Carbohydrate-mediated Phagocytic Recognition of Early Apoptotic Cells Undergoing Transient Capping of CD43 Glycoprotein*

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A novel mechanism of phagocytic recognition of apoptotic cells was found and characterized. Jurkat cells incubated with appropriate concentrations of etoposide or anti-Fas antibody transiently became susceptible to binding and phagocytosis by THP-1 cell-derived macrophages at 2 h. The bound Jurkat cells showed no chromatin condensation, but the binding was prevented by a caspase inhibitor, indicating that they were recognized at an early stage of apoptosis. The ligands recognized on the apoptotic cells were sialylpolyolactosaminyl sugar chains because 1) the binding was inhibited by an oligosaccharide preparation of erythrocyte membrane, and its inhibitory activity was destroyed by polyolactosaminoglycan-specific endo-β-galactosidase or neuraminidase; 2) Jurkat cells pretreated with endo-β-galactosidase or neuraminidase failed to be agglutinated by Datura stramonium agglutinin; and 3) the treatment of the apoptotic cells with polyolactosaminoglycan-binding Datura stramonium agglutinin prevented recognition. The sialylpolyolactosaminyl chains involved were most likely those of a sialolglycoprotein CD43 because anti-CD43 antibody inhibited recognition. CD43 on apoptotic Jurkat cells was found to form a cap at 2 h, and the cap disappeared at 4 h. This transient capping of CD43 coincided with the transient increase in the susceptibility of the cells to macrophage recognition, suggesting that CD43 capping is responsible for generation of the carbohydrate ligands for recognition. Furthermore, microscopic observation suggested that the apoptotic cells were recognized in part by the CD43 cap. Taken together, we conclude that apoptotic Jurkat cells transiently undergo CD43 capping at an early stage of apoptosis and are recognized by macrophages through the cluster of sialylpolyolactosaminyl chains of the capped CD43.

Cells dying by apoptosis are swiftly ingested by phagocytes before they rupture and release injurious and immunogenic contents into the surrounding tissue, and thus clearance of apoptotic cells by phagocytes is important in maintaining tissue homeostasis (1–4). Moreover, phagocytosis of apoptotic cells by macrophages has long been suggested by inhibition with various carbohydrates in the recognition of apoptotic thymocytes by peritoneal macrophages (21, 22), that of apoptotic neutrophils by fibroblasts (23), that of apoptotic lymphocytes by Kupffer cells (24), and that of apoptotic liver cells by unidentified liver cells (25). However, little is known about carbohydrate chains and glycoconjugates involved and about the mechanism of generation of the carbohydrate ligands on apoptotic cells. In a series of studies, we have demonstrated that oxidized cells such as oxidized erythrocytes (26, 27), neutrophils (28), and human T lymphocyte cell line Jurkat cells (29) are recognized by macrophages and that the ligands on the oxidized cell surface recognized by macrophages are carbohydrate chains containing sialylated polyolactosaminoglycans (i.e. sialyl poly-N-acetyllactosaminyl chains) (26–29) of membrane glycoproteins. We also found that naturally occurring antibody to band 3 glycoprotein of human erythrocytes, which is directed to sialylated polyolactosaminoglycans of band 3 (30), binds to oxidized and senescent erythrocytes (31, 32) and to oxidized Jurkat cells (29). These observations have been reasonably explained by the hypothesis that membrane glycoproteins aggregate to form clusters upon cell oxidation, and the resultant clusters of their extracellular polyolactosaminoglycans provide multivalent high affinity ligands for macrophage receptors.

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† The abbreviations used are: PS, phosphatidylserine; Z, benzoyl oxy-acetylserine; fmk, fluoromethyl ketone; DSA, D. stramonium agglutinin; DPBS−, Ca2+-, Mg2+-free Dulbecco’s phosphate buffered saline; BSA, bovine serum albumin.

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and for anti-band 3 antibody (“glycoprotein clustering hypothesis”) (27–34).

