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Promotion of the Uptake of PS Liposomes and Apoptotic Cells by a Product of Growth Arrest–Specific Gene, *gas6*

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Gas6, a ligand of receptor tyrosine kinases Axl, Sky, and Mer, potentiates cell proliferation and prevents cell death. It also contains γ-carboxylglutamic acid residues that mediate the interaction of some blood coagulation factors with negatively charged phospholipids. In our previous study, we demonstrated that Gas6 specifically binds to phosphatidylserine (PS) and links Axl-expressing cells to the PS-coated surface. In this study, to further understand the biological role of the interaction of Gas6 with PS, we examined the effect of Gas6 on the uptake of PS liposomes by macrophages. *In vitro* phagocytosis studies showed that Gas6 enhanced the uptake of PS liposomes approximately threefold and that the interaction of Gas6 with the surface of macrophages was essential for this enhancement. Analyses of the mechanism of the uptake of PS liposome suggested that Gas6 interacts with PS liposome *via* its N-terminal Gla domain and with macrophages *via* its C-terminal domain. Like that of PS liposomes, the uptake of apoptotic cells by macrophages was also enhanced, approximately twofold, in the presence of Gas6. These findings suggest that Gas6 may help phagocytic cells recognize cells with PS exposed on their surfaces, which is considered to be one of the mechanisms for clearing away dying cells. Thus, Gas6 may play a critical role in homeostasis by facilitating the clearance of PS-expressing cells.

Key words: Axl, Gas6, macrophage, phagocytosis, phosphatidylserine.

Gas6 was initially identified as a gene product whose expression in fibroblasts increased during the growth-arrested state, although its biological activity was not clearly known (*1*). We purified Gas6 as a protein that is released from cultured vascular smooth muscle cells (VSMCs) and specifically potentiates VSMC proliferation mediated by Ca^{2+}-mobilizing receptors (*2*). Another biological activity of Gas6 is the prevention of cell death of VSMCs and fibroblasts (*3*, *4*). Gas6 has also been suggested to be a ligand for receptor tyrosine kinases Axl, Sky, and Mer (*5–8*). The biological activities of Gas6 are probably mediated by these receptor tyrosine kinases.

The outer leaflet of eukaryotic cell membranes contains most of the cholinephospholipids, whereas the aminophospholipids are mainly present in the inner leaflet of the cell (*9*). While asymmetry seems to be the rule for normal cells, loss of membrane lipid asymmetry, in particular the emergence of phosphatidylserine (PS) at the cell surface, seems to be involved in the recognition and elimination of apoptotic (*10*) and senescent cells (*11*).

Several mechanisms might be responsible for the clearance of PS-expressing cells by phagocytes. These include direct recognition *via* specific PS receptors (*10–13*) and class B scavenger receptors (*14–16*) or an indirect mechanism that might include thrombospondin-dependent vitronectin receptors (*17*) and antibody-dependent recognition *via* Fc receptors.

Gas6 is composed of definite structural motifs: a Gla domain, four epidermal growth factor-like repeats, and a C-terminal domain (*1*). The Gla domain is rich in γ-carboxylglutamic acid residues, which are found in some factors in blood coagulation. The function of the Gla domain is thought to be mediation of the Ca^{2+}-dependent binding of Gas6 to negatively charged phospholipids. In a previous study, we examined the interaction of Gas6 with phospholipids and found that it specifically bound to phosphatidylserine (PS) and that the interaction was dependent on Ca^{2+} and Gla residues (*18*). We demonstrated further that Gas6 functions as an adhesion molecule that binds Gas6 receptor–expressing cells to PS-coated surfaces (*18*). In this paper, we characterize the effect of Gas6 on the uptake of PS liposomes by macrophages. *In vitro* phagocytosis studies showed that Gas6 enhanced the uptake of PS liposomes by macrophages, and that the interaction of Gas6 with the surface of macrophages, probably with its specific receptor, Axl, is essential to this enhancement. Gas6 also enhanced

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Abbreviations: Gla, gamma-carboxylglutamic acid; PL, phospholipid; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DCP, dicetylphosphate; Chol, cholesterol; DMSO, dimethylsulfoxide; VSMC, vascular smooth muscle cell; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; HBS, Hank's buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; DMEM, Dulbecco's modified eagle medium; PMSF, phenylmethylsulfonylfluoride; ECD, extracellular domain.

the uptake of PS-expressing apoptotic thymocytes by macrophages. These findings suggest that Gas6 may help phagocytic cells recognize cells with PS exposed on their surfaces, which is considered to be one of the mechanisms for clearing away dying cells.

