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Carbohydrate chains and phosphatidylserine successively work as signals for apoptotic cell removal☆

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Abstract

At an early stage of apoptosis, Jurkat cells transiently become susceptible to binding and phagocytosis by macrophages through the polylactosamine-type carbohydrate chains of CD43 [J. Biol. Chem. 279 (2004) 5967]. Susceptibility of apoptotic Jurkat cells to macrophage recognition was studied over an extended time range of 0–24 h including a later stage. Jurkat cells incubated with appropriate concentrations of apoptosis-inducing agents etoposide or anti-Fas antibody became susceptible to macrophage-binding at 2 h, and the susceptibility fell to the control level at 4 or 6 h. However, it increased again at later hours (6–24 h). Flow cytometric analyses of CD43 and phosphatidylserine (PS) on the apoptotic cells indicated that CD43 began to degrade at around 4 h, and PS is externalized significantly at 4 or 6 h. The macrophage-binding at 2 h was prevented by glycosidase treatment of Jurkat cells, but not by annexin V. Conversely, the later binding at 12 or 18 h was not prevented by glycosidase treatment, but was done so by annexin V. These results suggest that Jurkat cells become susceptible to phagocytic removal at an early stage of apoptosis by the carbohydrate-mediated mechanism, and at a later stage by the PS-mediated mechanism.

Keywords: Apoptosis; Macrophage; Polylactosamine; Phosphatidylserine exposure; CD43; Eat-me signal

Apoptotic cells are rapidly recognized and ingested by phagocytes. This phenomenon is important for a body to prevent release of intracellular contents that may be damaging to the surrounding tissue or immunogenic to self [1–4]. Without this clearance mechanism, various disorders such as inflammation and autoimmunity are expected to occur [1,2,5].

Macrophages recognize the cell surface components or structures on apoptotic cells not existing on live cells [2–4]. These include phosphatidylserine (PS) that is exposed from the inner surface of plasma membrane to the outer surface during the course of apoptosis [6–9], carbohydrates that undergo poorly identified alterations on the cell surface [10–14], and some other membrane molecules [3,4].

We have recently demonstrated that human T lymphatic Jurkat cells undergoing apoptosis are recognized and phagocytosed by macrophages at an early stage of apoptosis depending on the cell surface carbohydrate chains containing sialylated polylactosaminoglycans (i.e., sialylpoly-*N*-acetyllactosaminyl chains) [15]. The susceptibility of the apoptotic Jurkat cells to the macrophage recognition was transiently increased at 1 or 2 h and decreased at 4 h after induction of apoptosis. Interestingly, the increased susceptibility of the apoptotic cells to the macrophage recognition was found to be due to transient cap formation of CD43, a mucin-like major sialoglycoprotein containing

☆ *Abbreviations:* BSA, bovine serum albumin; DPBS(−), Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline; FITC, fluorescein isothiocyanate; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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sialylpolylactosaminyl chains [16,17]. It was suggested that CD43 capping on the apoptotic cell membrane resulted in condensation of their carbohydrate chains into the capped area, which increased the binding avidity of the sialylpolylactosaminyl saccharide ligands to the macrophage receptors [15].

Although the ligands involved in the observed recognition of the apoptotic Jurkat cells by macrophages were found to be carbohydrates, it is also known that apoptosis of many cell types, including Jurkat cells, results in externalization of PS on cell surface [6,18,19].

We, then, studied the possibility of PS involvement in the macrophage recognition of apoptotic Jurkat cells under the same experimental conditions as used in the previous work but extending the time range of apoptosis to 24 h.

Materials and methods

Materials. Etoposide and bisbenzimide (Hoechst 33258) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA), PKH26 Red Fluorescent Cell Linker Kit, and phorbol myristate acetate were obtained from Sigma–Aldrich (St. Louis, MO). Endo-β-galactosidase (EC 3.2.1.103, *Escherichia freundii*) and neuraminidase (EC 3.2.1.18, *Vibrio cholerae*) were obtained from Seikagaku Fine Chemicals (Tokyo, Japan) and Behringwerke AG (Marburg, Germany), respectively. Anti-Fas monoclonal antibody (clone: CH-11), recombinant human annexin V, MEBCYTO apoptosis kit, and MEBSTAIN apoptosis kit direct were purchased from Medical and Biological Laboratories (Nagoya, Japan). Clone DF-T1 and clone L10 of anti-CD43 mouse monoclonal antibodies were obtained from DAKO (Glostrup, Denmark) and Southern Biotechnology Associates (Birmingham, AL), respectively. Alexa Fluor 488-goat anti-mouse IgG (H + L) conjugate was the product of Molecular Probe (Eugene, OR).