The above findings led us to investigate whether a similar carbohydrate-mediated mechanism participates in phagocytic recognition of apoptotic cells. We report here that apoptotic Jurkat cells transiently undergo capping of CD43 membrane glycoprotein at an early stage of apoptosis and that sialylated polyglycosaminoglycans of the capped CD43 that are condensed on the cell surface serve as ligands for recognition and phagocytosis by macrophages.

**EXPERIMENTAL PROCEDURES**

**Materials**—Etoposide, cycloheximide, bisbenzimide (Hoechst 33258), ethidium bromide, and trypsin (E.C. 3.4.21.4, porcine pancreas) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Vine serum albumin (BSA), RNase A, proteinase K, and phorbol myristate acetate were obtained from Sigma. Benzoylcarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk) was the product of the Peptide Institute (Osaka, Japan). Rhodamine-labeled concanavalin A and Datura stramonium agglutinin (DRA) were purchased from Vector Laboratories (Burlingame, CA). Enb-galactosidase (E.C.3.2.1.103, Escherichia freundii), α-galactosidase A (Melibiose) (E.C.3.2.1.22, Mortierella virosa), and endo-β-N-acetylglucosaminidase H (E.C. 3.2.1.19) were obtained from Seikagaku Fine Chemicals (Tokyo, Japan). Neuraminidase (E.C.3.2.1.18, Vibrio cholerae) was purchased from Behringwerke AG (Marburg, Germany). Anti-Fas antibody (clone CH-11) was purchased from Medical & Biological Laboratories (Nagoya, Japan). Anti-CD43 mouse monoclonal antibody (clone DF-T1) and control mouse IgG1 were obtained from DAKO (Glostrup, Denmark). Anti-CD2 and anti-CD3 mouse monoclonal antibodies were obtained from the Nichirei Corporation (Tokyo, Japan). Alexa Fluor-488 goat anti-mouse IgG (H+L) conjugate was the product of Molecular Probe (Eugene, OR). Oligosaccharides of human erythrocyte membrane glycoprotein, mainly those from band 3 glycoprotein, were prepared by hydrazinolysis of defatted human erythrocyte ghosts as described before (27).

**Induction and Measurement of Apoptosis**—Apoptosis of Jurkat cells (Riken Cell Bank, Tsukuba, Japan) was induced by incubation of the cells (4.0 × 10⁶ cells/ml) in RPMI 1640 medium containing 5% fetal bovine serum with appropriate concentrations of etoposide, anti-Fas antibody (clone CH-11), or cycloheximide at 37 °C in 5% CO₂ atmosphere for various hours. Apoptosis of the treated cells was assessed by chromatin condensation and by DNA fragmentation. For measurement of chromatin condensation, etoposide-treated Jurkat cells were fixed with 1% glutaraldehyde in Ca⁺⁺-,Mg⁺⁺-free Dulbecco’s phosphate-buffered saline (DPBS(−)) at room temperature for 30 min followed by staining with 6.1 μM bisbenzimide (Hoechst 33258) in DPBS(−) at room temperature overnight. Morphology of the nuclei of Jurkat cells was observed under a fluorescence microscope. For measurement of DNA fragmentation, etoposide-treated Jurkat cells were washed twice with DPBS(−). The cell pellets (4 × 10⁶ cells) were resuspended in 100 μl of DPBS(−), and then 10 μl of 10 μg/ml RNase, 10 μl of 10 mg/ml proteinase K, and 20 μl of 10% sodium dodecyl sulfate were sequentially added to this solution. After incubation at 37 °C for 30 min, 300 μl of NaI solution (26 mM Tris/HCl, pH 8.0, 6 mM Na⁺, 13 mM EDTA, 0.5% sodium N-lauroylsarcosinate, 10 mg/ml glycogen) was added to the solution. The solution was incubated at 37 °C for 30 min and then chilled on ice for 20 min. DNA was precipitated by addition of 500 μl of isopropanol and followed by centrifugation at 15,000 rpm for 15 min at 4 °C. The DNA pellet was sequentially washed with 50 and 100% isopropanol and then with diethylther. The air-dried DNA pellet was dissolved in 100 μl of TE buffer (10 mM Tris/HCl, pH 7.4, 5 mM EDTA) and electrophoresed on 2% agarose gels at 50 V for 2 h, and DNA bands were visualized by staining with ethidium bromide.