MATERIALS AND METHODS

Materials—Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and PS were purchased from Avanti. Methylprednisolone, cholesterol, and dicetylphosphate were from Sigma. FITC-annexin V was from Bender MedSystems, and FITC-streptavidin was from ZYMED.

Preparation of Recombinant Rat Gas6, Gla Domain-Deficient Gas6, and Axl Extracellular Domain (Axl-ECD)—The cDNA constructs were subcloned into the PUC-SR_α expression vector. CHO cells were transfected with rat Gas6 expression plasmid or His-tagged human Axl extracellular domain (Axl-ECD) expression vector. Confluent CHO cells were cultured in protein-free culture medium PM-1000 (Eiken, Tokyo) in the presence of 4 μM vitamin K_2. Recombinant Gas6 was purified from culture medium as described elsewhere (*2*).

The culture supernatants of CHO cells transfected with plasmid containing the His-tagged cDNA of Axl-ECD were pooled and concentrated. This sample was then applied to a Ni-charged chelating column equilibrated with 10 mM imidazole, 0.5 M NaCl, and 20 mM sodium phosphate buffer, pH 7.4. The column was washed with the same buffer and eluted with 500 mM imidazole, 0.5 M NaCl, and 20 mM sodium phosphate buffer, pH 7.4. Peak fractions were dialysed against 1 mM PMSF and 20 mM Tris-HCl buffer, pH 7.4, and loaded on a Q-sepharose column equilibrated with 1 mM PMSF and 20 mM Tris-HCl buffer, pH 7.4. The column was washed with the same buffer. The bound proteins were eluted with a gradient of NaCl from 0 to 0.5 M in 1 mM PMSF and 20 mM Tris-HCl buffer, pH 7.4. The His-tagged Axl-ECD was eluted with 0.25 M NaCl. The protein was pure as judged by SDS-PAGE.

Gla domain-deficient Gas6 expression plasmid was transfected into COS-7 cells by lipofection. The cells were cultured for 3 days in PM-1000. Gla domain-deficient Gas6 was purified from the culture medium by use of an anti-rat Gas6 antibody column with monitoring by Western blot analysis using anti-Gas6 antibody.

Binding to Phospholipids—The binding of Gas6 to lipids was examined by enzyme-linked immunosorbent assay (ELISA). Lipids were dissolved in ethanol, and 50 μl each of the individual lipids was used to coat each well of the ELISA plate (Corning). Wells coated with ethanol alone were used as the control. The wells were kept at 25°C for 18 h to evaporate the ethanol, then blocked by incubation with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 3% bovine serum albumin (BSA) for 2 h at room temperature. Next, the wells were washed with TBS containing 5 mM $CaCl_2$ and incubated with Gas6 or Gla domain-deficient Gas6 in TBS containing 1% BSA and 5 mM $CaCl_2$. The binding of Gas6 or Gla domain-deficient Gas6 was determined using rabbit anti-Gas6 IgG and peroxidase-conjugated anti-rabbit IgG (Chemicon International). The anti-Gas6 IgG detected Gas6 and Gla domain-deficient Gas6 equally by ELISA and Western blotting (data not shown).

Liposomal Preparation—The liposomes were composed of phosphatidylcholine (PC)/phospholipid (PL)/dicetylphosphate (DCP)/cholesterol (Chol)/rhodamine-phosphatidylethanolamine (PE) (molar ratio 80:20:10:75:2). We used PC, phosphatidylserine (PS), PE, and phosphatidylinositol (PI) as phospholipids. The indicated lipids (3.74 μmol) were suspended in chloroform and evaporated under N_2 gas to make lipid films. Each lipid film was resuspended in 1 ml of PBS and sonicated. We used this solution as a 3.74 mM liposome solution.