Glycosidase treatment of Jurkat cells. A suspension of Jurkat cells (Riken Cell Bank, Tsukuba, Japan) in RPMI1640 medium bufferized with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.2 (RPMI1640–Hepes) (4.0×10^6 cells/ml), was incubated with 100 mU/ml of endo-β-galactosidase or 100 mU/ml of neuraminidase at 37 °C for 1 h. The cells were washed twice in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline [DPBS(−)], and subjected to flow cytometric analysis and macrophage-binding assay.

Preparation of apoptotic cells. Apoptosis was induced in Jurkat cells by incubation of the cells (4.0×10^6 cells/ml) in RPMI1640 medium containing 5% fetal bovine serum (RPMI1640-5% FBS) with appropriate concentrations of etoposide or anti-Fas antibody (clone: CH-11) at 37 °C in 5% CO_2 atmosphere for various hours. The cells were washed three times with DPBS(−), resuspended in RPMI1640–Hepes at 4×10^6 cells/ml, and subjected to the macrophage-binding assays. For measurement of apoptosis, the resuspended cells were incubated for additional 2 h without apoptosis-inducing agents and serum at 37 °C before measurement to adjust the duration of cell incubation to that of binding assays that take 2 h.

Macrophage-binding assays. Binding of apoptotic Jurkat cells to macrophages was performed for 2 h in the absence of serum to avoid influence of any serum factor on the binding using monolayers of THP-1 cells (Japanese Cancer Research Resources Bank, Osaka, Japan) differentiated into macrophages by phorbol myristate acetate as described previously [15]. Data are expressed as the number of bound Jurkat cells per 100 macrophages as counting more than 300 macrophages.

Measurement of apoptosis-associated cellular changes. Chromatin condensation was assessed by staining the cells with bisbenzimide (Hoechst 33258) as described [15].

Externalization of PS was assessed by binding of fluorescein isothiocyanate-labeled annexin V (FITC-annexin V) to the cells using a commercial assay kit (MEBCYTO apoptosis kit). Briefly, Jurkat cells (2×10^6 cells/ml) suspended in a kit buffer containing appropriate concentrations of FITC-annexin V and propidium iodide were incubated for 15 min at room temperature in the dark according to the manufacturer's instruction. The cell suspension was then diluted with another buffer and immediately analyzed by a flow cytometer (FACSCalibur, Becton–Dickinson) using a software CELLQUEST, gating for FSC and SSC region of intact Jurkat cells. Propidium iodide-positive cells were regarded as necrotic cells.

Apoptosis-induced DNA fragmentation was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method using a commercial assay kit (MEBSTAIN apoptosis kit direct). Briefly, Jurkat cells (1×10^6 cells) were fixed in DPBS(−) containing 4% paraformaldehyde (4 °C, 30 min), washed in DPBS(−) containing 0.2% BSA [0.2% BSA–DPBS(−)], and permeabilized in 70% ethanol at −20 °C for 30 min. After washing in 0.2% BSA–DPBS(−), the cells were treated with TdT and FITC-dUTP for 1 h at room temperature in the dark to label the fragmenting nuclear DNA at the 3′-hydroxyl ends with FITC. The cell suspension was diluted with 500 μl of 0.2% BSA–DPBS(−), and subjected to flow cytometric analysis, gating for FSC and SSC region of intact Jurkat cells.

The amount of cell surface CD43 was assessed by flow cytometry using anti-CD43 antibody. Jurkat cells (4×10^7 cells/ml) were treated with 10 μg/ml anti-CD43 mouse monoclonal antibody (clone: DF-T1) in RPMI1640–Hepes–0.2% BSA at 0 °C for 30 min, and washed twice with DPBS(−) at 0 °C. The cells were then incubated with 10 μg/ml Alexa Fluor 488-goat anti-mouse IgG (H + L) conjugate in RPMI1640–Hepes–0.2% BSA at 0 °C for 30 min. After washing twice in DPBS(−), the stained cells were subjected to flow cytometry. FSC and SSC region of intact Jurkat cells was gated for the measurement.

Results

Time course change in the susceptibility of apoptotic Jurkat cells to macrophage binding

The susceptibility of apoptotic Jurkat cells to the macrophage recognition was assessed as a function of time of apoptosis induction using human monocytic THP-1 cells differentiated into adherent cells as macrophages. Apoptosis was induced by etoposide at 10 μM or anti-Fas antibody at 2 ng/ml because these concentrations gave maximal binding of early apoptotic Jurkat cells in the previous study [15]. As shown in Fig. 1A, Jurkat cells became susceptible to binding to macrophages when treated with etoposide for 2 h, and the susceptibility fell to the control level at 4 h, which confirmed the previous results [15]. The susceptibility was increased again at 6 h and later hours up to 24 h. Chromatin condensation was observable at 4 h and increased thereafter.

