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Tethering of Apoptotic Cells to Phagocytes through Binding of CD47 to Src Homology 2 Domain-Bearing Protein Tyrosine Phosphatase Substrate-1

Kazutoshi Tada,* Masato Tanaka,*† Rikinari Hanayama,* Keiko Miwa,*‡ Azusa Shinohara,*‡ Akihiro Iwamatsu,*§ and Shigekazu Nagata*‡¶

Apoptotic cells are swiftly phagocytosed by macrophages and immature dendritic cells. In this study, we found that one mouse macrophage cell line (BAM3) engulfed apoptotic thymocytes, but not a lymphoma cell line (WR19L), mAbs that inhibited the phagocytosis of apoptotic thymocytes by BAM3 were identified. Purification of the Ag revealed that it was Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1 (SHPS-1). CD47, the ligand for SHPS-1, was expressed in mouse thymocytes, but not in WR19L. When WR19L was transformed with CD47, the transformants, after induction of apoptosis, could be phagocytosed by BAM3. The WR19L transformants expressing CD47 were more efficiently engulfed in vivo by splenic dendritic cells than the parental WR19L. Masking of the phosphatidylserine exposed on apoptotic thymocytes inhibited the engulfment, whereas the anti-SHPS-1 mAb inhibited not only the engulfment, but also the binding of apoptotic cells to phagocytes. These results indicate that macrophages require CD47 and phosphatidylserine on apoptotic cells for engulfment, and suggest that the interaction between CD47 and SHPS-1 works as a tethering step in the phagocytosis.

Phagocytes engulf apoptotic, but not healthy cells, indicating that the apoptotic cells present an “eat me” signal (or signals) to the phagocytes (10), and the phagocytes recognize the signal using a specific receptor. In the early stage of the apoptotic process, the surface of the plasma membrane dramatically changes, and phosphatidylserine (PS)3 that is on the inner leaflet of the plasma membrane in living cells is exposed to the cell surface, suggesting that PS is an “eat me” signal presented by apoptotic cells (11, 12). Other possible ligands in apoptotic cells are exposed sugars such as galactose, mannose, and N-acetylgalactosamine, oxidized low density lipoprotein (LDL), and ICAM-3 (8, 13). Various molecules have also been proposed to act as a receptor for apoptotic cells or as a bridging molecule between apoptotic cells and phagocytes. These include αvβ3 integrin, αvβ5 integrin, class A scavenger receptor, CD36, CD14, PS receptor (PSR), a receptor tyrosine kinase called MER, thrombospondin, and milk fat globule epidimal growth factor 8 (MFG-E8) (8, 14, 15). The identification of a large number of candidate molecules suggests significant redundancy in apoptotic cell recognition and engulfment. To explain this redundancy and understand the role of individual receptors, Hoffmann et al. (16) recently proposed a two-step model for phagocytosis. In this model, apoptotic cells are first tethered to phagocytes through the interaction between putative ligands and receptors, then engulfed by phagocytes through PS-stimulated macropinocytosis.

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Abbreviations used in this paper: PS, phosphatidylserine; CMFDA, 5′chloromethyllnatoce diacetate; FasL, Fas ligand; ICAD, inhibitor of caspase-activated DNase; LDL, low density lipoprotein; MFG-E8, milk fat globule epidermal growth factor 8; PSR, PS receptor; PVDF, polyvinylidene difluoride; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1; sSHPS-1, soluble SHPS-1; dm, double mutant.

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In this study, we screened mouse macrophage cell lines for their ability to engulf apoptotic cells, and found one SV40-transformed cell line (BAM3) that engulfed apoptotic thymocytes, but not a T cell lymphoma line (WR19L). mAbs against BAM3 that inhibited the phagocytosis of apoptotic thymocytes were established. Purification of the Ag recognized by the mAbs revealed that it was Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1 (SHPS-1) (17). The thymocytes were found to express CD47, the ligand for SHPS-1, also known as integrin-associated protein (18, 19), while WR19L did not express CD47. When WR19L cells were transformed with CD47, the transformants bound to SHPS-1-expressing phagocytes, and if they were induced to undergo apoptosis, they were engulfed by phagocytes in a PS-dependent manner. These data indicate that the interaction between CD47 expressed on the apoptotic cells and SHPS-1 expressed in phagocytes is involved in the tethering step, which is followed by PS-mediated engulfment.