Induction of Apoptosis in Thymocytes and Flow Cytometry—Thymocytes were obtained from C57BL/6J mice. Thymocytes (5×10^7 cells/ml) in 10% FCS/DMEM were incubated with 10 μM methylprednisolone (diluted from a 10 mM stock in DMSO) at 37°C for 4 h. Control thymocytes were incubated with identical concentrations of DMSO alone. Cells (1×10^6) were stained with Gas6, biotinylated-anti-Gas6, FITC-streptavidin or FITC-annexin V in Hanks' buffered saline (HBS) containing 0.5% BSA and 0.1% NaN_3 for 30 min on ice, washed, and analyzed using a FACScan (Becton-Dickinson, Mountain View, CA).

Macrophage Recognition and Uptake—Macrophages were obtained from the peritoneal cavity of C57BL/6J mice 4 days after an intraperitoneal injection of 2 ml of thioglycollate broth. The cells were washed, resuspended in Dulbecco's modified eagle medium (DMEM) containing 0.1% BSA, and plated in 24-well tissue culture plates (Coaster) (0.5×10^6 cells/well). The macrophage monolayer was washed with PBS and incubated with 50 μM fluorescence-labeled liposomes or ^{51}Cr-labeled thymocytes in DMEM containing 0.1% BSA. After incubation for 1 h, macrophages were washed twice with HBS containing 0.2% BSA and twice with HBS. The cells were then lysed with 0.1 N NaOH containing 0.1% SDS, and the accumulation of fluorescence or ^{51}Cr by cells was quantified by measurement of fluorescence intensity or radioactivity of cell lysates.

Preparation of [^{125}I]Gas6—[^{125}I]Gas6 (6,000 cpm/fmol) was prepared by a chloramine T method as described elsewhere (*19*).

Binding Experiments—For binding assay with macrophages, the macrophages in 12-well dishes were washed twice with binding medium (Hank's solution, pH 7.4, 0.1% bovine serum albumin), then incubated with 0.3 nM [^{125}I]-Gas6 and 300 nM non-labeled Gas6 or Axl-ECD at 4°C for 2 h in 0.3 ml of binding medium. The reaction was stopped by rapid removal of the medium, and the cells were washed three times with ice-cold binding buffer, then solubilized in 1 N NaOH. The radioactivity was measured with a γ-counter. Specific binding was defined as the difference between binding in the presence and absence of the unlabeled Gas6 (300 nM).

RESULTS

Interaction of Gas6 with PS—To determine the binding specificity of Gas6 to liposomes, we analyzed the binding activity of Gas6 to lipids with the same lipid composition as liposomes. ELISA plates were coated with the indicated lipids, and the binding activity of Gas6 was examined by ELISA as described above. The result (Fig. 1) shows that Gas6 binds specifically to PS-containing lipids, but does not bind to other phospholipid-containing lipids.

Effect of Gas6 on the Uptake of PS-Liposomes by Macrophages—Because Gas6 binds specifically to PS, it seemed reasonable to assume that Gas6 might serve as a specific marker for macrophages to recognize PS-expressing cells. To model PS-dependent phagocytosis, we investigated the effect of Gas6 on the uptake of PS-containing liposomes. We incubated mouse macrophages for 1 h in media containing 50 μM fluorescence-labeled liposomes at 37°C in the presence or absence of 40 nM Gas6 and examined them using fluorescence micrographs (Fig. 2). Macrophages incubated with PS-liposomes exhibited only a slight fluorescence in the absence of Gas6. But in the presence of Gas6, the macrophages accumulated a massive amount of fluorescence. On the other hand, macrophages incubated with PC-liposomes exhibited no significant fluorescence. For quantitative analysis, we lysed cells incubated in liposome-containing media and measured the fluorescence intensity of the cell lysate (Fig. 3). Only slight uptake of PS-liposomes by macrophages was noted in the absence of Gas6. On the other hand, in the presence of 40 nM Gas6, the uptake of PS-liposomes was about threefold higher than that in its absence. This result shows that Gas6 promotes the uptake of PS liposomes by macrophages. Gas6 had no effect on the uptake of PC, PE, or PI-liposomes (Fig. 3). These results are consistent with the binding specificity of Gas6 to phospholipids shown in Fig. 1.