Similarly, anti-Fas-induced apoptotic cells showed the transient increase in the susceptibility to the macrophage binding at an early stage (0–6 h), and the second increase at 12 h up to 24 h (Fig. 1B). Chromatin condensation in this case was very weak or moderate until 24 h.

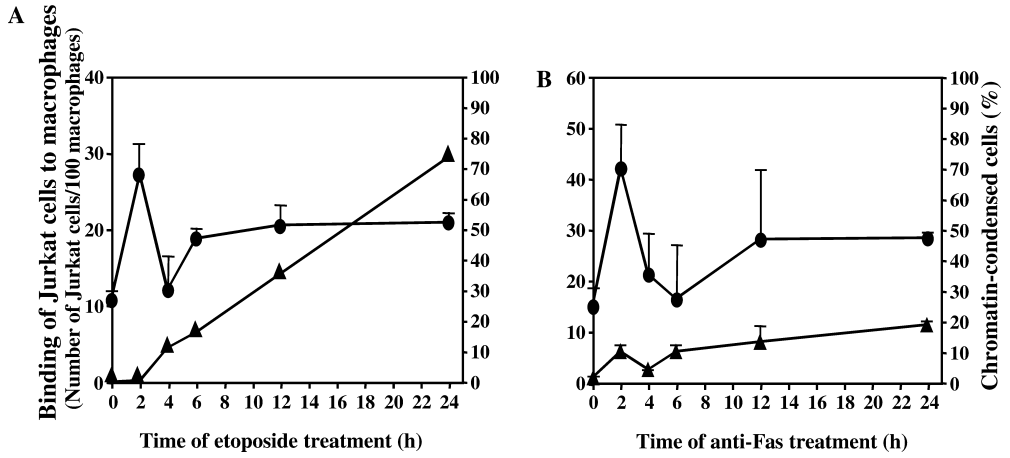

Fig. 1. Time course change in the susceptibility of apoptotic Jurkat cells to macrophage binding. Jurkat cells were treated with 10 μM etoposide (A) or 2 ng/ml anti-Fas antibody (B) at 37 °C for the indicated hours, and subjected to the macrophage-binding assay as described in Materials and methods (filled circles). Each point represents the mean ± SD of triplicate determinations. The etoposide-treated (A) and the anti-Fas-treated (B) Jurkat cells were further incubated at 37 °C for 2 h in RPMI1640–Hepes after removal of the apoptosis-inducing agents, and their chromatin condensation was measured (filled triangles). Data are expressed as the percentage of chromatin-condensed cells in total cells. Each point represents the mean ± SD of triplicate determinations.

To confirm the time course nuclear changes of the apoptotic cells, DNA fragmentation of Jurkat cells similarly treated with the apoptosis-inducing agents was analyzed by TUNEL assays using flow cytometry. As shown in Fig. 2, etoposide-treated cells were TUNEL negative until 4 h, and a significant increase in the number of positive cells was observed at 6 h. Therefore, the time before 6 h will be referred to as an early stage. Based on the same criterion, the time before 12 h will be referred to as an early stage for the anti-Fas-treated cells because a significant number of TUNEL positive cells were not observed until 12 h. The early stage of anti-Fas-treated cells is longer than that of the etoposide-treated cells.

Time course change in the cell surface-CD43 level of apoptotic Jurkat cells

During the study on CD43 capping of apoptotic cells under the fluorescence microscope, we noticed that CD43 staining with anti-CD43 antibody was weaker at the late stage than at the early stage. To see whether cell surface CD43 level changes during the course of apoptosis, flow cytometric analysis was performed. The number of CD43 positive Jurkat cells was found to begin decreasing at 4 h for the etoposide-induced apoptotic cells, and at 2 h for the anti-Fas-induced apoptotic cells (Fig. 3). Although initiation of the decrease was earlier for the anti-Fas-induced apoptosis than for the etoposide-induced apoptosis, the rate of decrease was much slower for the former than for the latter. The observed decrease in the CD43-expressing cells suggests that CD43 is degrading during the course of apoptosis.