**Binding and Phagocytosis Assays**—THP-1 cells (Japanese Cancer Research Resources Bank, Osaka, Japan) were cultured in RPMI 1640 with 5% fetal bovine serum and plated on 12-well plates at 1 × 10⁶ cells/well in which round coverslips (15-mm diameter) were placed and cultured with 50 nM phorbol myristate acetate at 37 °C in 5% CO₂ atmosphere for 4–5 days. The monolayers of THP-1 cells differentiated into macrophages on coverslips were washed in DPBS(−) before use. Jurkat cells (4 × 10⁶ cells/ml) treated with an apoptosis-inducing agent were prepared with appropriate concentrations for various hours at 37 °C in RPMI 1640 with 5% fetal bovine serum were washed three times with DPBS(−), resuspended in RPMI 1640 medium buffered with 20 mM HEPES, pH 7.2 (RPMI 1640-HEPES) at 4 × 10⁶ cells/ml, and coincubated with macrophage monolayers for 1 h with gentle shaking. In the case of time course experiments, the beginning of the Jurkat cell incubation with an apoptosis-inducing agent was staggered for each time group so that all of the binding assays may be done at one time. Unbound cells were removed by gentle washing, and then bound Jurkat cells and macrophages were fixed with 1.25% glutaraldehyde and stained with Mayer’s hematoxylin solution. The number of bound Jurkat cells and macrophages was counted under a light microscope (>400 magnification). The data are expressed as the number of bound Jurkat cells/100 macrophages as counting more than 300 macrophages. For phagocytosis assay, etoposide-treated Jurkat cells were labeled with fluorescein isothiocyanate (4 μg/ml) at 37 °C for 10 min. The labeled Jurkat cells were coincubated with macrophage monolayers similarly to the binding assay. After coincubation, unbound and lightly attached Jurkat cells were removed by washing and by treatment with 0.5 mg/ml trypsin at room temperature for 2 min. Then the cell surface facing the medium was stained with rhodamine-labeled concanavalin A (20 μg/ml) at 0 °C for 10 min, and Jurkat cells taken up by macrophages were identified under a confocal laser scanning fluorescence microscope (μ-Radiance; Bio-Rad).

**Measurement of Cell Surface CD43**—Jurkat cells or Jurkat cell-bound macrophage monolayers were treated with 10 μg/ml anti-CD43 mouse monoclonal antibody (clone DF-T1) in RPMI 1640-HEPES with 0.2% BSA at 0 °C for 30 min and washed several times with DPBS(−) at 0 °C. Bound antibody was detected by treatment of the cells with 10 μg/ml Alexa Fluor-488 goat anti-mouse IgG (H+L) conjugate in RPMI 1640-HEPES with 0.2% BSA at 0 °C and washing several times with DPBS(−) at 0 °C. The cells were resuspended in Hanks’ balanced salt solution at 0 °C and immediately subjected to microscopic observation by a fluorescence microscope (Axiovert 200M; Carl Zeiss) or a confocal laser scanning fluorescence microscope and flow cytometry analysis by a flow cytometer (FACSCalibur; Becton Dickinson) using CELLQUEST software. Three-dimensional analysis of confocal images was performed using a software LaserSharp. Throughout the immunofluorescence staining process, the cell suspensions were kept at 0 °C to prevent antibody-induced antigen redistribution that may occur at higher temperature.

**RESULTS**

**Phagocytic Recognition of Early Apoptotic Jurkat Cells**—First, we tried to determine conditions for induction of apoptosis that are suitable for macrophage recognition assay, using etoposide as an apoptosis-inducing agent and Jurkat cells as target cells. Human monocytic THP-1 cells differentiated by phorbol myristate acetate into adherent cells were used as macrophages. Fig. 1A demonstrates the relationship between concentrations of etoposide or the time of etoposide treatment and binding of the etoposide-treated Jurkat cells to macrophages. Jurkat cells became susceptible to binding to macrophages when treated with the proper concentrations of etoposide for proper periods. Maximal binding was observed when treated with 10 μM of etoposide for 1 or 2 h (Fig. 1A, left and right panels, filled circles). Treatment with etoposide at higher concentrations or for longer periods resulted in decreased binding. Thus, we hereafter used Jurkat cells treated with etoposide at 10 μM for 2 h, unless otherwise indicated.

