Expression of Developmental Endothelial Locus-1 in a Subset of Macrophages for Engulfment of Apoptotic Cells

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A major function of macrophages is to engulf apoptotic cells to prevent them from releasing noxious materials as they die. Milk fat globule-EGF-factor 8 (MFG-E8) is a glycoprotein secreted by activated macrophages that works as a bridge between apoptotic cells and phagocytes by specifically recognizing phosphatidylserine exposed on apoptotic cells. In this study, we found that developmental endothelial locus-1 (Del-1), originally identified as an embryonic endothelial cell protein that binds αβ3 integrin, is structurally and functionally homologous to MFG-E8. That is, both consist of a signal sequence, two epidermal growth factor domains and two factor VIII-homologous domains (C1 and C2). Del-1 bound to the apoptotic cells by recognizing phosphatidylserine via the factor VIII-homologous domains with an affinity similar to that of MFG-E8. The phagocytic activity of NIH 3T3 cells against apoptotic cells was enhanced by Del-1 through an interaction between the epidermal growth factor domain in Del-1 and αβ3 integrin expressed in the NIH 3T3 cells. Screening of primary macrophages and macrophage cell lines for the expression of MFG-E8 and Del-1 indicated that MFG-E8 and Del-1 are expressed in different sets of macrophages. These results suggest the existence of macrophage subsets that use MFG-E8 or Del-1 differently to engulf apoptotic cells. The Journal of Immunology, 2004, 172: 3876-3882.

Abbreviations used in this paper: MFG-E8, milk fat globule epidermal growth factor 8; EGF, epidermal growth factor; Del-1, developmental endothelial locus-1; wt, wild type; MFG-E8L, MFG-E8 long; MFG-E8S, MFG-E8 short.
Materials and Methods

Mice, Armenian hamsters, and cell lines

The transgenic mice (ICAD-Sdm) expressing a caspase-resistant form of inhibitor of caspase-activated DNase-short form were described previously (21). C57BL/6 mice and Armenian hamsters were purchased from Japan SLC (Shizuoka, Japan) and Oriental Yeast (Tokyo, Japan), respectively. Macrophages were prepared from the thymus and liver of mouse embryos as described previously (24). Bone marrow-derived macrophages were obtained by culturing bone marrow cells for 7 days in the presence of M-CSF. Mouse NIH 3T3 transformants expressing integrins α₁β₁, were described previously (21). The NIH 3T3 cells were further transformed with DNase II to enhance the TUNEL reactivity.

Production of rDel-1 and rMFG-E8

A DNA fragment for the full-length coding sequence of mouse Del-1 was prepared by RT-PCR with RNA from E15.5 mouse embryo using primers whose sequences have been published (22). After joining a FLAG epitope at the 3′ end, the Del-1 cDNA was inserted into the pEF-BOS-EX vector (25). The expression plasmids for Del-1 mutants carrying a series of point mutations were generated by means of recombinant PCR. The primers used were: 5′-GAAGCTTACGAGGAGAAACATCATAGGC-3′ for D98E, 5′-TATCTCCTAATCTGCTGCTTCTAAATAGAG-3′ for R195A, 5′-AATGCAAGTCTAGTATGAGCCTTCTACC-3′ for R295A, and 5′-CGAAGCCATTGATCTTGGACCTGAGAAT-3′ for R308A. The expression plasmids were introduced into human 293T cells by the calcium phosphate precipitation method, and Del-1 secreted into the medium was purified using ANTI-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO). The production of mouse MFG-E8 long (MFG-E8L) was described previously (21).