Anti-CD43 monoclonal antibody used here is clone DF-T1, the epitope for which was reported to be dependent on terminal sialyl residues [20]. To exclude the possibility that the observed decease in the CD43 positive cells after induction of apoptosis is due to desialylation of terminal sialyl residues by endogenous neuraminidases, another anti-CD43 monoclonal antibody (clone L10), that is sialic acid-independent [21], was tested for the binding ability. As shown in Fig. 4A, DF-T1 monoclonal antibody failed to bind to the neuraminidase-treated Jurkat cells, while L10 monoclonal antibody did not. Binding profiles of L10 monoclonal antibody to apoptotic Jurkat cells were basically the same as those of DF-T1 (Fig. 4B). Accordingly, the observed decrease in the cell surface CD43 level of apoptotic cells was not due to desialylation, but due to degradation of CD43 molecules.

Time course of PS exposure on apoptotic Jurkat cells

Externalization of PS on apoptotic Jurkat cells was assessed by flow cytometry using FITC-annexin V. In the case of etoposide-treated Jurkat cells, the number of PS-exposed cells began to increase at 2 h, and significantly increased at 4 h and thereafter (Fig. 5, left). Anti-Fas-induced apoptotic cells showed a small proportion of PS-exposed cells at 1 h, but a significant increase was observed at 6 h (Fig. 5, right). PS-exposure and CD43 degradation appear to be proceeding in parallel (Figs. 3 and 5).

Cells undergoing secondary necrosis were observed significantly at 24 h for the etoposide-treated Jurkat cells, but not for the anti-Fas-treated cells, as measured

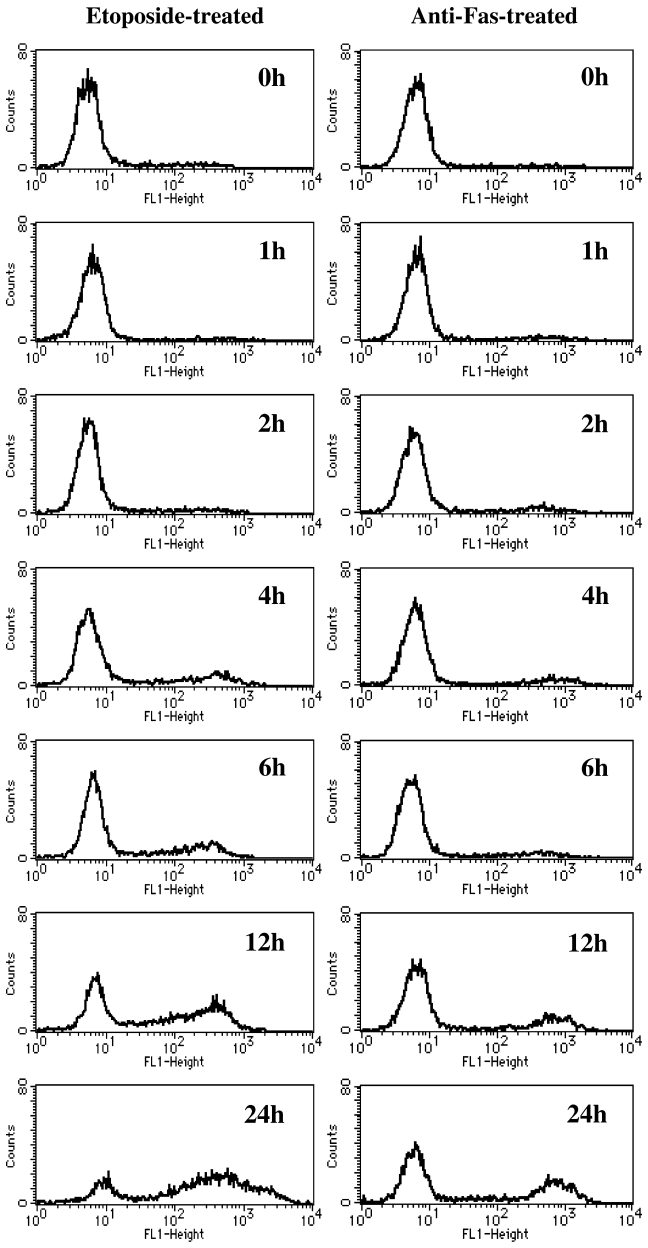

Fig. 2. Time course of DNA fragmentation in apoptotic Jurkat cells. Jurkat cells were treated with 10 μM etoposide or 2 ng/ml anti-Fas antibody at 37 °C for the indicated hours, washed, and incubated again at 37 °C for additional 2 h in RPMI1640–Hepes without etoposide and anti-Fas antibody. Cells undergoing DNA fragmentation were measured by TUNEL method using a flow cytometer as described in Materials and methods.