The extent of apoptosis was assessed by chromatin condensation staining with Hoechst 33258. The percentage of chromatin-condensed cells in Jurkat cells treated with various concentrations of etoposide for 2 h (Fig. 1A, left panel, filled squares) and those treated with 10 μM etoposide for various hours (Fig. 1A, right panel, filled squares) increased with dose of etoposide (left panel) and with time of treatment (right panel), respectively. The percentage of the chromatin-condensed cells at each point increased slightly after an additional 2 h of incubation without etoposide (filled triangles), the same conditions under which the Jurkat cell-macrophage binding assay was performed. DNA fragmentation was not significant for the Jurkat cells treated with 10 μM etoposide for 2 h and was slightly detectable after an additional 2 h of incubation without etoposide (data not shown). Thus, during etoposide treatment and the following binding assay without etoposide, only small pro-
portions of Jurkat cells underwent nuclear changes. The Jurkat cell specimens of the binding assay were then examined for chromatin condensation by staining with Hoechst 33258. None of the Jurkat cells binding to the macrophages showed chromatin condensation (data not shown), indicating that etoposide-treated Jurkat cells were recognized by macrophages at an early stage of apoptosis before chromatin condensation occurred. To exclude the possibility that recognition occurred independently on the apoptotic process, the effect of the caspase inhibitor Z-VAD-fmk (35) on recognition was tested. As shown in Fig. 1B, recognition by macrophages and chromatin condensation of etoposide-treated Jurkat cells were prevented by Z-VAD-fmk to the level of etoposide-untreated cells, indicating that the observed susceptibility of the etoposide-treated Jurkat cells to macrophage recognition was dependent on the apoptotic process.

We examined whether the apoptotic cell recognition observed herein resulted in phagocytosis. After the binding assay procedure using fluorescein isothiocyanate-labeled apoptotic Jurkat cells and the following removal of lightly attached Jurkat cells by trypsin treatment, the cell surface was labeled with rhodamine-labeled concanavalin A and then observed under a confocal laser scanning fluorescence microscope. As shown in Fig. 1C, Jurkat cells remaining on macrophage surface were observed (left panel, arrowheads), suggesting that these cells began being phagocytosed. Jurkat cells being inside the macrophages were also observed (left and right panels, arrows), which obviously indicated that phagocytosis was taking place. Then the values of binding and phagocytosis (the mean number of Jurkat cells binding and phagocytosed per 100 macrophages ± S.D., respectively) were determined for the same assay. When the binding was 29.7 ± 5.1, the phagocytosis...
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Fig. 2. Involvement of carbohydrate chains of CD43 in the binding of early apoptotic Jurkat cells to macrophages. Apoptosis of Jurkat cells was induced by treatment with 10 μM etoposide for 2 h. All the data are the means ± S.D. of triplicate experiments. A, effect of oligosaccharides from erythrocyte membrane glycoproteins on the binding of apoptotic Jurkat cells to macrophages. Binding of apoptotic Jurkat cells to macrophages was carried out in the presence or the absence of oligosaccharides (100 μg/ml) prepared from human erythrocyte membrane or those digested with endo-β-galactosidase (100 milliunits/ml) or neuraminidase (100 milliunits/ml) in 50 mM acetate buffer, pH 5.8, 0.1 M NaCl at 37 °C for 48 h followed by heat inactivation (100 °C, 2 min) of the enzymes. Binding assay was performed as described under “Experimental Procedures.” B, effect of pretreatment with glycosidases on the binding of apoptotic Jurkat cells to macrophages. Jurkat cells were pretreated with endo-β-galactosidase (50 milliunits/ml) or neuraminidase (50 milliunits/ml) in RPMI 1640-HEPES at 37 °C for 1 h. The cells were washed, treated with etoposide, and subjected to the binding assay. C, effect of DSA on the binding of apoptotic Jurkat cells to macrophages. After etoposide treatment, Jurkat cells were incubated with or without the indicated concentrations of DSA in RPMI 1640-HEPES containing 0.2% BSA at 0 °C for 30 min, washed, and subjected to the binding assay. D, effect of anti-CD43 antibody on the binding of apoptotic Jurkat cells to macrophages. After etoposide treatment, the Jurkat cells were incubated with or without 10 μg/ml of anti-CD43 mouse monoclonal antibody (clone DF-T1) or control mouse IgG1 in RPMI 1640-HEPES containing 0.2% BSA at 0 °C for 30 min, washed, and subjected to the binding assay.