Materials and Methods

Mice, Armenian hamsters, cell lines, recombinant proteins, and Abs

There are two alternatively spliced forms for the inhibitor of caspase-activated DNase (ICAD), ICAD-L for a long form and ICAD-S for a short form. The transgenic mice (ICAD-Sdm) expressing a caspase-resistant double mutant (dm) of ICAD-S were described previously (20). C57BL/6 mice and Armenian hamsters were purchased from Japan SLC (Hamamatsu, Japan) and Japan Oriental Yeast (Tokyo, Japan), respectively. A derivative of mouse T cell lymphoma, WR19L, expressing mouse Fas (W3), was described previously (21), and cultured in RPMI 1640 containing 10% FCS, or W3/Ildm were treated at 37 °C for 2 h with 0.2 ml of FasL.

Identification of SHPS-1

Molecules recognized by the 15D9 mAb were purified from mouse J774.A.1 cells by immunoprecipitation. In brief, the 15D9 mAb was covalently linked to protein A-Sepharose (2 mg protein/ml bed volume) using dimethyl pimelimidate (Pierce). J774.A.1 cells (2 x 10^9) were lysed in 200 ml of protein A-Sepharose buffer and the lysate was preincubated with 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 15 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM p-aminodiphenylmethane, 1 mg/ml leupeptin, and 1 μM pepstatin. The lysate was preincubated with 40 μl of human IgG-Sepharose (Amersham-Pharmacia), followed by incubation for 2 h with 10 μg mAb and 15 μl of protein A-Sepharose 4F7 beads. Proteins bound to the beads were eluted by boiling for 5 min in 0.017 M Tris-HCl buffer (pH 6.8) containing 0.5% SDS and 2% 2-ME, and were separated by PAGE. After the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), the membrane was probed with HRP-conjugated streptavidin (Roche Molecular Biochemicals), and the biotin-streptavidin complex was visualized by an ECL system (Renaissance; DuPont-NEN Research Products, Boston, MA).

Cloning of cDNAs for SHPS-1 and CD47

To isolate mouse SHPS-1 cDNA, two oligonucleotides (5'-GGCGCGGAGATGTGGCCCTT and 5'-CGCGCGGAGATGTGGCCCTT) and 5'-TACCTCCTCCTGACCTCGAG) were prepared based on the published sequence (27) (GenBank, D87968), and RT-PCR was conducted with RNA from BAM3. A cDNA for CD47 (19) was obtained from mouse thymocytes by RT-PCR using primers (5'-GGCGCGGAGATGTGGCCCTT and 5'-CAGTCCCTCCTGACCTCGAG). The amplified DNA fragments were inserted into pGEM-T-EASY (Promega, Madison, WI), and verified by DNA sequencing.

Production of soluble SHPS-1 in human 293T cells

The soluble form of mouse SHPS-1 (sSHPS-1) was produced in human 293T cells, as described (15). In brief, a DNA fragment coding for the extracellular region of SHPS-1 (aa 1–365) was ligated with a DNA fragment coding for an interleukin zipper motif and a FLAG epitope at the C-terminal end, and inserted into the pEF-BOS-EX vector (28). An expression plasmid for sSHPS-1-M14t11V mutant lacking the IgV-like domain (aa 29–147) was generated by means of PCR. The expression plasmids were introduced into human 293T cells, and SHPS-1 secreted into the

In vitro phagocytosis

In vitro phagocytosis was conducted essentially as described, (24, 25). In brief, W3/Ildm cells (2 x 10^5 cells/ml) were incubated with 1 μM 5′-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR) at 37°C for 30 min in the serum-free RPMI 1640 medium, and treated with FasL, as described above. The CMFDA-labeled apoptotic cells (5 x 10^3 cells in 0.5 ml PBS) were administered i.v. into 8-wk-old female C57BL/6 mice. One hour after injection, the spleens were treated with collagenase D (Roche Molecular Biochemicals), and the splenic dendritic cells were enriched by magnetic cell sorting using anti-CD11c microbeads (Miltenyi Biotec, Auburn, CA).