Binding of Gas6 to Macrophages—Using reverse transcription PCR (RT-PCR), we found that the mouse macrophages express mRNA of Axl, one of the specific receptors of Gas6 (data not shown). We next analyzed binding of [^{125}I]Gas6 to macrophages in the absence or presence of

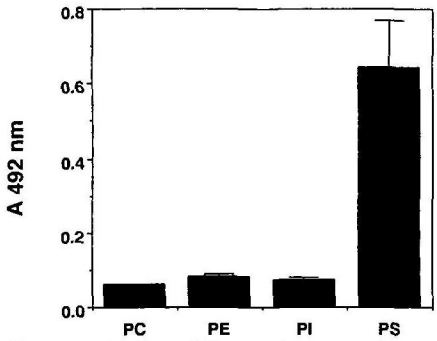

Fig. 1. **Binding specificity of Gas6.** The wells of an ELISA plate were coated with lipids and incubated with 4 nM Gas6. As described in "MATERIALS AND METHODS," the binding of Gas6 to lipids was determined by ELISA with rabbit anti–rat Gas6 IgG and peroxidase-conjugated anti–rabbit IgG. Data are mean ± SD (n = 3).

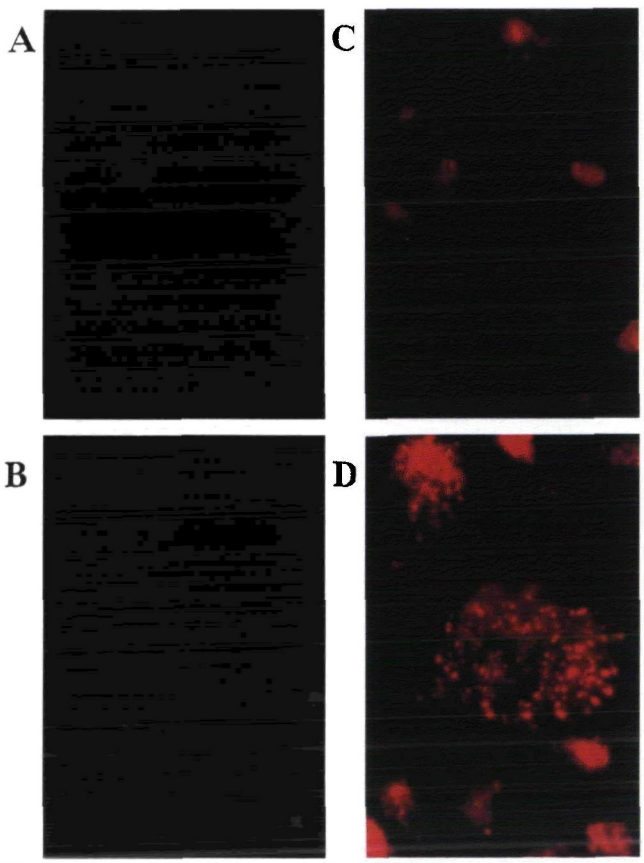

Fig. 2. **Promotion of the uptake of PS liposomes by macrophages in the presence of Gas6.** Macrophage monolayers were incubated for 1 h at 37°C with fluorescence-labeled PC liposomes (A, B) or PS liposomes (C, D) in the absence (A, C) or presence (B, D) of 40 nM Gas6. The monolayers were then washed, and accumulation of liposomes by macrophages was observed using fluorescence micrography (× 400).