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was 7.6 ± 2.5 for the etoposide-treated Jurkat cells, whereas the binding and the phagocytosis for control cells were 3.8 ± 0.8 and 2.6 ± 1.0, respectively. The data suggest that approximately 20–25% of the apoptotic cells bound to macrophages were taken up during the 2-h binding assay. Phagocytosis of etoposide-treated cells increased up to 11.0 ± 1.7 when the binding assay was prolonged to 4 h.

Involvement of Carbohydrate Chains of CD43 in Recognition—We next examined whether recognition of the early apoptotic Jurkat cells by macrophages occurred through carbohydrate chains on Jurkat cells, as was the case with recognition of oxidized Jurkat cells (29). Recognition was inhibited when oligosaccharides prepared from human erythrocyte membrane were present during the binding assay (Fig. 2A). The inhibitory activity of the oligosaccharides was destroyed when treated with endo-β-galactosidase, an enzyme that specifically cleaves poly-N-acetyllactosaminyl structure (i.e. Gal[β1–4GlcNAcβ1–3 repeats) at the β-galactosidic bond (36) or with neuraminidase that removes sialic acid residues from the nonreducing termini of carbohydrate chains (Fig. 2A). As shown in Fig. 2B, when Jurkat cells had been pretreated with endo-β-galactosidase or neuraminidase prior to induction of apoptosis, recognition did not occur. These data suggest that sialylated polylactosaminoglycans are recognition sites on apoptotic cells, although the possibility that polylactosaminoglycans and other sialylated chains independently contributed to recognition cannot be ruled out. In support of these results, DSA, a lectin known to preferentially bind to polylactosamine-type carbohydrate chains (37), significantly inhibited the recognition of apoptotic cells at concentrations of 0.05 and 0.1 μg/ml (Fig. 2C) without causing cell agglutination. Pretreatment of Jurkat cells with α-galactosidase A, a glycosidase that hydrolyzes nonreducing terminal α-galactosyl linkages, was ineffective on recognition as expected because polylactosamines do not have α-galactosyl linkages (data not shown).

It is known that CD43 (leukosialin/sialophorin), a mucin-like major sialoglycoprotein on various types of hematopoietic cells (38, 39) that shows multiple functions such as cell adhesion, anti-adhesion, signal transduction, and cytoskeletal interaction (40, 41), possesses sialylated polylactosaminoglycans (42).
To see whether sialyl residues or/and polylactosaminyl chains of CD43 on apoptotic Jurkat cells are recognized by macrophages, the effect of anti-CD43 monoclonal antibody (clone DF-T1), an antibody directed against a sialic acid-dependent unidentified epitope of CD43 (43), on the recognition of apoptotic cells was tested. Recognition was effectively blocked by the anti-CD43 antibody, whereas irrelevant control IgG1 had no effect (Fig. 2D), suggesting the involvement of carbohydrate chains of CD43 in recognition. The antibody bound to endo-β-galactosidase-treated Jurkat cells as equally well as to untreated cells, although it did not bind to neuraminidase-treated cells, as analyzed by flow cytometry (data not shown). This suggests that the carbohydrate ligands of CD43 involved in the macrophage recognition are distinct from the epitope for the anti-CD43 monoclonal antibody. Because sialylpolylactosaminyl sugar chains of CD43 were shown to be O-glycans (42), the effect of pretreatment of Jurkat cells with endo-β-N-acetylglucosaminidase H, an enzyme that specifically cleaves the di-N-acetylchitobiose (GlcNAcβ1–4GlcNAc) linkages of some types of N-glycans in glycoprotein (44) but does not cleave O-glycans, was tested. The enzyme pretreatment did not affect the increased susceptibility of the etoposide-treated cells to the macrophage recognition (data not shown), which is consistent with the above observations suggesting the involvement of sialylpolylactosaminyl sugar chains of CD43 in recognition.