Preparation of mAbs

mAbs were prepared by immunizing Armenian hamsters with rDel-1. In brief, the purified Del-1 (100 μg) was injected s.c. into hamsters twice in a 2-wk interval. A final booster was performed by injecting 40 μg of Del-1 into the footpads. Three days later, the hamsters were sacrificed, and lymphocytes were harvested from the lymph nodes and fused with P3U1 mouse myeloma cells. Hybridomas were tested by ELISA for binding activity to Del-1, and positive clones were screened further to identify clones that were suitable for Western blotting (clone 8-29) and immunoprecipitation (clone 4-9). After they were subjected to limiting dilution, the hybridomas were cultured in GIT medium (Nihon Seiyaku, Tokyo, Japan), and the mAb was purified by protein A-Sepharose 4FF beads (Amersham Biosciences, Piscataway, NJ). The biotinylation of mAb and chemical cross-linking of mAb to protein A-Sepharose were performed according to standard protocols (26, 27).

Immunoprecipitation and Western Blotting

Macrophages (1 × 10⁶ cells) from various sources were cultured in AIM V medium (Invitrogen, Carlsbad, CA) for 48 h. The culture medium was collected, and adjusted to 1% Triton X-100 as described (28). Del-1 was then immunoprecipitated with 10 μL of anti-Del-1 mAb (clone 4-9)-protein A-Sepharose, and eluted from the beads with 30 μL of triethylamine (pH 11.7) containing 0.1% Triton X-100. An aliquot (20 μL) of the eluate was mixed 1:1 with 2× sample buffer without a reducing agent, separated by 10% PAGE, and the proteins were transferred to a nitrocellulose filter. The filter was subjected to Western blotting with biotinylated anti-Del-1 mAb (clone 8-29) and peroxidase-conjugated streptavidin (Roche, Indianapolis, IN). Proteins recognized by the Ab were visualized using a chemiluminescence reaction (Western Lightning Chemiluminescence Reagent; PerkinElmer, Wellesley, MA).

Protein lipid overlay assay and solid-phase ELISA

1-α-phosphatidylinositol, 1-α-phosphatidylcholine, 1-α-phosphatidylethanolamine, and 1-α-phosphatidylinositol were purchased from Sigma-Aldrich. The protein lipid overlay assay was performed as described (29). In brief, each phospholipid (500 pmol) was spotted onto HydroB-C extra membrane (Amerham Biosciences, Piscataway, NJ) and allowed to dry at room temperature. After blocking nonspecific binding sites by incubating the membranes in PBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 2 mg/ml BSA, it was incubated for 2 h at room temperature with 10 nM FLAG-tagged MFG-E8 or Del-1. The membrane was washed with TBST, and the bound proteins were detected with peroxidase-conjugated anti-FLAG mAb (M2; Sigma-Aldrich), followed by a chemiluminescence reaction.

The solid phase ELISA for Del-1 and MFG-E8 binding to phospholipids was conducted as previously described (21). In brief, phospholipid (100 μl, 3 μg/ml) in methanol was added to 96-well Immulon 1B microtiter plates (Thermo Environmental Instruments, Franklin, MA) and air-dried. Nonspecific binding sites were blocked by treating the wells with 10 mg/ml BSA in PBS. Del-1 and MFG-E8 were diluted to the appropriate concentrations with PBS containing 0.05% Tween 20 and 10 mg/ml BSA, added to the wells, and incubated at room temperature for 1 h. After the wells were washed with PBS containing 0.05% Tween 20, the bound proteins were quantified by ELISA using biotinylated anti-FLAG mAb and peroxidase-conjugated streptavidin. The peroxidase activity was detected using a peroxidase-detecting kit (Sumitomo Chemical, Tokyo, Japan), and quantified by measuring the absorbance at 450 nm with a microELISA reader.