Preparation of mAbs, immunoprecipitation, and Western blotting

mAbs against BAM3 were prepared by immunizing Armenian hamsters, as described (15). In brief, 1.5 x 10^5 BAM3 cells were injected s.c. into hamsters twice with a 4-wk interval. Three days after a final booster (1.5 x 10^5 cells) into the footpads, lymphocytes from the popliteal and inguinal lymph nodes were fused with P3U1 mouse myeloma and selected in hypoxanthine/aminopterin/thymidine medium, and the supernatants were tested by an in vitro phagocytosis assay. After cloning, hybridomas were cultured in GIT medium (Nihon Seiyaku, Tokyo, Japan), and Abs were purified by protein A-Sepharose 4FF beads (Amersham-Pharmacia Biotech, Tokyo, Japan).

To identify molecules recognized by mAbs, proteins on the cell surface were labeled with EZ-Link sulfosuccinimidyldi(6-iodotinamido)-6-hexanamidohexanate (Pierce, Rockford, IL), and the cells were lysed in buffer A (50 mM HEPES-NaOH buffer, pH 7.6, containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10% glycerol, 1 mM p-aminodiphenylmethane, 1 μg/ml leupeptin, and 1 μM pepstatin). The lysate was preincubated with 40 μl of human IgG-Sepharose (Amersham-Pharmacia), followed by incubation for 2 h with 10 μg mAb and 15 μl of protein A-Sepharose 4FF beads. Proteins bound to the beads were eluted by boiling for 5 min in 0.017 M Tris-HCl buffer (pH 6.8) containing 0.5% SDS and 2% 2-ME, and were separated by PAGE. After the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), the membrane was probed with HRP-conjugated streptavidin (Roche Molecular Biochemicals), and the biotin-streptavidin complex was visualized by an ECL system (Renaissance; DuPont-NEN Research Products, Boston, MA).

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medium was purified using anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO).

**Results**

A mouse macrophage cell line with a limited phagocytic activity

Cells that express the caspase-resistant form of ICAD (ICAD-Ldm or ICAD-Sdm) do not undergo DNA degradation during apoptosis, but their DNA can be degraded by DNase II in macrophages after the apoptotic cells are phagocytosed (20, 29). Using this knowledge, we previously established a quantitative assay for the phagocytosis of apoptotic cells (15). This assay was used to examine the phagocytic activity of macrophage cell lines against two types of cells that were treated with apoptotic stimuli: thymocytes from ICAD-Sdm mice that were treated for 4 h with 10 μM dexamethasone, and mouse T cell lymphoma WR19L transformants expressing mouse Fas and ICAD-Ldm (W3/Ildm) that were treated for 2 h with FasL. Under these conditions, ~50% of the thymocytes and >90% of the W3/Ildm cells underwent apoptosis, as judged by annexin V staining. Of the several macrophage cell lines tested, BAM3 and J774A.1 were found to phagocytose thymocytes, but they phagocytosed W3/Ildm cells less efficiently.

BAM3 is a mouse macrophage cell line established by transformation of peritoneal macrophages with SV40 (30). As shown in Fig. 1, when BAM3 cells were incubated with freshly prepared thymocytes from ICAD-Sdm mice, few of the macrophages became TUNEL positive (b). In contrast, 41.5% of the BAM3 cells became TUNEL positive when they were incubated with the apoptotic thymocytes (c). This value was comparable to that observed with thioglycolate-elicited peritoneal macrophages (b), indicating that BAM3 cells could engulf apoptotic thymocytes as efficiently as peritoneal macrophages. In contrast, when the apoptotic W3/Ildm cells were used as prey, BAM3 did not phagocytose them at all (e), although peritoneal macrophages efficiently phagocytosed the W3/Ildm cells (f). These results suggested that WR19L cells lack molecules that were necessary to permit their phagocytosis by BAM3 cells.