**Transient Capping of CD43 on Early Apoptotic Jurkat Cells**—The increased susceptibility of polylactosaminyl chains of CD43 on apoptotic Jurkat cells to macrophage recognition may be explained by the “glycoprotein clustering hypothesis” that has been proposed for oxidized cells (27–34). We then investigated whether CD43 molecules form clusters upon apoptosis and thereby contribute to recognition by macrophages. CD43 on some of the Jurkat cells treated with etoposide for 2 h was actually found to undergo capping (Fig. 3C, arrow), in which CD43 molecules localized to one pole of the cell and formed a large cluster of the molecules, whereas unincubated (Fig. 3A) or untreated (Fig. 3B) control cells unchanged. CD43 on most of control Jurkat cells was uniformly distributed forming micropatches (Fig. 3D). Upon the induction of apoptosis, the cells undergoing various extents of CD43 capping appeared (Fig. 3E). Visualization of CD43 distribution by two-dimensional and three-dimensional confocal fluorescence microscopy clearly demonstrated the uniform distribution of CD43 micropatches on nonapoptotic cells (Fig. 3F), and cap formation of CD43 on apoptotic cells (Fig. 3G). The proportion of CD43-capped cells in the etoposide-treated cells was nearly 8% at the time of microscopic observation (Fig. 4A). The CD43 cap formation was inhibited by Z-VAD-fmk (Fig. 4A), confirming that the cap formation was dependent on caspase activation and therefore associated with apoptotic process. Interestingly, the increased CD43 capping observed at 2 h dropped to the level of untreated cells at later hours (Fig. 4B). The time of this transient change of CD43 distribution coincided well with the time course of the susceptibility of etoposide-treated cells to macrophage recognition (Fig. 1A, right panel), suggesting that CD43 capping is responsible for the increased susceptibility of apoptotic cells to the carbohydrate-mediated recognition by macrophages. Furthermore, immunostaining of CD43 on Jurkat cells attached to the macrophage monolayer showed that the capped CD43 on Jurkat cell surface was localized at the sites where the cells attached to the macrophages (Fig. 5, arrows). This also suggests that apoptotic Jurkat cells are recognized by macrophages through the capped CD43 on their surface.

To see the distribution of other T-cell surface antigens at the early stage of apoptosis, distribution of CD2 (sheep red blood cell receptor/leukocyte function associated antigen-2) and a mature T-cell marker CD3 on early apoptotic Jurkat cells was observed under the conditions causing CD43 capping. Their distribution, as detected by anti-CD2 or anti-CD3 monoclonal antibodies, was uniform, and no capping was observed (data not shown), indicating that membrane protein antigens do not necessarily undergo capping during apoptosis.

**Carbohydrate-mediated Recognition of Early Apoptotic Cells Induced by Other Apoptotic Stimuli**—The above findings are not confined to the etoposide-induced apoptosis. As shown in Fig. 6A, Jurkat cells treated with the proper concentrations of anti-Fas antibody for the proper number of hours became susceptible to macrophage recognition very similarly to the case...
for the etoposide-induced apoptosis. Treatment with 2 ng/ml (left panel) of anti-Fas antibody for 1 or 2 h (right panel) gave maximal recognition by macrophages. This recognition was dependent on polyactosaminyl chains and neuraminic acid residues on Jurkat cells (Fig. 6B) and CD43 on the cell surface (Fig. 6C) as was the case for the etoposide-induced apoptosis. Moreover, increased CD43-capped cells were observed for the anti-Fas antibody-treated cells (Fig. 7B, arrows). Equivalent results were obtained again for cycloheximide-induced apoptosis (data not shown). Hence, the transient CD43 capping and the resultant carbohydrate-mediated recognition by macrophages are comparable with three different apoptotic stimuli.