In vitro phagocytosis assay

Phagocytosis of apoptotic cells was performed as described previously (21). In brief, NIH 3T3 cells or their transformants (2 × 10⁴ cells) were
cultured in 8-well Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY) that had been coated with 0.1% gelatin. Thymocytes from 4- to 8-wk-old ICAD-Sdm mice were treated with 10 μM dexamethasone to induce apoptosis. The apoptotic thymocytes were added to the well, and phagocytosis was allowed to proceed for 2 h. After fixation with 1% paraformaldehyde, the adherent cells were subjected to the TUNEL reaction using an Apoptag kit (Intergen, Purchase, NY) and DAB-black (Zymed Laboratories, South San Francisco, CA) and observed by light microscopy. The TUNEL-positive thymocytes in a total of 150 NIH 3T3 cells were counted, and the number of engulfed apoptotic thymocytes per NIH 3T3 cell (phagocytosis index) was determined.

Results

Binding of Del-1 to apoptotic cells

Del-1 is a protein of 480 amino acids, and consists of a signal sequence, three EGF-like domains including one with an Arg-Gly-Asp motif, and two factor VIII-homologous (discoidin-I-like) domains (C1 and C2) (Fig. 1). This structure is similar to that of MFG-E8, except that the third EGF domain in Del-1 is not found in MFG-E8. There are two forms for MFG-E8 (MFG-E8 short (MFG-E8S) and MFG-E8L for MFG-E8 short and long forms, respectively) that are generated by alternative splicing (30). In MFG-E8S, there is no domain corresponding to the third EGF domain of Del-1, but in MFG-E8L the third EGF repeat is replaced by a proline-threonine-rich (P/T-rich) region (Fig. 1). The overall identity and similarity between Del-1 and MFG-E8L are 48% and 54%, respectively, at the amino acid sequence level.

We previously showed that MFG-E8, and in particular MFG-E8L, binds to apoptotic cells by recognizing phosphatidylserine (21). To examine whether Del-1 also binds to apoptotic cells, rDel-1 was produced in human 293T cells and purified. The Del-1 did not bind to freshly isolated thymocytes (Fig. 2A). In contrast, when thymocytes were treated for 6 h with dexamethasone, ~70% of them underwent apoptosis, as judged by TUNEL staining, and Del-1 specifically bound to these apoptotic cells (Fig. 2A). Annexin V binds to apoptotic cells by recognizing phosphatidylserine (31). Del-1 inhibited the binding of Annexin V to the apoptotic cells in a dose-dependent manner, and its inhibition was complete at a concentration of 1.0 μg/ml (Fig. 2B). The binding of MFG-E8L to apoptotic cells was also inhibited by Del-1. These results suggested that Del-1 binds to apoptotic cells by recognizing phosphatidylserine.

Binding of Del-1 to phosphatidylserine

The possibility that Del-1 binds directly to phosphatidylserine was examined using the protein lipid overlay assay (29). When a nitrocellulose filter spotted with various phospholipids was incubated with MFG-E8L or Del-1, both MFG-E8L and Del-1 specifically bound to phosphatidylserine (Fig. 2C). In contrast, neither of MFG-E8L nor Del-1 bound to phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine. The affinities of MFG-E8L and Del-1 to phosphatidylserine, determined with microtiter plates coated with phosphatidylserine, were comparable (Fig. 2D). Considering that the affinity of MFG-E8 to phosphatidylserine is ~10 times less than that of MFG-E8L (21), it seems that the third EGF domain in Del-1 may have a similar function as the P/T-rich domain of MFG-E8L in the binding of phosphatidylserine.

The C1 and C2 domains of Del-1 and MFG-E8 belong to the discoidin-I family (32), which includes the C1 and C2 domains of factor V and factor VIII. Factors V and VIII are phosphatidylserine-binding cofactors for the factor IX serine protease in blood coagulation (33). The tertiary structure of the C2 domains of factor V and VIII has been determined by x-ray analysis (34, 35). They have a jelly-roll β-barrel motif consisting of 19 β-strands including 8 core β-strands. The amino acid sequences of the C1 and C2 domains of Del-1 and MFG-E8 are well conserved with those of factors V and VIII, particularly for the 8 β-strand core region (Fig. 3, A and B). Among the conserved amino acids, the arginine residues at amino acid positions 440, 443, and 446 of Del-1 (Fig. 3, A and B) correspond to the arginine residues at amino acid positions 447, 450, and 453 of factor V (32) and 441, 444, and 447 of factor VIII (37).

