Correction

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The authors note that, due to a printer’s error, the keyword “pospholipid asymmetry” should instead appear as “phospholipid asymmetry,” and the keyword “pagocytosis” should instead appear as “phagocytosis.” The online version has been corrected.

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Constitutive exposure of phosphatidylserine on viable cells

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Apoptotic cells are quickly recognized and engulfed by phagocytes to prevent the release of noxious materials from dying cells. Phosphatidylserine (PS) exposed on the surface of apoptotic cells is a proposed “eat-me” signal for the phagocytes. Transmembrane protein 16F (TMEM16F), a membrane protein with eight transmembrane segments, has the Ca-dependent phospholipid scramblase activity. Here we show that when lymphoma cells were transformed with a constitutively active form of TMEM16F, they exposed a high level of PS that was comparable to that observed on apoptotic cells. The PS-exposing cells were morphologically normal and grew normally. They efficiently responded to interleukin 3 and underwent apoptosis upon treatment with Fas ligand. The viable PS-exposing cells bound to peritoneal macrophages at 4 °C, but not at 25 °C. Accordingly, these cells were not engulfed by macrophages. When apoptotic cells were injected i.v. into mice, they were phagocytosed by CD11c+CD8+ dendritic cells (DCs) in the spleen, but the PS-exposing living cells were not phagocytosed by these DCs. Furthermore, when PS-exposing lymphoma cells were transplanted s.c. into nude mice, they generated tumors as efficiently as parental lymphoma cells that did not expose PS. These results indicated that PS exposure alone is not sufficient to be recognized by macrophages as an eat-me signal.

phospholipid asymmetry | phagocytosis

Many harmful and unnecessary cells are generated during animal development, and they undergo apoptosis (1). In adults, senescent cells and virus- or bacteria-infected cells are removed by apoptosis (2). Apoptotic cells are swiftly engulfed by macrophages or immature dendritic cells (DCs) to prevent the release of noxious materials from the dying cells, which may activate the immune system, leading to autoimmune disease (3, 4).

Macrophages specifically engulf apoptotic cells, but not living ones, indicating that the dying cells present an “eat-me” signal(s) to the phagocytes. Phosphatidylserine (PS), which is present on the inner leaflet of plasma membranes in healthy cells, is quickly exposed to the outer plasma membrane of apoptotic cells and has been proposed as an eat-me signal (5). In fact, masking the PS on apoptotic cells inhibits their engulfment in vitro and in vivo (6, 7), strongly supporting the identification of PS as an eat-me signal.

The asymmetrical distribution of phospholipids on the plasma membrane is mediated by various transporters (8): ATP-dependent amino phospholipid translocase or flipase transports amino phospholipids from the outer leaflet to the cytoplasmic side. In addition, a Ca-dependent scramblase bidirectionally transports phospholipids. We recently identified transmembrane protein 16F (TMEM16F), also called anocerin 6, as this phospholipid scramblase (9). During that study, we noticed that an Asp-to-Gly point mutation at amino acid position 409 renders the molecule constitutively active; cells that express this mutant form of TMEM16F constitutively expose PS on their surface.

In this report, we found that there is an alternatively spliced variant of mouse TMEM16F. Mouse lymphoma cells expressing the TMEM16F splice variant with the Asp-to-Gly mutation exposed more PS than those expressing the authentic TMEM16F splice form with the same point mutation. These high-PS-exposing lymphoma cells grew normally and responded normally to interleukin (IL)-3 by proliferating and to Fas ligand (FasL) by becoming apoptotic. The level of PS exposed on these cells was comparable to that observed on apoptotic cells. However, peritoneal macrophages did not engulf the viable PS-exposing cells, even though they efficiently engulfed apoptotic cells in a PS-dependent manner. These living PS-exposing cells were transplanted into nude mice, they grew as aggressively as the parental lymphoma cells. These results indicate that although PS exposure is essential as an eat-me signal, it is not sufficient for the apoptotic cells to be engulfed.