**DISCUSSION**

The present study has revealed a novel carbohydrate-mediated mechanism by which macrophages recognize and ingest apoptotic cells. The results demonstrated that Jurkat cells transiently became susceptible to recognition and phagocytosis at an early stage of apoptosis at which nuclear changes such as chromatin condensation and DNA fragmentation were not significant and that recognition was mediated by the apoptotic cell surface carbohydrate chains having sialyl residues and polylactosaminyl structure. The responsible sialylated polylactosaminyl chains were most likely those of CD43 transiently undergoing capping.

Involvement of carbohydrate chains on apoptotic Jurkat cells was demonstrated by the inhibition of recognition by the oligosaccharides from erythrocyte membrane, by the loss of their inhibitory activity by treatment with neuraminidase or endo\(\beta\)-galactosidase, by the loss of recognition by treatment of Jurkat cells with the glycosidases, and by blocking the ligand activity on the cells with a polylactosamine-binding lectin DSA. The loss of the inhibitory activity of the erythrocyte oligosaccharides by endo-\(\beta\)-galactosidase also indicated the requirement for the \(N\)-acetyllactosaminyl repeating structure (Gal\(\beta\)-1\(\rightarrow\)4GlcNAc\(\beta\)-1\(\rightarrow\))\(n\) for the ligand activity. Because these results coincided very well with those obtained for the recognition of various types of oxidized cells (24–28), apoptotic cells and oxidized cells appear to display similar carbohydrate ligands on cell surface.

Some early reports on the involvement of carbohydrates in macrophage recognition of apoptotic cells (21, 22) speculated that the carbohydrate ligands on apoptotic cells to be recognized by phagocytes were asialosaccharide chains exposed by the removal of the nonreducing terminal sialic acids from the sialosaccharide chains during apoptosis, although no evidence was available for the postulated desialylation of sialosaccha-
ride chains during apoptosis. However, the present results indicate, on the contrary, that terminal sialyl residues are essential moieties for the dying cells to be recognized, and the masking role of the terminal sialyl residues are unlikely. Furthermore, measurement of the amount of the Jurkat cell surface sialyl residues releasable by neuraminidase treatment indicated that the amount of terminal sialyl residues of the cell surface did not change at least during the early stage of apoptosis studied here.2

The transient increase in the susceptibility of Jurkat cells to the macrophage recognition at an early stage of apoptosis was clearly explained by the findings that CD43 on Jurkat cells transiently forms a cap at the nearly same stage of apoptosis and this sialylpolylactosamine-containing membrane glycoprotein is involved in recognition. The carbohydrate moieties of CD43 are approximately 70% by weight, consisting of a few N-glycans and approximately 90 O-glycans (38), and these carbohydrate moieties have been suggested to be involved in the various functions of CD43 (40, 41). The present finding re-
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...how does the cap disappear? Because CD43 capping was dependent on caspase activity, it is likely that caspase-dependent cytoplasmic events causing cytoskeletal changes are involved. One possibility may be involvement of moesin, one of the cytoskeletal proteins of the ERM (ezrin/radixin/moesin) family (43) that link cross-link intracellular domain of CD43 and actin filaments. This protein was reported to be dephosphorylated and detach from plasma membrane depending on caspase activation (47). This event was induced at an early stage of apoptosis and resulted in the disappearance of the microvilli of the cell (47). Therefore, CD43 capping may be one of the consequences of this cytoplasmic disintegration. Otherwise, some cytoskeletal machinery may actively induce the movement of CD43 to form a cap.

The number of CD43-capped cells peaked at 2 h and dropped to the level of the control at 4 h. The duration of the CD43-capped cell appearance is therefore less than 4 h, but the life of the CD43 cap on each cell should be shorter than this duration. Following the CD43 movement on a single cell would be necessary for determination and analysis of the life of the CD43 cap. Disappearance of the CD43 cap from the apoptotic cell surface may be due to proteolytic cleavage of the protein, because CD43 is known to be proteolytically down-regulated upon stimulation (48–50) or spontaneously (50). It is also known that extracellular fragment of CD43 is released into plasma (51, 52).

