed by blocking the cysteine residues with IAA, a sulfhydryl-reactive alkylating agent. The data in Fig. 4C show that IAA treatment of protein S prevented dimer formation during incubation with PS vesicles.
The source of the oxidation responsible for protein S oxidation was investigated next. Previous studies with CD36 indicated that its prophagocytic activity is stimulated by oxidized fatty acyl chains in PS and, to a lesser extent, PC (36). The possibility that lipid oxidation was also responsible for protein S oxidation in our experiments was considered unlikely given that the phospholipids vesicles that induce protein S cysteine oxidation (dimerization) are composed of dioleoyl PS and PC; oleic acid has only one unsaturated bond and is extremely difficult to oxidize. This was confirmed in our experimental system by exposing DOPS and DOPC vesicles to the potent lipid oxidant AAPH and measuring formation of conjugated dienes, which are a good measure of fatty acyl lipid peroxidation (31). Almost no conjugated diene formation was detected in dioleoyl vesicles (data not shown). A role for lipid oxidation in promoting protein S dimerization was also ruled out by creating oxidized lipid vesicles containing palmitoyl-linoleoyl PS (oxPLPS) and palmitoyl-linoleoyl PC; linoleic acid has two unsaturated bonds and is much more susceptible to oxidation than oleic acid. Treatment of these vesicles with AAPH resulted in roughly 18% of the fatty acid chains being oxidized to conjugated dienes. However, incubation of protein S with oxidized PLPS vesicles resulted in the same level of protein S dimers as seen with nonoxidized DOPS vesicles (Fig. 4D). In contrast, a significant increase in protein S oligomer formation was observed when protein S bound to DOPS-LMV was treated either with the cysteine oxidant TnCl or with the sulfhydryl-specific cross-linker BM(PEO)₉ (Fig. 4E). Both the cross-linker and TnCl-induced oxidative oligomerization were dependent on the presence of PS-LMV; little oligomerization was observed when free protein S in solution was treated with TnCl.

**FIGURE 4.** Dimerization/oligomerization of protein S (proS) after binding to apoptotic cells or phospholipid LMV. A, Analysis of proS incubated with apoptotic BL-41 cells. As described in Materials and Methods, BL-41 cells were induced to undergo apoptosis with etoposide (200 μg/ml) and then mixed with purified human protein S (8 μg/ml) in binding buffer for 30 min on ice. The membrane fraction was isolated and 10 μg of this fraction was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with rabbit anti-protein S. AC + proS denotes the membrane fraction from apoptotic cells incubated with protein S. Samples in the left two lanes were nonreduced and in the right two lanes were reduced with 50 mM DTT. B, Analysis of proS incubated with phospholipid LMV. proS (8 μg/ml) was incubated with 0.5 mM vesicles made from PS-LMV or PC-LMV in binding buffer containing 0.1% BSA for 1 h on ice. Following centrifugation, bound proteins were eluted with LDS-loading buffer and analyzed by Western blot immunoassay with anti-protein S Ab as described for A. C, Effect of IAA modification on protein S dimerization induced by PS-LMV. Left panel, Native protein S or IAA-treated protein S (proS-IAA) was incubated with PS-LMV. Bound protein S was isolated and analyzed as described for B. D, Effect of PS oxidation on protein S oligomerization. PLPS was oxidized (oxPLPS) with 2 mM AAPH as described in Materials and Methods. proS was incubated with either PLPS, oxPLPS, or DOPS and analyzed as described for B above. E, Protein S dimerization/oligomerization induced by chemical oxidation or cross-linking. Protein S was incubated with TnCl or the cysteine cross-linker BM(PEO)₉ in the presence or absence of DOPS-LMV as described in Materials and Methods. The data are representative of three independent experiments.

**FIGURE 5.** Protein S (proS) oligomerization by oxidized or nonoxidized phospholipid vesicles causes preferential binding to Mer-Fc. Western blot immunoassay of protein S coprecipitated with Mer-Fc in the presence or absence of different phospholipid vesicles (PL-LMV). PLPS denotes oxidizable, but unoxidized, palmitoyl linoleoyl PS vesicles; oxPLPS are the same vesicles oxidized with AAPH (see Materials and Methods). DOPS and DOPC are nonoxidizable dioleoyl PS and PC, respectively. SDS-PAGE was run under nonreducing conditions. The 68-kDa band corresponds to protein S monomer, which binds nonspecifically to protein G beads. IAA-modified protein S was used for the sample in lane 9. The data are representative of three independent experiments.
dimer/oligomers that formed during incubation with PS vesicles bound preferentially to Mer-Fc. These bands are only observed in coprecipitates from samples containing protein S, Mer-Fc, and PS vesicles. There was some background binding of monomeric protein S to the protein G beads in the absence of Mer-Fc (Fig. 5, lane 3) despite extensive washing and the use of different detergents (dehyocholate and SDS; data not shown). There is some specific binding of monomeric protein S to Mer-Fc (Fig. 5, lanes 1 and 4) despite reversible washing and the use of different detergents (deoxycholate and SDS; data not shown).