Del-1 FOR ENGULFMENT OF APOPTOTIC CELLS

FIGURE 2. Binding of Del-1 to phosphatidylserine exposed on apoptotic cells. A, Binding of Del-1 to apoptotic cells. Freshly prepared wt thymocytes (1 × 10⁶ cells) (left panel) or thymocytes treated with dexamethasone for 6 h (right panel) were incubated for 30 min with 0.1 μg/ml Del-1, followed by staining with biotinylated anti-Del-1 mAb (clone 8-29) and PE-conjugated streptavidin. After fixation with 1% paraformaldehyde, the cells were subjected to TUNEL staining with FITC-labeled dUTP, and analyzed by FACS. B, Effect of Del-1 on the binding of annexin V or MFG-E8 to apoptotic thymocytes. Apoptotic thymocytes were stained in the presence of 1.0 μg/ml Del-1 with 10 μg/ml PE-conjugated annexin V (left panel) or with 0.1 μg/ml MFG-E8 followed by the biotinylated anti-MFG-E8 mAb (clone 2422) and PE-conjugated streptavidin (right panel), and analyzed by FACS. The annexin V- and MFG-E8-staining profiles without Del-1 are shown by dotted lines. C, Specific binding of Del-1 and MFG-E8 to phosphatidylserine. A nitrocellulose filter was spotted with phosphatidylcholine (PC), phosphatidylcholine (PI), phosphatidylethanolamine (PE), or phosphatidylserine (PS) and incubated at room temperature for 2 h with 10 nM FLAG-tagged MFG-E8 or Del-1. Proteins that bound to the phospholipids were detected by an ANTI-FLAG mAb as described in Materials and Methods. D, Dose-dependent binding of Del-1 or MFG-E8 to phosphatidylserine. Microtiter plates coated with PS or PC were incubated with increasing concentrations of Del-1 or MFG-E8, and proteins that bound to the wells were quantified by ELISA using ANTI-FLAG mAb. The absorbance at 450 nm is shown. Assays were done in triplicate, and the average values are plotted with SD.
acid positions 195, 295, and 308 are essential for the function of factors V and VIII (36). To confirm that the tertiary structure of the C1 and C2 domains of Del-1 is similar to that of factors V and VIII, these arginine residues were replaced by alanine. As shown in Fig. 3C, none of the Del-1 mutants could bind to apoptotic cells, suggesting that these arginine residues are involved in the binding of phosphatidylserine or in stabilizing the protein fold.

Enhancement of phagocytosis of apoptotic cells by Del-1

We previously showed that MFG-E8 stimulates NIH 3T3 cells to engulf apoptotic cells. To examine whether Del-1 has a similar activity, the phagocytosis assay with NIH 3T3 cells was conducted using thymocytes from the ICAD-Sdm mice as prey (21). In this assay system, DNA of the apoptotic thymocytes becomes TUNEL-positive only after the thymocytes are phagocytosed. When freshly prepared thymocytes from ICAD-Sdm mice were cocultured with NIH 3T3 cells, no thymocytes were engulfed by the NIH 3T3 cells in the presence or absence of Del-1, as judged by the TUNEL-staining of the NIH 3T3 cells (data not shown). In contrast, when apoptotic thymocytes were cocultured with NIH 3T3 cells (3T3/wt), a significant percentage of NIH 3T3 cells engulfed the apoptotic thymocytes, and this percentage was enhanced by Del-1. That is, the phagocytosis index (the average number of apoptotic cells per NIH 3T3 cell) was 0.18 in the absence of Del-1 and increased to 1.06 following the addition of Del-1 (Fig. 4A). This effect of Del-1 on the engulfment of apoptotic cells was pronounced when the NIH 3T3 cells expressing α3β1 integrin were used as phagocytes (Fig. 4A), and many of these NIH 3T3 cells contained >8 apoptotic cells (Fig. 4B). The ability of Del-1 to stimulate the engulfment of apoptotic cells was comparable to that of MFG-E8. The Del-1 mutants (R195A, R295A, and R308A) that could not bind apoptotic cells did not enhance the engulfment, confirming the specific effect of Del-1 on the engulfment of apoptotic cells. When the aspartic acid at aa 98 in the RGD motif was mutated to glutamic acid, the mutant Del-1 (D98E) bound to apoptotic cells (data not shown) but did not stimulate their engulfment by NIH 3T3 cells (Fig. 4). These results indicated that, as
with MFG-E8, the RGD motif in the second EGF domain of Del-1 is essential for the binding of Del-1 to the integrin-expressing cells.