Identification of a cell surface protein that regulates the phagocytosis by BAM3 cells

To identify the molecules involved in the phagocytosis of apoptotic cells by BAM3, mAbs against BAM3 cells were prepared in the Armenian hamster. By screening 2500 hybridomas with the assay described above, we identified two mAbs (15D9 and FG2) that inhibited phagocytosis of apoptotic thymocytes by BAM3 (Fig. 2A). They inhibited the phagocytosis in a dose-dependent manner, and the percentage of BAM3 engulfing dead cells was reduced to 9% in the presence of 10 μg/ml of 15D9 (Fig. 2B). Microscopic observation demonstrated that not only the number of cells that engulfed apoptotic cells, but also the number of engulfed apoptotic cells per macrophage decreased in the presence of 15D9 (Fig. 2C). The 15D9 and FG2 mAbs also inhibited the phagocytosis of apoptotic thymocytes by J774A.1 cells (Fig. 2D), suggesting that BAM3 and J774A.1 use the same molecule(s) for phagocytosis of apoptotic cells.

A FACS analysis with 15D9 indicated that BAM3 and J774A.1 strongly expressed the Ag(s) for 15D9 (data not shown). The cell surface proteins of BAM3 and J774A.1 were labeled with biotin, and immunoprecipitation with mAb 15D9 brought down two proteins of 120 and 47 kDa (Fig. 3A). J774A.1 cells were then cultured on a large scale, and the 120- and 47-kDa proteins were affinity purified using beads coupled to mAb 15D9 (Fig. 3A). A mass spectrometry analysis of the purified proteins indicated that they were mouse SHPS-1. SHPS-1 consists of an IgV and two IgC1 domains in the extracellular region, a transmembrane region, and a cytoplasmic region containing two immunoreceptor tyrosine-based inhibitory motifs (Fig. 3B) (31). Two different cDNAs, one coding for a long form of SHPS-1 containing all three Ig domains in the extracellular region and the other coding for a short form of SHPS-1 carrying only IgV domain (Fig. 3B), were isolated by RT-PCR from BAM3 with a 3:1 ratio of abundance. When the long form of SHPS-1 cDNA was introduced into NIH3T3, the stable transformants stained with both the 15D9 and FG2 mAbs (Fig. 3C), indicating that both mAbs recognized mouse SHPS-1.

**FIGURE 1.** Phagocytosis of apoptotic cells by BAM3 and peritoneal macrophages. BAM3 (a–e) or thioglycolate-elicited peritoneal macrophages (pMac) (f–j) were incubated with freshly prepared (b and g) or dexamethasone-treated (c and h) thymocytes from ICAD-Sdm mice, or living (d and i) or FasL-treated (e and j) W3/Ildm. In a and f, macrophages were incubated alone. The cells were stained for Mac-1 and TUNEL, and the TUNEL-staining profiles in the Mac-1-positive population are shown. The number indicates the percentage of TUNEL-positive cells in the Mac-1-positive population (% phagocytosis).
FIGURE 2. Establishment of mAbs that inhibit the phagocytosis by BAM3. A, Inhibitory effects of mAbs on the phagocytosis by BAM3. BAM3 was pretreated for 30 min with 10 μg/ml of 15D9 mAb, FG2 mAb, or hamster IgG (hIgG), and incubated with freshly prepared (living) or dexamethasone-treated (apoptotic) thymocytes from ICAD-Sdm mice. The cells were stained for Mac-1 and TUNEL, and the percentage of TUNEL-positive cells in the Mac-1-positive population was determined. The assay was performed in triplicate. B, Dose-dependent inhibition of phagocytosis by 15D9 mAb. The phagocytosis of apoptotic thymocytes from ICAD-Sdm mice was performed in triplicate in the presence of the indicated concentrations of mAb 15D9, and the percentage of phagocytosis was determined. C, Microscopic observation of phagocytosis by BAM3 cells. Phagocytosis of apoptotic thymocytes by BAM3 cells was carried out in chamber slides in the absence (b) or presence of 10 μg/ml normal hamster IgG (c) or mAb 15D9 (d). The BAM3 cells were stained for TUNEL and observed by light microscopy. a, Shows BAM3 cells without apoptotic thymocytes. Original magnifications, ×400. D, The effect of the mAbs on phagocytosis by J774A.1 cells. J774A.1 cells were incubated with apoptotic thymocytes from ICAD-Sdm mice in the absence or presence of 10 μg/ml 15D9 or FG2 mAb. The cells were stained for Mac-1 and TUNEL, and the percentage of phagocytosis was determined by FACS. Assays were performed in triplicate, and the average values are shown.
The 15D9 mAb recognized the short form of SHPS-1 as well (data not shown), suggesting that the epitope for 15D9 was localized to the IgV domain. A Northern hybridization detected a SHPS-1 mRNA of ~4 kb in thioglycolate-elicited peritoneal macrophages, bone marrow-derived macrophages, or macrophage cell lines, but not in lymphoid, myeloid, and fibroblast cell lines (Fig. 3D), supporting the macrophage-restricted expression of SHPS-1 (32).