Inhibition of disulfide bond formation by pretreating the protein S with IAA significantly inhibited the level of dimers and trimers bound to Mer-Fc. The low level of dimers that bound even with IAA treatment were present in the starting protein S preparation (see Fig. 4A) and are actually enriched by binding to Mer-Fc. There was no difference in Mer-Fc binding when oxPLPS vesicles or nonoxidizable DOPS vesicles were used, and only background levels of oligomerization and Mer-Fc binding were seen with DOPC. Oxidation of the PS linoleoyl fatty acids with AAPH did not increase the level of protein S oligomers bound to Mer-Fc (Fig. 5, lane 6).

Second, we prevented protein S oligomerization by pretreating the protein with IAA and then measured its ability to stimulate the phagocytosis of apoptotic cells. Fig. 6A shows that IAA modification of protein S blocked completely the ability of protein S to stimulate the phagocytosis of apoptotic cells. IAA modification also blocked protein S-dependent phospholipid uptake by macrophages (Fig. 6B). A different sulfhydryl-blocking reagent, NEM, also completely inhibited the ability of protein S to stimulate phagocytosis (data not shown). Consistent with the role of MerTK in mediating protein S-stimulated phagocytosis, IAA modification inhibited the ability of protein S to stimulate MerTK phosphorylation during incubation with apoptotic cells (Fig. 6C).

**Discussion**

Protein S is present in the serum at concentrations (~25 μg/ml; Ref. 25) that well exceed the concentrations required to activate macrophage phagocytosis in vitro (<0.5 μg/ml; H. Uehara and E. Shacter, unpublished observations). Roughly 60% of protein S in the plasma is bound to C4BP, which inactivates the procoagulant activity of protein S (24). However, this still leaves an excess of protein S to stimulate monocyte/macrophages via Mer-Fc. Until now, a molecular mechanism for regulating the prophagocytic activity of protein S had not been well-understood. The findings presented here demonstrate that some macrophages, protein S must first undergo oxidative oligomerization on the apoptotic cell surface. This requirement likely prevents circulating protein S from constitutively stimulating monocyte/macrophage activities in tissues and in the circulation. Thus, protein S does not serve simply as a passive bridge between apoptotic cells and macrophages. Rather, a more complex mechanism exists in which protein S must first undergo Cys oxidation and oligomerization on the surface of apoptotic cells before it can stimulate phagocytosis. A potent cysteine oxidant such as taurine chloramine does not induce oligomerization of protein S in solution (see Fig. 4E). It only occurs on the surface of apoptotic cells or PS vesicles. Hence, three essential components are required for serum to stimulate the phagocytosis of apoptotic cells: PS as the apoptotic cell receptor, MerTK as the macrophage receptor, and disulfide-linked protein S dimers or oligomers as the catalysts. Interference with any of these components inhibits apoptotic cell clearance by macrophages. According to this model, PS exposed on the outer surface of apoptotic cells is not only an “eat me” signal (37) but also provides a scaffold for intermolecular protein S interactions and oxidative oligomerization of the protein. In this way, apoptotic cells enhance their own uptake by providing a platform for protein S activation. A model for the proposed mechanism through which protein S stimulates phagocytosis of apoptotic cells is shown in Fig. 7. In this model, the binding of protein S dimers induces the dimerization and activation of the MerTK. MerTK dimerization and activation has been demonstrated in studies using rCD8-MerTK chimeric molecules (38).

Formation of protein S dimers occurs through a spontaneous, nonenzymatic auto-oxidation of cysteine residues and is independent of lipid oxidation. Dimerization occurs when protein S is bound to DOPS vesicles that are resistant to oxidation, and the use
of experimentally oxPLPS vesicles does not enhance dimer formation. Based upon these data, and the absence of any other oxidizing reagents in the experiments with phospholipid vesicles, we conclude that protein S undergoes auto-oxidation when bound to membranal PS. The data do not rule out the possibility that oxidizing species in apoptotic cells also contribute to protein S oxidation in the cell system, as has been proposed for other experimental systems (32, 36). Addition of the protein disulfide isomerase inhibitor bacitracin to the cell incubations did not inhibit either protein S dimer formation or phagocytosis of apoptotic cells, suggesting that this key cell surface enzyme is not responsible for catalyzing the observed disulfide bond formation (data not shown). Taken cumulatively, the results suggest that binding of protein S monomers to a PS scaffold aligns the cysteine residues in a way that promotes the dimerization/oligomerization by auto-oxidation. The free cysteine residues that can potentially participate in the intermolecular disulfide bond are at positions 47, 72, 247, and 527 in the mature, secreted form of protein S. The Cys residues at 47 and 72 are localized between the PS-binding Gla domain and the epidermal growth factor-like domain. Cys-247 is localized between the fourth EGF-like domain and the SHBG domain, and Cys 527 is within the SHBG domain. Additional studies will be required to determine the specific disulfide bonding pattern that leads to activation of protein S phagocytic activity.