Expression of Del-1 in macrophages that do not express MFG-E8

The above results indicated that Del-1 and MFG-E8 have a similar structure and function. However, the expression profile of Del-1 was strikingly different from that of MFG-E8. As shown in Fig. 5A, Del-1 mRNA was expressed in fetal thymic and fetal liver macrophages, and weakly in bone marrow-derived macrophages. These macrophages did not express MFG-E8. The macrophage cell lines BAM3 and J774A.1 expressed Del-1, but not MFG-E8. In contrast, MFG-E8 mRNA was detected in thiglycolate-elicited peritoneal macrophages and the P388D1 macrophage cell line, which did not express Del-1. Neither Del-1 nor MFG-E8 was expressed in thymocytes, the NIH 3T3 fibroblast cell line, or in lymphoid or myeloid cell lines (Fig. 5A, and data not shown). MFG-E8 is secreted from macrophages as a component of exosomes (28)(K. Miyasaka, R. Hanayama, S. Nagata, unpublished results). Similarly, when conditioned medium from fetal thymic macrophages, fetal liver macrophages, or the BAM3 cell line was spun, no Del-1 protein was detected in the supernatant (data not shown). In contrast, when the conditioned medium of these macrophages was treated with 1.0% Triton X-100 before centrifugation, a large amount of Mr 53K Del-1 protein was detected in the supernatants (Fig. 5B), suggesting that Del-1 is also produced by macrophages as a component of exosomes.

Discussion

Del-1 was originally identified by an enhancer trap event in one transgenic mouse line as a protein that is transiently expressed in endothelial cells during early mouse embryogenesis (22). Del-1
inhibits the formation of the vascular-like structure from the endothelial-like yolk sac cells (22). It also stimulates angiogenesis in the in ovo chick chorioallantoic membrane assay (37). These activities of Del-1 are mediated by its binding to $\alpha_\beta_3$ integrin expressed on endothelial cells. In this report, we showed that Del-1 is structurally and functionally homologous to MFG-ESL, which works as a bridge between apoptotic cells and phagocytes. That is, Del-1 specifically binds apoptotic cells by recognizing phosphatidylinerse via its C1 and C2 domain. Del-1 that was engaged by phosphatidylinerse on microtiter plates recruited NIH 3T3 cell expressing $\alpha_\beta_3$ integrin via the RGD motif (data not shown). Del-1 also stimulated the engulfment of apoptotic cells by NIH 3T3 fibroblasts. It is possible that the functions of Del-1 in the engulfment of apoptotic cells and angiogenesis in the fetus are independent and that Del-1 has dual functions. However, the vascular remodeling in normal developmental, as well as pathological, neovascularization is often associated with endothelial cell apoptosis (38, 39). Del-1 expressed in the epithelial cells in the fetus may have a role in the engulfment of the dying endothelial cells, thus enhancing neovascularization.