To confirm that SHPS-1 is involved in the phagocytosis of apoptotic cells, the extracellular region of SHPS-1 (sSHPS-1) and a variant lacking the IgV domain (sSHPS-1/IgV) were prepared. As shown in Fig. 3E, sSHPS-1 inhibited the phagocytosis of apoptotic thymocytes by BAM3 in a dose-dependent manner. In contrast, sSHPS-1/IgV had no effect on phagocytosis by BAM3. These results indicated that sSHPS-1 is required for phagocytosis of apoptotic cells by BAM3, and that the 15D9 and FG2 mAbs worked as antagonists for this process by binding to the IgV domain of SHPS-1.

Requirement of CD47 for the engulfment of apoptotic cells
CD47, also called integrin-associated protein, can bind SHPS-1 (18, 19, 33). A FACS analysis with anti-CD47 revealed that freshly prepared thymocytes expressed CD47 (Fig. 4Aa). The expression level of CD47 on apoptotic thymocytes was comparable to that on the fresh thymocytes (b). The sSHPS-1 bound to the living as well as apoptotic thymocytes (c and d), and preincubation of thymocytes with sSHPS-1 inhibited the binding of anti-CD47 (e). These results indicated that primary mouse thymocytes express CD47 that has the ability to bind to SHPS-1, and that the expression of CD47 did not change during apoptosis. In contrast, WR19L cells expressed little CD47 on their surface (f), and did not bind sSHPS-1 (g).

We then examined the effect of CD47 on the phagocytosis by BAM3. An expression vector for CD47 was introduced into W3/Ildm cells, and several transformants expressing the functional
CD47 (W3/Ildm/CD47) were established (Fig. 4B). When these transformants were added to BAM3 cells after treatment with FasL, a significant percentage (15–17%) of the BAM3 cells became TUNEL positive, indicating that BAM3 engulfed the apoptotic W3/Ildm/CD47 cells (Fig. 4C). The engulfment of apoptotic W3/Ildm/CD47 cells by BAM3 was dependent on SHPS-1 because the 15D9 anti-SHPS-1 mAb completely inhibited the process. In contrast, when W3/Ildm3/CD47 was added to BAM3 without inducing apoptosis, no engulfment was observed.

The apoptotic cells are engulfed by marginal zone dendritic cells in the spleen (24, 25). To examine the effect of CD47 on the clearance of apoptotic cells in vivo, W3/Ildm and W3/Ildm/CD47 cells were labeled with CMFDA, treated with FasL, and injected i.v. into mice. As shown in Fig. 4D, ~15% of the CD11c-positive dendritic cells contained the apoptotic W3/Ildm cells after 1 h. This percentage of the dendritic cells carrying the apoptotic cells increased to 22% when W3/Ildm/CD47 cells were injected into the mice. These results indicated that the surface expression of CD47 contributed to the efficient clearance of apoptotic cells in vivo.