The requirement for a PS scaffold for protein S activation mirrors the role of protein S as a cofactor in the protein C anticoagulation system, which was the only known activity defined for protein S before its phagocytic activity was discovered (39). When serving as an anticoagulant cofactor, the interaction between protein S and activated protein C also requires the presence of a membrane surface containing PS (40). In both cases, the binding of protein S to PS involves the N-terminal Gla domain of the protein. It will be interesting to determine whether protein S oligomerization is essential for its role in supporting activation of protein C under physiological conditions. In this regard, it is notable that noncovalent protein S multimers that exist in some purified protein S preparations show a higher PS binding affinity and a 100-fold higher APC-independent anticoagulant activity (41). Annexin V, which is another PS-binding protein, also forms noncovalent oligomers upon binding to PS vesicles. Thus, oligomerization may be a common outcome for proteins bound to a PS surface (42).

The data presented in this report show that disulfide-linked protein S dimers (and possibly oligomers) stimulate phagocytosis by activating macrophage MerTK and that protein S must undergo dimerization to stimulate MerTK. These conclusions are supported by the findings that a neutralizing Ab to MerTK inhibited protein S-stimulated phagocytosis, and that prevention of dimerization with SH-blocking agents (IAA or NEM) completely inhibited the ability of protein S to stimulate both phagocytosis and MerTK tyrosine phosphorylation. In addition, activation of MerTK by protein S was only observed when the macrophages were coincubated with both protein S and apoptotic cells; very little MerTK activation was observed when the macrophages were incubated either with apoptotic cells alone or with protein S alone. These findings are in contrast to the homologous serum protein and MerTK ligand gas6, which activated MerTK even in the absence of apoptotic cells, thereby revealing a significant difference in biological activity between protein S and gas6.

Recently, Sather et al. (43) described the presence of a soluble form of Mer (sMer) in human plasma and culture medium, indicating that this might be a possible mechanism for regulating gas6-stimulated phagocytosis. sMer is a proteolytic cleavage product of the MerTK receptor and consists of the extracellular domain of MerTK. It sequesters gas6 and blocks its ability to activate MerTK and stimulate phagocytosis. This observation highlights an interesting and important difference between regulation of the biological activities of protein S and gas6. As demonstrated here, protein S oligomers are much more effective ligands for MerTK protein than protein S monomers. It may therefore be predicted that circulating protein S monomers can bypass the inhibitory effects of sMer and stimulate engulfment of apoptotic cells upon binding to PS on the cell surface.

The involvement of MerTK, gas6, and protein S in the phagocytosis of apoptotic cells in vivo has been studied in two different rodent models. A role for MerTK was initially identified from studies of naturally occurring human and rat MerTK mutations that caused a failure of phagocytosis of the outer segment by retinal pigment epithelium, resulting in retinal degeneration (44–46). Subsequent studies using MerTK knockout mice revealed that macrophages lacking MerTK are unable to ingest apoptotic thymocytes, either in vivo or in vitro (8, 47). Previous work showed that gas6 is a ligand for MerTK, Tyro3, and Axl tyrosine kinases (34, 48, 49) and suggested that it stimulates photoreceptor outer segment phagocytosis by the retinal pigment epithelium (50). However, more recent studies found that gas6 knockout mice develop normal retina, suggesting the involvement of another molecule in MerTK-mediated phagocytosis of outer segment (18, 19). Consistent with this finding and our previous findings, Hall et al. (18) showed that protein S stimulates outer segment uptake and that protein S-dependent stimulation is abrogated in retinal pigment epithelial cells that lack MerTK activity. Furthermore, mouse protein S was found to activate MerTK in isolated eye cups devoid of anterior eye and neural retina (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such asyers and neural retina (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such as Fordson and colleagues (19). Our findings provide the molecular basis for protein S binding to MerTK. Interestingly, the results may explain why previous studies failed to detect human protein S-mediated activation of any of the human TAM kinases such.
as MerTK and Tyro3 (51, 52); those experiments were conducted in the absence of a PS scaffold.

Disregulation of protein S may have clinical impact that relates to its role in stimulating the phagocytosis of apoptotic cells, most notably autoimmunity and sepsis (26). Protein S deficiency is a marker for the autoimmune disease systemic lupus erythematosus (29). Whether this deficiency is the cause or the effect of lupus symptoms is not clear, but the relationship is consistent with the view that the efficient phagocytosis of apoptotic cells is required to prevent autoimmune reactions to intracellular molecules released by dying cells. According to our data, a possible cause and effect relationship between protein S deficiency and autoimmune disease should be examined, possibly through targeted disruption of the protein S gene in an experimental animal model. In a different clinical setting, the binding of protein S to apoptotic cells in the circulation may contribute to the progression of disseminated intravascular coagulation during sepsis. In this situation, both proteins S and C would be depleted through their binding to apoptotic cells and subsequent removal by monocytic cells (26). If this is correct, then supplementation of sepsis patients with both protein S and activated protein C would be a more effective therapy than administration of activated protein C alone (53).

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References