Another important point to be addressed is whether or not PS-mediated recognition is involved in the present experiments. In another study, we have found that PS exposure and PS-mediated recognition occurred at a relatively later stage of apoptosis than the carbohydrate-mediated recognition demonstrated here. It is interesting to note that CD43 capping can create a better cell surface microenvironment for other cell surface components to interact with other cells (40) because CD43 has long and extended mucin-like extracellular domain that is disturbing, when uniformly distributed, for interaction of the cell surface components with other cells. Thus, CD43 capping on apoptotic cells may facilitate other “eat me” signals, including PS, to interact with their respective receptors on macrophages by withdrawing from the overall cell surface to one pole of the cell.

The present study was carried out using human leukemic cell lines, Jurkat and THP-1 cells, but the major profiles of the present findings (i.e. transient capping of CD43 at an early stage of apoptosis and macrophage recognition of the apoptotic cells through the sialylated polyglactosaminoglycans of CD43) were again true in the primary cell system using mouse splenic macrophages. Hence, the carbohydrate-mediated recognition of early apoptotic cells by macrophages found here may be a generally observable event.

Finally, the present observations suggested the presence on macrophages of a new lectin-like receptor for sialylated polyglactosaminoglycans that is involved in the recognition of early apoptotic cells. A candidate protein for this receptor (53) is currently under investigation.

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REFERENCES

1. Savill, J., Fadok, V., Henson, P., and Haslett, C. (1993) Immunol. Today 14, 131–136
2. Platt, N., da Silva, R. P., and Gordon, S. (1998) Trends Cell Biol. 8, 365–372
3. Savill, J., and Fadok, V. (2000) Nature 407, 784–788
4. Fadok, V. A., Bratton, D. L., and Henson P. M. (2001) J. Clin. Invest. 108, 957–962
5. Ren, Y., and Savill, J. (1998) Cell Death Differ. 5, 563–568
6. Vol, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalderon, J. D., and Girkontaite, I. (1997) Nature 386, 350–351
7. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson P. M. (1998) J. Clin. Invest. 101, 890–898
8. Nakamura, K., Yuh, K., Sugyo, S., Kuroki, M., Shijo, H., and Tamura, K. (1999) Cell Immunol. 183, 147–154
9. Steinman, R. M., Turley, S., Mellman, I., and Inaba, K. (2000) J. Exp. Med. 191, 411–416
10. Gregory, C. D. (2000) Curr. Opin. Immunol. 12, 27–34
11. Savill, J., Hogg, N., Ren, Y., and Haslett, C. (1992) J. Clin. Invest. 90, 1513–1522
12. Stern, M., Savill, J., and Haslett, C. (1996) Am. J. Pathol. 149, 911–921
13. Takakawa, P., Tousi, S., and Nishizuka, S. (1998) FEMS Lett. 159, 269–272
14. Mechador, D., Maccarellis, J. O., Gershov, D., and Elkon, K. B. (1998) J. Exp. Med. 188, 2313–2320
15. Taylor, P. R., Caragati, A., Fadok, V. A., Cook, H. T., Andrews, M., Carroll, M. C., Savill, J. S., Henson P. M., Botto, M., and Waller, M. P. (2000) J. Exp. Med. 192, 359–366
16. Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A., and Henson, P. M. (2001) J. Exp. Med. 194, 781–785
17. Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002) Nature 417, 182–187
18. Sato, S. J., Heutelingsperger, C. P. M., McGahan, A. J., Rader, J. A., van Schie, R. C. A. A., and deVries, G. M. (1996) Am. J. Pathol. 148, 1469–1476
19. Yamanaka, M., Yamanaka, and M. Beppu, unpublished observation.

Y. Nakano, M. Yamanaka, and M. Beppu, unpublished observation.

M. Yamanaka, S. Eda, and M. Beppu, manuscript in preparation.

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