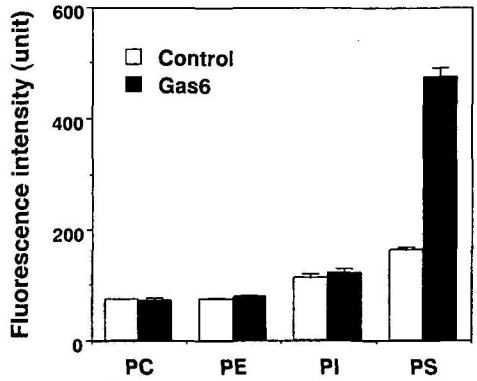

Fig. 3. **Uptake of liposomes by macrophages.** Macrophages monolayers were incubated with fluorescence-labeled liposomes for 1 h at 37°C in the absence (open bars) or presence (solid bars) of 40 nM Gas6. The plates were then washed, and the uptake was quantified by fluorescence intensity. Data are mean ± SD (n = 3).

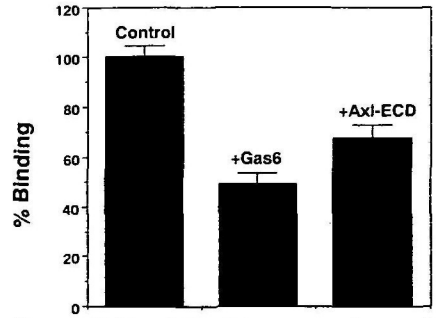

Fig. 4. **Binding activity of Gas6 to macrophages.** Macrophage monolayers were incubated for 2 h at 4°C with 0.3 nM [^{125}I]-Gas6 and 300 nM non-labeled Gas6 or Axl-ECD. The monolayers were then washed, and binding of [^{125}I]Gas6 was quantified by measuring the radioactivity of the cell lysate. Data are mean ± SD (n = 3).

non-labeled Gas6. Figure 4 showed that binding of [^{125}I]-Gas6 was inhibited by excessive (1,000-fold) non-labeled Gas6, indicating that Gas6 specifically bound to macrophages. Furthermore, we examined the effect of Axl extracellular domain (Axl-ECD) on the specific binding of [^{125}I]Gas6 to macrophages and found that Axl-ECD inhibited the binding (Fig. 4). Since Gas6 interacts with Axl *via* its C-terminal domain, the above data strongly suggest that Gas6 binds to the surface of macrophages *via* its C-terminal domain.

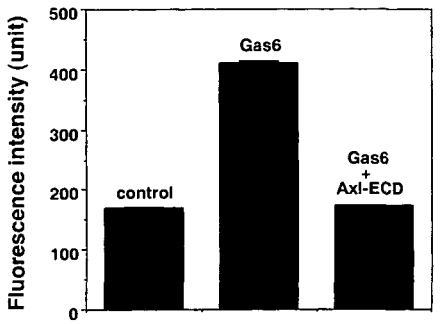

Fig. 5. **Inhibition of Gas6-stimulated uptake of PS liposomes by Axl-ECD.** Macrophage monolayers were incubated with fluorescence-labeled PS liposomes for 1 h at 37°C with or without 60 nM Axl-ECD in the absence or presence of 40 nM Gas6. The plates were then washed, and the macrophages were lysed. The uptake was quantified by measurement of fluorescence intensity of cell lysate. Data are mean ± SD (n = 3).

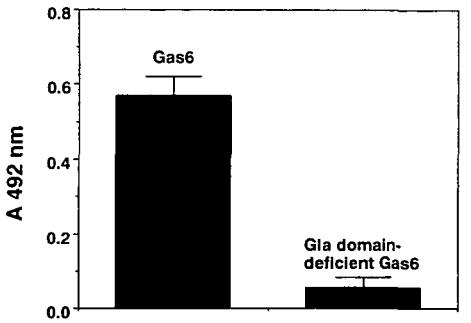

Fig. 6. **Binding activity of Gla domain–deficient Gas6 to PS.** PS (30 pmol/well)-coated plates were incubated with 14 nM Gas6 or Gla domain–deficient Gas6. Gas6 and Gla domain–deficient Gas6 which did not bind to the wells were removed, and the bound protein was quantified with rabbit anti–Gas6 IgG. Data are means ± SD (n = 3).