We previously observed weak but significant binding of MFG-E8 to phosphatidylyethanolamine (21). Kagen et al. (15) recently analyzed the phospholipids in human cell lines that were exposed during Fas-induced apoptosis, and reported that although the exposure of phosphatidylinerse is strictly regulated by the apoptotic process, a significant amount of phosphatidylyethanolamine is exposed even in living cells. Therefore, we re-evaluated the binding of MFG-E8 and Del-1 to phospholipids, and found that the addition of 0.05% Tween 20 to the assay mixture completely inhibited the binding of MFG-E8 or Del-1 to phosphatidylyethanolamine. In contrast, this concentration of Tween 20 had no effect on the binding of MFG-E8 or Del-1 to phosphatidylinerse. MFG-E8 did not bind phosphatidylcholine or phosphatidylinositol even in the absence of the detergent. Therefore, we have no clear explanation for the binding of Del-1 or MFG-E8 to phosphatidylyethanolamine in the absence of the detergent. The C1 and C2 domains of factors V and VIII in the discoidin family bind phosphatidylinerse but not other phospholipids (40, 41). In this study, we showed that the arginine residues conserved among the discoidin family members are essential for the binding of Del-1 to apoptotic cells, and thus phosphatidylinerse. It is therefore reasonable to conclude that Del-1 and MFG-E8 preferentially bind to phosphatidylinerse.

Lymphocytes have been progressively subdivided into a large number of subtypes with distinct functions. Mouse mononuclear phagocytes are defined as blood mononuclear cells that express CD11b and F4/80, and the heterogeneity of macrophages and dendritic cells has been recognized (42–45). Very recently, Geissmann et al. (46) reported the existence of monocyte subsets that have different surface markers. According to them, the Cx3Crl+/Gr1low subset of monocytes is short-lived and represents the immediate circulating precursors for dendritic cells and CD11c-myeloid cells in inflammatory conditions (inflammatory subset). The second subset of monocytes (Cx3Crl++/Gr1–) persists longer in tissues and serves as a precursor for resident myeloid cells in noninflamed tissues (resident subset). Del-1 was expressed in macrophages in the fetal liver, fetal thymus, and bone marrow, whereas MFG-E8 was expressed in thiglycolate-elicited macrophages, suggesting that Del-1 and MFG-E8 are expressed in resident and inflammatory subsets of macrophages, respectively. It is likely that these resident and inflammatory subsets of macrophages correspond to “unnactivated and activated” macrophage subsets defined by Pradhan et al. (45). If the differential expression of Del-1 and MFG-E8 in the resident and inflammatory macrophages can be confirmed, Del-1 and MFG-E8 will be good surface markers for distinguishing these two subsets of monocytes in the blood, and macrophages and dendritic cells in tissues. In this regard, it is noteworthy that the $\delta$ promoter sequences of Del-1 and MFG-E8 differ significantly compared with their conserved coding sequence. The murine and human Del-1 gene promoters contain binding sites for NF-AT, $\delta$-EF1, and TAL1 transcription factors that are necessary for the development of blood cells (47–49) while binding sites for the NF-$\kappa$B and CREB transcription factors can be found on the promoters of murine and human MFG-E8. Studies using the Del-1 and MFG-E8 gene promoters in a transgenic and/or knockin mouse strategy may help characterize the functions of subsets of mononuclear phagocytes.

In this study, we described that Del-1 as well as MFG-E8 bind phosphatidylinerse exposed on apoptotic cells with high affinities, and can work as a bridge between apoptotic cells and phagocytes. We recently established a mouse line that is deficient in MFG-E8.4 The ability of thiglycolate-elicited peritoneal macrophages to engulf apoptotic cells was significantly reduced in MFG-E8-null mice, suggesting a nonredundant role of MFG-E8 in thiglycolate-elicited peritoneal macrophages for engulfment of apoptotic cells. To determine whether Del-1 also plays a nonredundant role in the engulfment of apoptotic cells in fetal macrophages, it will be necessary to establish mice deficient in the Del-1 gene.

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