Results

PS Exposure by TMEM16F Mutants. We previously established a subline of Ba/F3 that strongly exposes PS in response to a Ca ionophore (9). Expression cloning of the gene responsible for the PS exposure in this cell line identified a mutant TMEM16F that carried an Asp-to-Gly point mutation at codon 409 (D409G) (Fig. 1D). Further screening of the library led to the identification of the cDNA for a splice variant with the same point mutation generated by the insertion of an extra exon from intron 1 of the TMEM16F gene (Fig. 1F). The protein D430G-long (D430G-L), which carried an extra peptide of 21 amino acids at amino acid position 24 (Fig. 1C), showed scramblase activity that was apparently stronger than that of the D409G. As shown in Fig. 1D, introduction of the wild-type TMEM16F into Ba/F3 did not induce the cells to expose PS. When the D409G mutant was expressed in Ba/F3, about 70% of the cells constitutively exposed PS. On the other hand, Ba/F3 cells expressing D430G-L exposed PS more strongly than those expressing D409G, indicating that the 21 aa insertion in the first cytoplasmic region of mouse TMEM16F has a positive effect on its scramblase activity. A similarly high level of PS exposure was observed when D430G-L was expressed in mouse WR19 cell transformants (W3-1dmn) expressing mouse Fas and the caspase-resistant form of inhibitor of caspase-activated DNase (ICAD) (10) (Fig. 1D).

Effect of Constitutive PS Exposure on Cell Growth and Apoptosis. The asymmetrical distribution of phospholipids on the inner and outer plasma membrane is thought to be important for maintaining the integrity of plasma membranes (8). However, the Ba/F3–D430G-L cells, which constitutively exposed PS, grew as efficiently as the parental Ba/F3 cells; the doubling time of both Ba/F3 and Ba/F3–D430G-L cells in the presence of 100 units/mL mouse IL-3 was 11.5 h (Fig. 2A). Accordingly, the dose-dependent response of the Ba/F3–D430G-L cells to IL-3 was comparable to that of the parental Ba/F3 cells (Fig. 2B).

The expression of the TMEM16F mutant or constitutive exposure of...
PS also had no effect on the growth of W3-Ilmd cells (Fig. 2C). Treating the parental W3-Ilmd and their D430G-L transformants grew at a comparable rate (doubling time, 12.5 h). The W3-Ilmd cells overexpress Fas, and are sensitive to FasL-induced apoptosis (10, 11). The dose-dependent response to recombinant FasL was comparable between W3-Ilmd and its transformants expressing D430G-L (Fig. 2D). These results suggested that the constitutive exposure of PS had little effect on the binding of IL-3 to promote cell growth or by FasL to promote apoptosis occurs normally.

**Strong PS Exposure on Living Cells.** We then compared the level of PS exposed on the cell surface of the living W3-D430G-L cells with that on apoptotic cells. As shown in Fig. 3A, the forward scatter (FSC) and side scatter (SSC) profiles were similar between the W3-Ilmd and W3-D430G-L cells. A small percentage of the W3-Ilmd cells were annexin V+ and Sytox blue+, indicating that they underwent necrosis under normal growth conditions. Except for these necrotic cells, more than 90% of the W3-Ilmd cells were annexin V+. The W3-D430G-L cells also included a small percentage of necrotic cells, but nearly 95% of them were annexin V+ (Fig. 3A).

When cells undergo apoptosis, their size decreases and cellular granularity increases (12). Accordingly, when the W3-Ilmd and W3-D430G-L cells were treated with FasL for 2 h, the FSC decreased from 118 to 68, and SSC increased from 66 to 127 in both cell lines (Fig. 3A), confirming that the constitutive PS exposure did not inhibit the FasL-induced apoptosis. The induction of apoptosis by FasL treatment caused PS to the cell surface of W3-Ilmd without increasing the amount of annexin V+ and Sytox blue+ necrotic cells. On the other hand, the treatment of W3-D430G-L with FasL did not increase the staining intensity of the annexin V binding, indicating that PS was exposed on the living W3-D430G-L cells to the same extent as on those undergoing apoptosis. MFG-E8 specifically recognizes PS, independent of Ca(2+) (13), and it bound to the W3-D430G-L cells about as well as to the apoptotic W3-Ilmd cells, confirming that both populations exposed PS at comparable levels (Fig. 3B).

The PS exposure was then examined by confocal fluorescence microscopy. As shown in Fig. 3C, almost none of the living W3-Ilmd cells were stained much, if at all, by FITC-labeled MFG-E8. In contrast, the shrunk and rounded FasL-treated W3-Ilmd cells uniformly bound MFG-E8. The lack of microvilli on the cell surface of the FasL-treated cells confirmed that they had undergone apoptosis (14). The living nonapoptotic W3-D430G-L cells were also uniformly labeled with MFG-E8, but the cells were distorted, and their microvilli were visible (Fig. 3D). Treatment of the W3-D430G-L cells with FasL caused them to shrink and become rounded, accompanied by smoothing of the cell surface, but it did not enhance their ability to bind MFG-E8, confirming that the level of exposed PS did not differ between the living and apoptotic W3-D430G-L cells.