Mechanism of Gas6-Mediated Phagocytosis—To investigate whether the enhancement of the uptake of PS liposomes by Gas6 requires the interaction of Gas6 with the surface of macrophages, we examined the effect of Gas6 on the uptake of PS-liposomes in the presence of Axl-ECD, which binds to receptor-binding site of Gas6 and inhibits the interaction of Gas6 to the surface of macrophages. Figure 5 shows that in the presence of Axl-ECD, Gas6 did not enhance the uptake of PS-liposomes by macrophages. These data suggest that the interaction of Gas6 with the surface of macrophages is essential to the enhancement of the uptake of PS-liposomes.

To confirm that the enhancement of the uptake of PS-liposome by Gas6 is mediated by the interaction of Gas6 with PS, we prepared Gla domain-deficient Gas6 and examined the effect of the mutant on the uptake of PS liposome by macrophages. The Gla domain is rich in γ-carboxy-

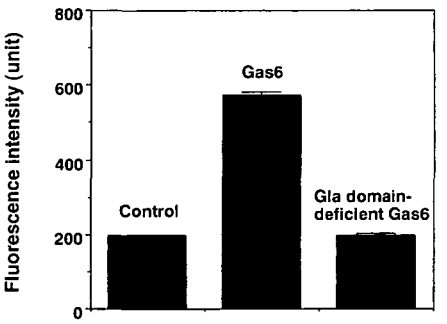

Fig. 7. **Uptake of PS liposomes by macrophages in the presence of Gla domain–deficient Gas6.** Macrophage monolayers were incubated for 1 h with fluorescence-labeled PS liposomes in the presence of 40 nM Gas6, 40 nM Gla domain–deficient Gas6, or none. The plates were then washed, and the macrophages were lysed. The uptake of liposomes was quantified by measurement of fluorescence intensity of cell lysate. Data were means ± SD (n = 3).

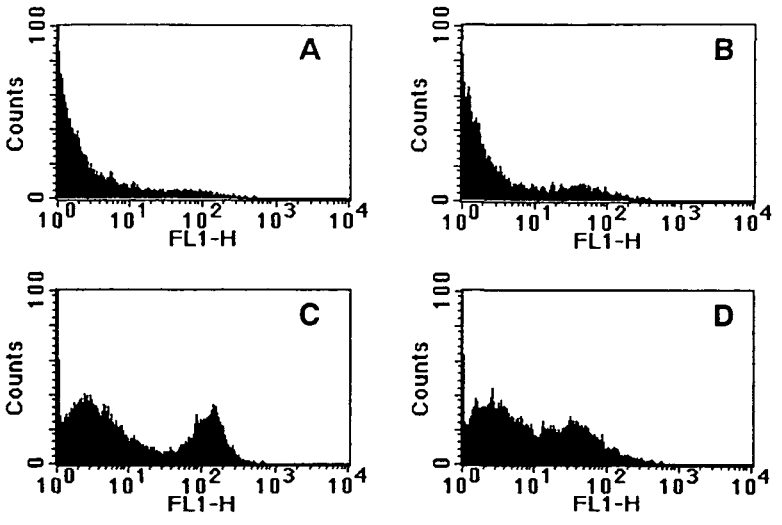

Fig. 8. **Flow cytometry analysis of the binding of Gas6 to apoptotic thymocytes.** Thymocytes were incubated for 4 h in the absence (A, C) or presence (B, D) of 10 μM methylprednisolone (MPS). The cells were then stained with Gas6, biotinylated anti-Gas6, and FITC-Streptavidin (C, D), or with FITC-annexin V (A, B), and analyzed by flow cytometry.

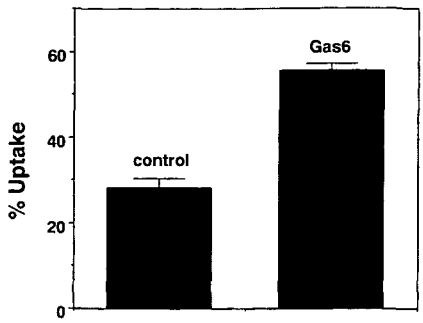

Fig. 9. **Promotion of uptake of apoptotic thymocytes by macrophages in the presence of Gas6.** ^{51}Cr-labeled apoptotic thymocytes were incubated with macrophage monolayers for 1 h at 37°C in the presence or absence of 40 nM Gas6. The plates were then washed, and the macrophages were lysed. The uptake of apoptotic thymocytes was quantified by measuring the radioactivity of the cell lysate. Data are means ± SD (n = 3).