**Corequirement of PS and CD47 for engulfment**

PS is known to be exposed on apoptotic cells and to work as a signal for their engulfment (8, 11). We previously identified MFG-E8-L as a bridging molecule between apoptotic cells and macrophages (15). A mutant MFG-E8-L, D89E, which has a point mutation in an RGD motif, can tightly bind PS on apoptotic cells, but fails to bind to macrophages. Thus, D89E works as a dominant-negative form of MFG-E8-L, or it masks PS exposed on the apoptotic cells (15). To examine whether PS is required for the phagocytosis of apoptotic cells by BAM3, D89E was added to the phagocytosis assay with BAM3 cells. As shown in Fig. 5A, 10 μg/ml D89E strongly inhibited the phagocytosis of apoptotic thymocytes by BAM3. A similar complete inhibition was observed when apoptotic W3/Ildm/CD47 cells were used as prey (data not shown). Because the 15D9 anti-SHPS-1 mAb also efficiently inhibited the phagocytosis of apoptotic cells by BAM3 (Fig. 5A), these results suggested that BAM3 recognized PS as well as CD47 for the engulfment of apoptotic thymocytes.

To examine how CD47 and PS are involved in phagocytosis, we reconstituted phagocytosis using mouse NIH3T3 cells.

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**FIGURE 4.** Efficient engulfment of CD47-expressing cells. **A**, Expression of CD47 in mouse thymocytes, but not in W3/Ildm cells. a, b, and f, The freshly prepared thymocytes (a), dexamethasone-treated thymocytes (b), and W3/Ildm cells (f) were stained with FITC-conjugated rat anti-mouse CD47 (filled area) or isotype-matched control Ab (IgG2a) (open area). c, Thymocytes were stained with anti-CD47 in the absence (open area) or presence (red filled area) of 10 μg/ml sSHPS-1. d, CD47, c, d, and g, The freshly prepared thymocytes (c), apoptotic thymocytes (d), and W3/Ildm cells (g) were incubated with 10 μg/ml of FLAG-tagged sSHPS-1 (filled area) or sSHPS-1-IgV (open area), and their binding to cells was detected with FITC-labeled anti-FLAG mAb. B, Establishment of the W3/Ildm transformants expressing CD47. W3/Ildm cells were transformed with the expression vector for mouse CD47. One of the stable transformants (filled area) and parental W3/Ildm cells (open area) were subjected to staining with anti-CD47 (left panel) and to binding assay for sSHPS-1 (right panel). C, Effects of CD47 expression on the phagocytosis of apoptotic cells by BAM3. W3/Ildm cells and three transformant clones expressing CD47 (CD47-A, -B, and -C) were treated with FasL or left untreated, and incubated with BAM3 cells in the presence or absence of 10 μg/ml 15D9 mAb. The cells were stained for Mac-1 and TUNEL, and the percentages of TUNEL-positive cells in the Mac-1-positive population are shown. D, Effect of CD47 on the engulfment of apoptotic cells. The apoptotic CMFDA-labeled W3/Ildm or W3/Ildm/CD47 cells were injected into the mice. One hour later, the dendritic cells were enriched from the spleen, and the percentages of the CMFDA-positive dendritic cells in the CD11c+ population were determined by a flow cytometry. The assay was done four times, and the data comparison was performed by Mann-Whitney U test.
NIH3T3 cells express a low level of DNase II (34), we first transformed the cells with DNase II (3T3/DNaseII), which allowed us to evaluate the engulfment of apoptotic cells by TUNEL staining. When 3T3/DNaseII cells were cocultured with freshly isolated thymocytes and incubated for 2 h with apoptotic thymocytes from ICAD-Sdm mice in the absence (a) or presence of 10 μg/ml of mAb 15D9 (b) or 1 μg/ml D89E (c). The cells were stained for Mac-1 and TUNEL, and the TUNEL-staining profiles in the Mac-1-positive population are shown. The number indicates the percentage of TUNEL-positive cells in the Mac-1-positive population. B. Phagocytosis assay with NIH3T3 transformants expressing SHPS-1. NIH 3T3/DNase II (3T3) or its transformant expressing SHPS-1 (3T3/SHPS-1) was grown in chamber slides and incubated for 2 h with apoptotic thymocytes from ICAD-Sdm mice in the absence (a and b) or presence of 10 μg/ml of mAb 15D9 (c) or 0.5 μg/ml of D89E (d). After vigorous washing, the cells were fixed with 1% paraformaldehyde and subjected to the TUNEL reaction. Original magnifications, ×400. The experiments were performed three times, and the numbers of thymocytes attached, but not engulfed (TUNEL negative), and those engulfed (TUNEL positive) by NIH3T3 were determined. The numbers per 100 NIH3T3 cells are shown in lower panel.