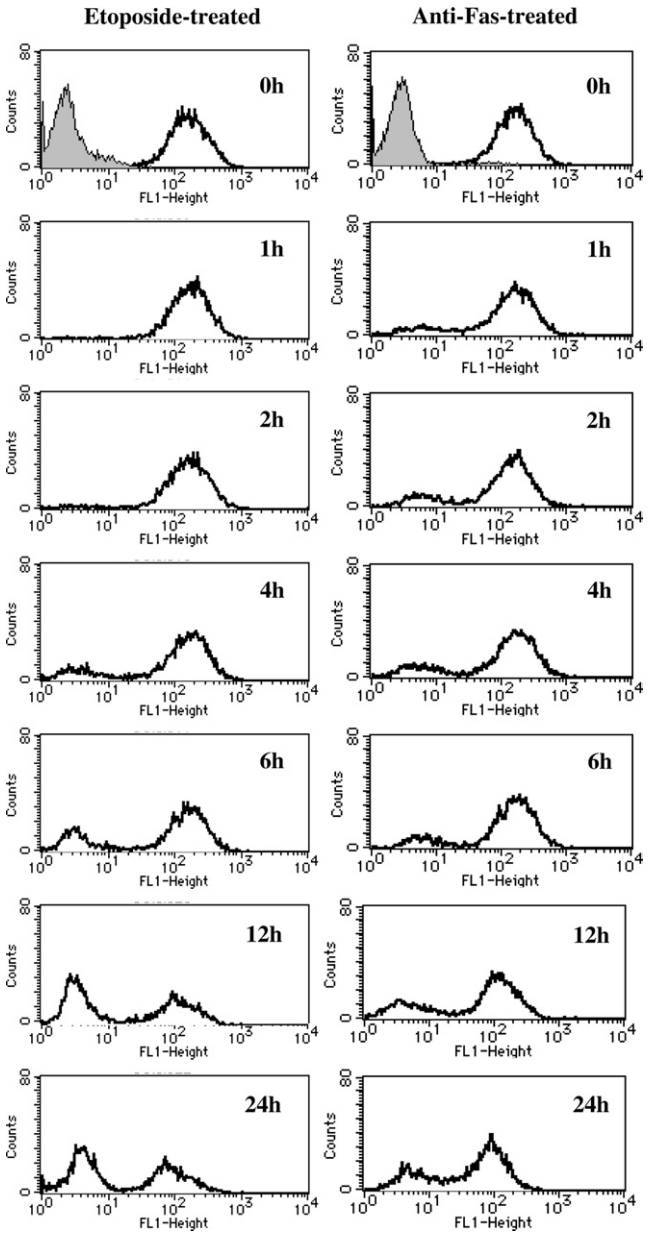

Fig. 3. Time course change in the cell surface-CD43 level of apoptotic Jurkat cells. Jurkat cells were treated with 10 μM etoposide or 2 ng/ml anti-Fas antibody at 37 °C for the indicated hours, washed, and incubated again at 37 °C for additional 2 h in RPMI1640–Hepes without etoposide and anti-Fas antibody. The cell surface-CD43 level was measured by flow cytometry using anti-CD43 antibody (DF-T1) as described in Materials and methods. Gray peaks at 0 h, stained with Alexa Fluor 488-conjugated secondary antibody alone.

by flow cytometry of propidium iodide-stained cells (data not shown).

Carbohydrate-dependent binding of the early apoptotic cells and PS-dependent binding of the late apoptotic cells to macrophages

The disappearance of CD43 cap at the end of the early stage of apoptosis [15], the degradation of CD43 on cell surface at about 4 h and thereafter (Fig. 3), and the significant increase in PS exposure at 4 or 6 h and thereafter (Fig. 5) suggest that the binding of apoptotic Jurkat cells at the late stage to macrophages is mediated not by carbohydrate chains but by the cell surface exposed PS. Binding inhibition studies were carried out to determine which is involved (Fig. 6). Macrophage-binding of etoposide-induced