glutamic acid (Gla) residues, which are thought to mediate its Ca^{2+}-dependent binding to negatively charged phospholipids. As expected, Fig. 6 shows that Gla domain-deficient Gas6 is unable to bind to PS, suggesting that the Gla domain is essential for Gas6 binding to PS. We then examined the effect of Gla domain-deficient Gas6 on the uptake of PS-liposomes by macrophages and found that it caused no enhancement (Fig. 7). The results indicated that the interaction of Gas6 with both PS-liposomes and the surface of macrophages is necessary for enhancement of the uptake of PS-liposomes. These data suggest a new phagocytosis pathway for PS-liposomes mediated by Gas6 and its specific receptor, probably Axl.

Interaction of Gas6 with Apoptotic Thymocytes—Many studies have shown that exposure of PS on the outer leaflet is a characteristic feature of cells undergoing apoptosis. Because Gas6 binds PS specifically and enhances the uptake of PS-liposomes by macrophages, it may mediate the recognition and uptake of apoptotic cells. To test this, we induced mouse thymocytes to undergo apoptosis with corticosteroid and examined the binding activity of Gas6 to these apoptotic thymocytes. Figure 8 shows that cultures incubated with corticosteroid developed a subpopulation of cells stained with annexin V. This indicates that apoptosis was efficiently induced. When cells were stained with Gas6 and anti-Gas6, the population of cells with fluorescence increased on induction of apoptosis. This shows that Gas6 binds to apoptotic cells.

To determine whether Gas6 influences the binding and uptake of these cells by macrophages, we induced ^{51}Cr-labeled thymocytes to undergo apoptosis and examined the influence of Gas6 on the uptake of these apoptotic cells by macrophages. Figure 9 shows that Gas6 enhanced the uptake approximately twofold. This result shows that Gas6 also mediates the uptake of apoptotic cells by macrophages. Therefore Gas6 may play an important role in the recognition and uptake of apoptotic cells.

DISCUSSION

Gas6 was first identified as a gene product whose expression in cells increased during the growth-arrested state, although its biological activity was not known (*1*). Later, we and other groups found that Gas6 promotes cell proliferation (*2*) and prevents cell death (*3*, *4*). As Gas6 has been found to act as a ligand of a receptor tyrosine kinase family (*5–8*), these biological activities of Gas6 are thought to be mediated by activation of the receptor tyrosine kinases. Studies using truncated mutants of Gas6 have demonstrated that the C-terminal region of Gas6 contains a receptor-interacting site and is involved in activation of the receptor tyrosine kinases (*20*).

At the N-terminal, Gas6 has a characteristic structure, the Gla domain. This domain, containing a cluster of negatively charged Gla residues, is found in some blood coagulation factors which exhibit their activity on lipid surfaces (*21*, *22*). The interaction of these coagulation factors with lipids is thought to be mediated through Ca^{2+}-dependent binding of Gla residues with negatively charged phospholipids (*23*, *24*). In the previous study, we examined the interaction of Gas6 with phospholipids and clarified that Gas6 specifically binds to PS, and that the binding is dependent on Ca^{2+} and Gla residues (*18*). These findings raised the possibility that Gas6 might function as a cell adhesion molecule that serves as a link between cells expressing PS and cells expressing Gas6 receptors. Based on this hypothesis, in this study, we examined whether Gas6 would stimulate the uptake of PS liposomes or PS-exposing cells by macrophages and demonstrated that it has remarkable activity in this regard. Therefore, stimulation of the recognition and uptake of PS-exposing cells by macrophages may be another important biological function of Gas6.

Normal cells do not have PS exposed on the outside of their cell membranes. The outer leaflet of the cell membranes usually contains most of the cholinephospholipids, while the aminophospholipids such as PS or PE are mainly present in the inner leaflet of the cells (*9*). However, in apoptotic cells or senescent erythrocytes, the membrane surface properties are altered: the membrane lipid asymmetry is lost and PS appears on the membrane surfaces. The presence of PS on the cell surfaces has several potential biological consequences. The most important one may be recognition and uptake by phagocytes. Several molecules, including class B scavenger receptor (*13*, *16*, *25*, *26*), CD36 (*27*), CD68 (*13*), CD14 (*28*, *29*) and β_2-glycoprotein I (*30*), have been proposed as candidate molecules for PS receptors.