Discussion

Many proteins expressed in phagocytes have been proposed as a candidate receptor involved in the engulfment of apoptotic cells (8, 10, 12, 35). PS is exposed on the surface during apoptotic cell death, and cells filled with PS by liposome transfer can be recognized and engulfed by phagocytes (36); indicating that some molecules on macrophages should recognize PS for engulfment. PSR (37), LDL receptor (38), and scavenger receptors (39) seem to

FIGURE 5. Requirement of CD47 and PS for engulfment by BAM3. A. Effect of D89E on the phagocytosis by BAM3 cells. BAM3 cells were incubated for 2 h with dexamethasone-treated thymocytes from ICAD-Sdm mice in the absence (a) or presence of 10 μg/ml of mAb 15D9 (b) or 1 μg/ml D89E (c). The cells were stained for Mac-1 and TUNEL, and the TUNEL-staining profiles in the Mac-1-positive population are shown. The number indicates the percentage of TUNEL-positive cells in the Mac-1-positive population. B. Phagocytosis assay with NIH3T3 transformants expressing SHPS-1. NIH 3T3/DNase II (3T3) or its transformant expressing SHPS-1 (3T3/SHPS-1) was grown in chamber slides and incubated for 2 h with apoptotic thymocytes from ICAD-Sdm mice in the absence (a and b) or presence of 10 μg/ml of mAb 15D9 (c) or 0.5 μg/ml of D89E (d). After vigorous washing, the cells were fixed with 1% paraformaldehyde and subjected to the TUNEL reaction. Original magnifications, ×400. The experiments were performed three times, and the numbers of thymocytes attached, but not engulfed (TUNEL negative), and those engulfed (TUNEL positive) by NIH3T3 were determined. The numbers per 100 NIH3T3 cells are shown in lower panel.
directly recognize PS on apoptotic cells. However, how these receptors are involved in the recognition and engulfment of apoptotic cells and whether any other molecules that cannot recognize PS are required for this process have not been clear. Macrophages and immature dendritic cells are a family of cells that have marked phenotypic heterogeneity and respond differently to various endogenous or exogenous stimuli (40). We reasoned that the apparent redundancy in the phagocytic system is at least in part due to this heterogeneity of phagocytes, and showed that a macrophage cell line (BAM3) has a simple system for the engulfment of apoptotic cells. This macrophage cell line seemed to recruit apoptotic cells by binding CD47 on target cells via SHPS-1, and then engulfs dying cells by recognizing PS exposed on their surface. These results strongly support the two-step model for engulfment proposed by Hoffmann et al. (16), with the CD47-SHPS-1 pair working as a tethering step in the engulfment process. A similar requirement of CD47 for the engulfment of apoptotic cells, although not absolute, was observed in another macrophage cell line, J774A.1, confirming the requirement of this type of tethering at least in a subset of macrophages.