**No Engagement of the PS-Exposing Living Cells by Macrophages.** Cells expressing the caspase-resistant form of ICAD do not undergo apoptotic DNA fragmentation (10). When these cells are engulfed by macrophages, DNase II in the lysosomes of the macrophages cleaves the DNA of the apoptotic cells. Using this system, we could distinguish the cells inside macrophages from those bound to the macrophage surface, to assay the engulfment of apoptotic cells (13). When FasL-treated W3-Ilmd cells were coincubated with thioglycollate-elicited peritoneal macrophages, 35–38% of the
Macrophages were stained with TUNEL (Fig. 4A). Microscopic observation of the macrophages confirmed that the apoptotic TUNEL+ cells were inside the macrophages (Fig. 4B). The engulfment of the apoptotic W3-Ildm cells was PS dependent, because it could be inhibited in a dose-dependent manner by the D89E mutant of MFG-E8 (Fig. 4C), which masks PS (13). On the other hand, like living W3-Ildm cells that did not expose PS, the PS-exposing living W3-D430G-L cells were not engulfed by the macrophages, although the cells could be engulfed after they were treated with FasL (Fig. 4A and B).

Mouse resident peritoneal macrophages express Tim-4, which specifically binds PS (15). To examine whether the PS-exposing living W3-D430G-L cells were macrophages, the cells were labeled with the cell-tracker 5-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMRA), and incubated with macrophages. As shown in Fig. 4D and E, when the coincubation was performed at 4°C, the apoptotic W3-Ildm cells and the living W3-D430G-L cells, but not the living W3-Ildm cells, slowly bound to the macrophages. On the other hand, when the incubation temperature was increased to 25°C, the apoptotic W3-Ildm and apoptotic W3-D430G-L cells quickly bound to the macrophages, but the ability of the living W3-D430G-L cells to bind the macrophages was lost. These results suggested that the affinity of PS-exposing living W3-D430G-L cells for macrophages is weaker than that of apoptotic cells.

**No Clearance of Phosphatidylserine-Exposing Cells in Vivo.** Circulating apoptotic cells are engulfed in the spleen by CD8α+ CD11c+ DCs in a PS-dependent manner (16, 17). To investigate whether the viable PS-exposing cells could be engulfed by DCs, the CMRA-labeled viable or apoptotic W3-Ildm and W3-D430G-L cells were injected into syngeneic BALB/c mice. As shown in Fig. 5A, about 20% of the CD8α+ DCs in the spleen carried apoptotic cells 30 min after the administration of dead cells. On the other hand, when the living W3-Ildm cells were injected, 6–8% of the CD8α+ DCs were CMRA+, suggesting that some population of W3-Ildm cells underwent apoptosis in vivo. The percentage of CMRA+ CD8α+ DCs obtained with the PS-exposing living W3-D430G-L cells was comparable to that obtained with the living W3-Ildm cells, indicating that the exposed PS was not sufficient for the viable cells to be engulfed by splenic CD8α+ DCs.

To confirm that the PS-exposing cells were not recognized by phagocytes in vivo, the W3-Ildm and W3-D430G-L cells were injected into BALB/c nude mice. As shown in Fig. 5B, both the W3-Ildm and W3-D430G-L cells generated tumors, which were of comparable size on day 28. Flow cytometry analysis of the
recovered day 28-tumor cells revealed that a significant population (about 33%) of W3-D430G-L but not W3-Ildm cells constitutively exposed PS (Fig. 5C). The apparent reduction of the PS-exposing cells could be due to the poor environmental conditions (hypoxia and acidic conditions) for the tumor cells.

Discussion

In this report, we found that the insertion of a 21-aa peptide at the N-terminal cytoplasmic region of TMEM16F strengthened the scramblase activity of its constitutively active mutant. Treating the cells with a Ca chelator blocked the PS exposure caused by the scramblase activity of its constitutively active mutant. We used these high-PS-exposing cells to study how the IL-3 receptor complex and FasL–Fas complex are internalized for the signal transduction in these cells.