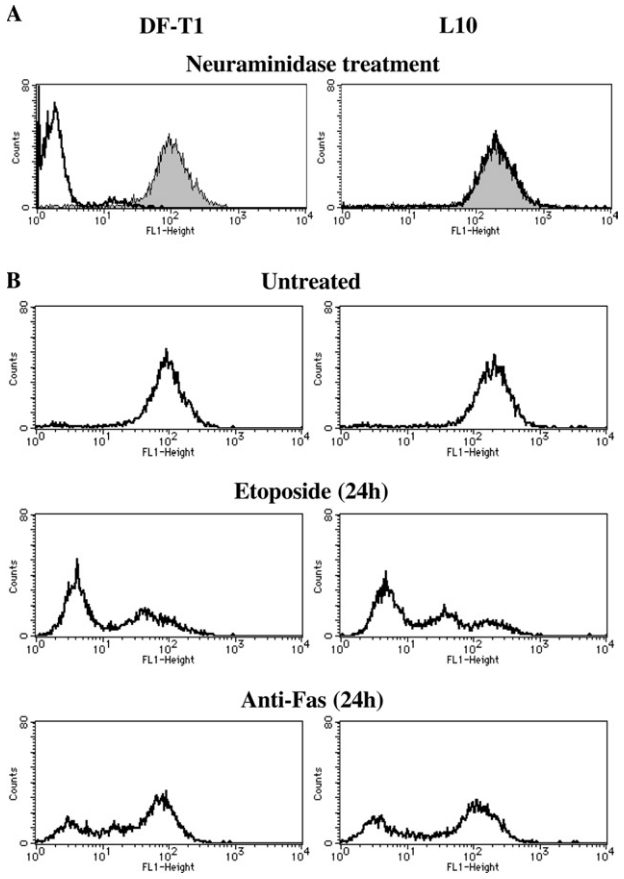

Fig. 4. Binding specificities of two anti-CD43 monoclonal antibodies DF-T1 (left) and L10 (right). (A) Effect of neuraminidase-treatment of Jurkat cells on their reactivity with DF-T1 and L10 antibodies. Jurkat cells were treated with neuraminidase as described in Materials and methods. Gray peaks, neuraminidase-untreated cells. Bold line, neuraminidase-treated cells. (B) Binding of DF-T1 and L10 antibodies to non-apoptotic and apoptotic Jurkat cells. Untreated and etoposide- or anti-Fas-induced apoptotic cells (24 h incubation) were prepared as described in Fig. 2.

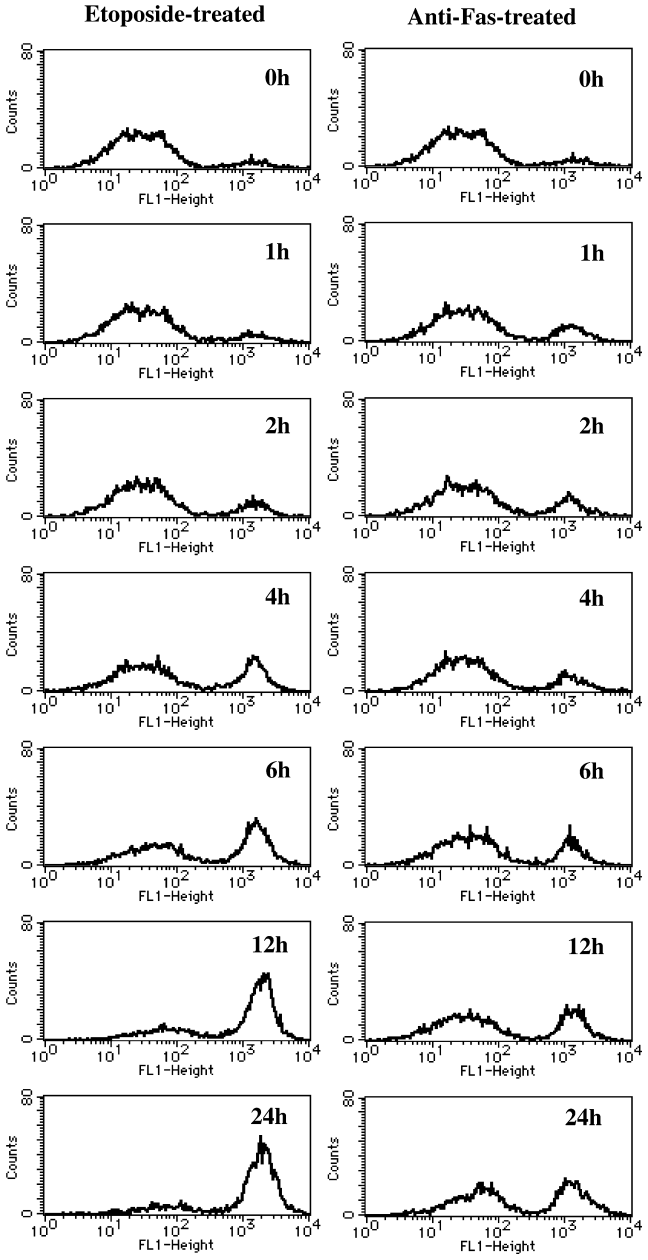

Fig. 5. Time course of PS exposure on apoptotic Jurkat cells. Jurkat cells were treated with 10 μM etoposide or 2 ng/ml anti-Fas antibody at 37 °C for the indicated hours, washed, and incubated again at 37 °C for additional 2 h in RPMI1640–Hepes without etoposide and anti-Fas antibody. Cells expressing PS on cell surface were measured by flow cytometry using FITC-labeled annexin V as described in Materials and methods.