CD47 is a peculiar transmembrane protein of the Ig superfamily, with a single IgV-like domain at its N terminus, and five transmembrane segments (41). It is broadly expressed in various cells, including lymphocytes and hemopoietic cells (42, 43). A variety of functions has been suggested for CD47, which include migration of neutrophils, platelet aggregation, costimulation for T-cell activation, and caspase-independent killing of B-cell lymphoma (44, 45). One of the well-supported functions of CD47 is to mediate cell-cell adhesion (18, 33), and the tethering effect of CD47 in the phagocytosis of apoptotic cells is likely to use similar mechanisms. In contrast, Oldenborg et al. (46) reported that the expression of CD47 prevents the engulfment of RBC by splenic macrophages, which apparently contradicts our results. Because CD47-expressing lymphocytes and thymocytes are efficiently engulfed by various macrophages in vitro and in vivo (this study, and see Ref. 47), it is unlikely that CD47 works as a “don’t eat me” signal in lymphocytes. CD47 in lymphocytes is known to be localized to the membrane rafts, specialized domains in the plasma membrane enriched in cholesterol and sphingolipids (48). Whether the apparent discrepancy between lymphocytes and erythrocytes is due to the cell specificity or a different conformation of CD47 on the plasma membrane remains to be studied. In any case, the finding of a T-cell lymphoma (WR19L) deficient in CD47 will provide an excellent experimental system to examine the function of this protein. In particular, it may be interesting to examine the role of CD47 in lymphomatogenesis in relation to the engulfment by macrophages.

SHPS-1, the receptor for CD47, is expressed in only limited cell types such as monocytes, macrophages, and immature dendritic cells that have a strong ability to engulf apoptotic cells (18, 32, 49). It is a receptor-type glycoprotein of the Ig superfamily, and carries two immunoreceptor tyrosine-based inhibitory motifs that can be tyrosine phosphorylated (17). The phosphorylated SHPS-1 recruits and activates tyrosine phosphatases such as SHP-1 and SHP-2 to regulate the signal transduction evoked by a variety of stimuli, including signals through receptor tyrosine kinase, Toll receptor, and integrins (31). Apoptotic cell clearance is known to be non-inflammatory (8), and apoptotic cells even actively suppress inflammation (50). The engagement of SHPS-1 with CD47 inhibits the endotoxin-induced production of proinflammatory cytokines in human immature dendritic cells (51). It is therefore likely that the interaction between CD47 and SHPS-1 in the phagocytosis process causes an anti-inflammatory effect, in at least some subsets of macrophages. In this study, we reconstituted the engulfment process with NIH3T3 cells expressing SHPS-1. Mutational analysis of SHPS-1 may allow us to examine in more detail the mechanism for the anti-inflammatory function of apoptotic cells.

We previously identified a protein (MFG-E8-L) that strongly binds to PS on apoptotic cells and has the ability to pass the dying cells to phagocytes (15). The thioglycolate-elicited peritoneal macrophages express MFG-E8-L abundantly, while BAM3 and J774A.1 cells do not express it (15). The anti-SHPS-1 mAb had no significant effect on the ability of thioglycolate-elicited peritoneal macrophages to engulf apoptotic thymocytes. Furthermore, the addition of MFG-E8-L rendered BAM3 capable to engulf the apoptotic WR19L cells to some extents (data not shown). These results indicate that binding of MFG-E8 to PS on apoptotic cells, and to αβ2 integrin on macrophages tethers the apoptotic cells to macrophages. Although we cannot rule out the possibility that peritoneal macrophages use a tethering mechanism other than CD47-SHPS-1, these results suggest that if the direct recognition of PS by its receptor (or bridging molecule) is strong enough to recruit the apoptotic cells to phagocytes, an extra tethering system may not be necessary at least in vitro. In contrast, the tethering process alone was not sufficient to promote phagocytes to engulf the cells. That is, BAM3 cells required PS to be exposed on the surface of apoptotic cells to engulf them. Whether any of the proposed receptors for PS such as PSR, CD36, CD14, LDL receptor, and scavenger receptors (12, 37–39, 52) is used in BAM3 cells for the PS-mediated engulfment of apoptotic cells remains to be clarified. In any event, establishing and screening macrophage cell lines will help to resolve whether each macrophage has a single unique system or redundant systems for the engulfment of apoptotic cells.

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References