PS is exposed during apoptotic cell death, and its exposure is necessary for the dead cells to be engulfed by macrophages (4, 21). PS is exposed in other situations, too. For example, activated platelets, mast cells, and neutrophils expose PS (22), whereas myocytes and trophoblasts transiently expose PS during their maturation (23, 24). Annexin V can bind to activated lymphocytes under some conditions (25–28), and to an ovarian carcinoma cell line (29), suggesting that these cells may also expose PS. Except for the activated neutrophils, these PS-exposing cells are not engulfed by macrophages. On the other hand, Fadok et al. reported that when the outer leaflets of living cells are enriched with PS by liposomes, the living cells are engulfed by macrophages (5). In contrast, we found that mouse lymphoma cells exposing PS were not engulfed if they were alive. This may agree with the previous report that the PS-exposure alone on the PS-exposing cells to study whether lipid rafts are present in the PS-exposing cells, and how the IL-3–IL-3 receptor complex and FasL–Fas complex are internalized for the signal transduction in these cells.
In conclusion, we showed here that PS exposed on apoptotic cells is required but not sufficient for the dead cells to be engulfed. Another mechanism(s), a modification of PS, or additional molecules that could be don’t-eat-me or eat-me signal(s), must be involved for the efficient engulfment of apoptotic cells. The cell line established in this report, which strongly exposes PS while remaining viable, will be useful for elucidating how macrophages recognize apoptotic cells for engulfment.

Materials and Methods

Mice, Cell Lines, Recombinant Proteins, and Reagents. C57BL/6J, BALB/c, and BALB/c nude mice were purchased from CLEA, Japan. All mouse studies were approved by the ethics review committee for animal experimentation of the Graduate School of Medicine, Kyoto University. Mouse IL-3-dependent Ba/F3 cells were maintained in Roswell Park Memorial Institute RPMI medium containing 10% FCS (Gibco), 100 units/mL mouse IL-3, and 50 μM 2-mercaptoethanol. W3-Ildm is a transformant of mouse T-cell lymphoma (W251R) that expresses mouse Fas and a caspase-resistant form of the inhibitor of ICAD (10) and was cultured in DMEM containing 10% FCS. Mouse IL-3 was produced by mouse C127/l cells transformed with a bovine papillomavirus expression vector bearing IL-3-cDNA as described (39). The biological activity of the mouse IL-3 was determined with Ba/F3 cells, and one unit is defined as the dilution that gives a half-maximum response. The recombinant soluble form of human FasL and the D89E mutant of mouse MFG-E8 were produced in COS7 and HEK293T cells, respectively, and purified as described (13, 40). The biological activity of FasL was determined with W3-Ildm cells, and one unit is defined as that dilution that gives a half-maximum response. The FITC-labeled bovine MFG-E8 (BLAC-FITC) was from Hematologic Technologies. CellTracker Orange CMRA was from Invitrogen. Rat mAbs against mouse Mac-1 (clone M1/70), FcγRIIb (clone 2.4G2), CD11c (clone H3L), Thy1.2 (clone S3-2-1), and CD8α (clone S3-6-7) were purchased from BD Pharmingen.

Transformation of Mouse Ba/F3 and W3-Ildm Cells. Ba/F3 and W3-Ildm cells were subjected to ecotropic and amphotropic retrovirus-mediated transfection, respectively. In brief, the retrovirus carrying the Flag-tagged TMEM16F cDNA was produced by introducing the pMX-puro vector into Plat-E cells expressing MuLV gag-pol and env (41) or into 293T cells with the amphotropic retrovirus-packaging construct pE-Ampho (Takara Bio) and pE-Fg-gag for MuLV gag-pol. The retroviruses were concentrated by centrifugation at 6,000 × g for 16 h at 4 °C and used to infect Ba/F3 and W3-Ildm cells. The transfectants were selected by culturing the cells in the presence of 1 μg/mL puromycin. If necessary, a population (1–5%) of the transfectants that was strongly stained with annexin V was sorted by FACS Aria (BD Bioscience) for further studies.

Induction of Apoptosis and Detection of Phosphatidylserine. To induce apoptosis, cells (1.0 × 10^6 cells/mL) were incubated with 100 units/mL Fasl at 37 °C for 2 h. The cell viability was assayed by WST-1 assay with 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (Roche Molecular Biochemicals) and 1-Methylenephenazine methosulfate as described (11). To detect PS, cells were stained at 25 °C for 5 min with 1,000-fold diluted Cy5-labeled annexin V (Biovision) or 800 ng/mL FITC-MFG-E8 in staining buffer [10 mM Hepes-KOH (pH 7.4) containing 140 mM NaCl and 2.5 mM CaCl_2], followed by incubation with 500 mM Systox blue, and analyzed by FACS Aria. For microscopic observation, 2 × 10^4 cells in eight-well Lab-Tek II chamber slides (Nalge Nunc) were incubated on ice for 15 min with 4 μg/mL FITC-MFG-E8 in staining buffer and observed by confocal fluorescence microscopy (FV1000-D; Olympus).