early apoptotic Jurkat cells (2 h) was prevented when Jurkat cells had been pretreated with endo-β-galactosidase, an enzyme that specifically cleaves poly-*N*-acetyllactosaminyl structure (i.e., (Galβ1-4GlcNAcβ1-3) repeats) at the β-galactosidic bond [22], or with neuraminidase that removes sialic acid residues from the non-reducing termini of carbohydrate chains (Fig. 6A), confirming the previous results [15]. However, macrophage-binding of etoposide-induced late apoptotic Jurkat cells (12 h) was not prevented by the same pretreatment (Fig. 6A). In contrast, annexin V that specifically binds to PS inhibited the macrophage-binding of etoposide-induced late apoptotic Jurkat cells (12 h), but not that of etoposide-induced early apoptotic Jurkat cells (2 h) (Fig. 6B). Similar results were obtained for anti-Fas-induced apoptotic cells (Figs. 6C and D). It is therefore likely that the macrophage recognition of the late apoptotic Jurkat cells is mediated by the cell surface-exposed PS, but not by carbohydrates.

Supporting these results, the early apoptotic Jurkat cells (2 h-treatment with etoposide) bound to macrophages were poorly stained with FITC-annexin V, whereas the late apoptotic cells (12 h-treatment with etoposide) bound to macrophages were stained with FITC-annexin V (Fig. 7).