As shown in this study, however, the uptake of PS liposomes by macrophages is very low in the absence of Gas6, and Gas6 significantly increased the uptake. The major differences between Gas6-dependent and independent mechanisms may lie in their time course and their lipid-specificity. Nishikawa *et al.* (*31*) reported that macrophages took up liposomes containing PS or PI in a time-dependent manner. The uptake continued for at least 36 h. Therefore, Gas6-independent mechanisms may be involved in long-term elimination of PS-exposing particles, and the Gas6-dependent mechanism in quick response.

To obtain insight into the molecular mechanism of Gas6-dependent uptake of PS-liposomes, we examined the effect of Axl-ECD on the specific binding of Gas6 to macrophages and on the uptake of PS liposomes. Figure 4 demonstrates that Axl-ECD inhibited specific binding of Gas6 to macrophages. Since Axl-ECD binds to the C-terminal domain of Gas6 (*20*), the result suggests that Gas6 binds specifically

to the surface of macrophages *via* its C-terminal domain. Axl-ECD also completely inhibited Gas6-stimulated uptake of PS liposomes (Fig. 5). These results indicate that interaction of the C-terminal domain of Gas6 with the surface of macrophages is necessary for the uptake of PS liposomes. Since macrophages express Axl mRNA (data not shown), it is most likely that Gas6 interacts with macrophages *via* Axl. However, it is possible that Sky or Mer, other receptors for Gas6, also bind Gas6 on macrophage surfaces, although the affinity of Sky or Mer with Gas6 is weaker than that of Axl (*8*).

Figure 7 demonstrates that Gla domain–deficient Gas6, which can activate its receptor (*20*) but does not interact with PS (Fig. 6), does not stimulate uptake of PS liposomes, indicating that the N-terminal Gla domain is necessary for the stimulation of PS liposome uptake. Thus, Gas6 seems to bridge macrophage membranes and PS liposomes by binding to the PS *via* the N-terminal Gla domain and to the receptor *via* the C-terminal domain, resulting in stimulation of uptake of PS liposomes by macrophages.

The above results using liposomes suggested that Gas6 might also recognize PS expressed on cells. Because apoptosis involves a reorientation of lipids in the cell plasma membrane that results in the surface expression of PS, we induced apoptosis in thymocytes and examined whether Gas6 would bind to PS-expressing apoptotic cells and how Gas6 affects the uptake of these cells by macrophages. Upon steroid-induced apoptosis, a higher fraction of the cells bound to Gas6, indicating increased expression of Gas6-binding sites at the cell surface (Fig. 8). The peak of Gas6-binding cells was smooth and the cells were distributed over a wide range of fluorescence intensity. This meant that the level of exposed PS on the surface of apoptotic cells was very diverse.

As in the case of PS liposomes, Gas6 enhanced the uptake of apoptotic cells by macrophages (Fig. 9). Macrophage uptake of the added thymocytes was approximately 60% in the presence of Gas6 and approximately 30% in its absence. As mentioned above, several reports have demonstrated that PS liposomes or PS-expressing cells are taken up by PS receptors in the absence of Gas6. However, our results suggest that Gas6-dependent phagocytosis may be more important than Gas6-independent phagocytosis. Fadok *et al.* reported that apoptotic cells were taken up *via* a PS-specific receptor by mouse peritoneal macrophages (*10*), but this receptor has not been identified. They incubated macrophages for a relatively long period with apoptotic cells. If Gas6 is released from the macrophages during the long incubation, the Gas6-dependent mechanism may explain the PS-specificity of the PS-specific receptor.

In summary, Gas6 seems to be involved in the recognition and clearance of cells that fail to preserve membrane lipid asymmetry. This would include not only cells undergoing apoptosis but also cells involved in related events such as activation of platelets and cell senescence.

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