Preparation of Macrophages and in Vitro Phagocytosis Assay. Resident peritoneal macrophages were prepared from 6- to 12-wk-old C57BL/6J mice as described (15). To prepare thioglycollate-elicited peritoneal macrophages, the mice were injected with 60 mg of thioglycollate, and the peritoneal macrophages were collected 4 d later. The in vitro phagocytosis assay was performed as described previously (13, 42). In brief, 6 × 10^5 thioglycollate-elicited peritoneal macrophages were grown overnight in 12-well cell culture plates (Corning). Apoptotic or PS-exposing cells (3 × 10^5) were added to the macrophages, and the mixture was incubated at 37 °C for 2 h in the presence of 1 μg/mL rat antimalharinum FcγRIII. Macrophages were detached from the plate by treatment with 0.25% trypsin in PBS containing 1 mM EDTA and stained with APC-conjugated antibody against mouse Mac-1, followed by wash steps with TrypsinStain with FITC-labeled DUTP (Roche Molecular Biochemicals). Flow cytometry was conducted using a FACS Aria, and the percentage of phagocytosis was defined as the percentage of TUNEL* cells in the Mac-1+ population. In some cases, peritoneal...
macrophages (6 × 10^6 cells) were incubated with living or apoptotic cells (3 × 10^5 cells/well) in eight-well Lab-Tek II chambers. After fixation with 1% paraformaldehyde, the cells were subjected to TUNEL staining using the Apoptag kit (Millipore), and observed by confocal fluorescence microscopy.

**Binding of PS-Exposing Cells to Macrophages.** CMRA-labeled living or apoptotic cells (2.5–5 × 10^5 cells) were coincubated in suspension with freshly prepared peritoneal cells (1 × 10^6 cells) in PBS supplemented with 10% FCS. The cells were stained with APC-conjugated anti-Mac-1, followed by 500 nM Sytox blue, and analyzed by FACS Aria. For microscopic observation, 1 × 10^5 peritoneal cells were seeded into eight-well Lab-Tek II chambers, incubated at 37 °C for 2 h, and washed with PBS containing 10% FCS. CMRA-labeled cells (5 × 10^5 cells) were added to the well, and the mixture was incubated on ice for 1 h. After the incubation, the cells were washed three times with prechilled PBS, stained with Alexa Fluor 488-conjugated anti-Mac-1 for 5 min on ice, and observed by fluorescence microscopy (Biorad BZ-9000, Keyence).

**In Vivo Phagocytosis Assay and Tumor Development.** The in vivo phagocytosis assay was carried out as described (17). In brief, 5 × 10^3 Wt-Ildm living cells were preincubated for 60 min with a caspase inhibitor, 10 μM Q-VD-OPh (R&D Systems), and stained at 25 °C for 30 min with 4 μM CMRA. Apoptosis was induced by treating the cells with 100 units/mL FasL for 90 min at 37 °C. The cells were then harvested, CMRA-labeled cells in 300 μL of PBS were injected i.v. into B6, 8-week-old female BALB/c mice. Thirty minutes later, the spleens were dissected, flushed with 100 units/mL collagenase D (Roche Diagnostics), teased apart with fine forceps, and digested with 400 units/mL collagenase D for 30 min at 37 °C. After hemolysis with EL buffer (Qiagen), the DCs were enriched by MACs sorting with anti-mouse CD11c microbeads (Miltenyi Biotech). The DCs were then stained with APC-conjugated rat anti-mouse CD11c and FITC-labeled rat anti-mouse CD16/CD32, followed by staining with 500 nM Sytox blue, and analyzed by FACS Aria. Some cases, the CMRA cells in the CD11c/CD16+ population were sorted by FACS Aria and observed by fluorescence microscopy.

To examine the ability of lymphoma to grow in vivo, cells (1 × 10^6) were injected into 8-week-old female BALB/c nude mice. Four weeks later, the tumors were dissected, and their weight was determined. The dissected tumors were further teased apart with fine forceps and digested with collagenase D as described above. After passing through mesh, the cells were washed with cold PBS and stained with Cy5-labeled annexin V, followed by staining with 1 μg/mL FITC-labeled rat anti-mouse Thy1.2 and Sytox blue, and analyzed by FACS Aria.

**Statistical Analysis.** All data were expressed as the mean ± SD. Differences between groups were examined for statistical significance using Student’s t test with Bonferroni correction.

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