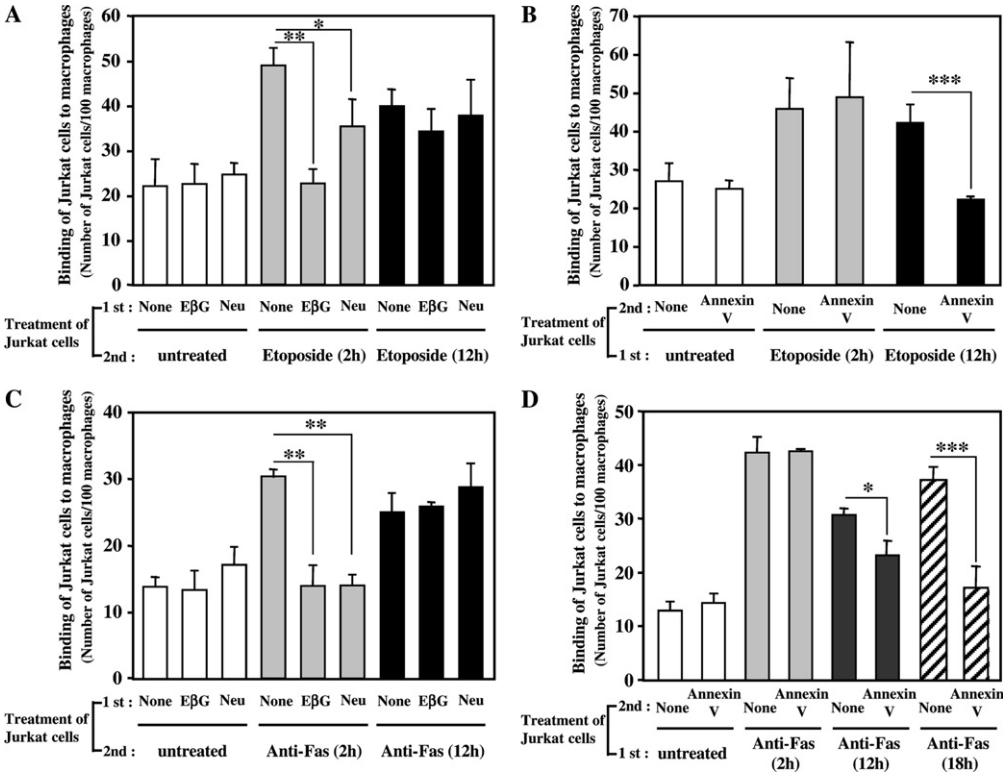

Fig. 6. Carbohydrate-dependent binding of the early apoptotic cells to macrophages and PS-dependent binding of the late apoptotic cells to macrophages. All the data are means ± SD of triplicate determinations. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in unpaired Student's t test. (A) Effect of pretreatment with glycosidases on the binding of etoposide-induced apoptotic Jurkat cells to macrophages. Jurkat cells were pretreated with endo-β-galactosidase (EβG) or neuraminidase (Neu) as described in Materials and methods, washed, treated with 10 μM etoposide at 37 °C for 2 or 12 h, and subjected to the macrophage-binding assay. (B) Effect of annexin V on the binding of etoposide-induced apoptotic Jurkat cells to macrophages. Jurkat cells were treated with 10 μM etoposide at 37 °C for 2 or 12 h and washed. The cells were then incubated with 10 μg/ml recombinant human annexin V in 10 mM Hepes–150 mM NaCl–5 mM KCl–1 mM $MgCl_2$–2 mM $CaCl_2$, pH 7.2, at 0 °C for 30 min, washed twice in DPBS(−), and subjected to the macrophage-binding assay. (C) Effect of pretreatment with glycosidases on the binding of anti-Fas-induced apoptotic Jurkat cells to macrophages. Experiments were carried out as described in (A) using 2 ng/ml anti-Fas antibody instead of etoposide. (D) Effect of annexin V on the binding of anti-Fas-induced apoptotic Jurkat cells to macrophages. Experiments were carried out as described in (B) using 2 ng/ml anti-Fas antibody instead of etoposide.

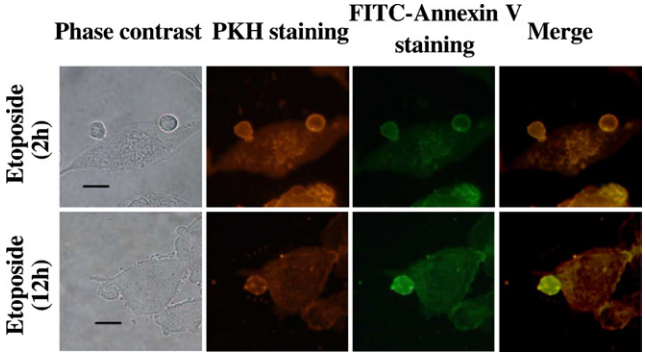

Fig. 7. Detection of cell surface PS of late apoptotic cells attached to macrophages. Jurkat cells were treated with 10 μM etoposide at 37 °C for 2 or 12 h and subjected to the macrophage-binding assay. After washing the bound cells with DPBS(−), cell surface PS was stained with FITC-annexin V as described in Materials and methods. The cells on the coverslips were then stained with a membrane-labeling fluorescent dye PKH26 (10 μM) at room temperature for 5 min. The labeling was quenched by addition of 1% BSA in DPBS(−). The cells were washed twice with DPBS(−) and subjected to microscopic observation by a fluorescence microscope (Axiovert 200 M, Carl Zeiss). Scale bars, 10 μM.

Discussion

The present study demonstrated that at an early stage of apoptosis carbohydrate chains of CD43 of apoptotic cells work as a signal for phagocytic recognition, and at a late stage of apoptosis cell surface-exposed PS works as another signal for the recognition. In other words, signals on apoptotic cell surface for phagocytic recognition (''eat-me signals'' [1,2]) switch from carbohydrates to PS during the course of apoptosis.

So far, many works have been done to characterize the cell surface changes of apoptotic cells that are recognized by phagocytes, but most works have focused on a particular membrane component, such as PS and carbohydrates, or on a particular stage of the apoptotic cells. Thus, little attention has been paid to the possibility that different mechanisms work at different stages of apoptosis. The present work may have demonstrated the first example that different recognition mechanisms or eat-me signals operate at different stages of apoptosis.

The presence of more than one mechanism of phagocytic recognition at different stages of apoptosis is obviously advantageous to the body to remove dying cells thoroughly. It is not known how much each mechanism contributes to the clearance of apoptotic Jurkat cells within the total range of apoptosis. As far as estimated by the macrophage-binding data (Fig. 1), the long-lasting PS-mediated mechanism may contribute more than the transient carbohydrate-mediated mechanism, although the macrophage-binding efficiency by the carbohydrate-mediated mechanism appears to be higher than that by the PS-mediated one. Nevertheless, the early recognition and removal by the carbohydrate-mediated mechanism may be much safer removal than recognition by the later PS-mediated mechanism because small proportions of apoptotic cells, at 12 h of etoposide treatment and at 24 h of anti-Fas treatment, underwent secondary necrosis as judged by propidium iodide-staining.

Another point is generality of the ligands. PS is a ubiquitous membrane component, and therefore can work as an eat-me signal on various types of cells. On the other hand, polylactosaminyl chains are not major carbohydrates on cell surface, and therefore they may be less available for the ligand on various types of cells. In this context, it is important to see whether the cell types other than blood cells can use this carbohydrate-mediated mechanism.

Decrease in the CD43 level itself on apoptotic cell surface at the end of the early stage of apoptosis explains why CD43 capping is transient, disappearing in 2 or 3 h after capping. Although the possibility that dispersion of CD43 molecules of a CD43 cap resulted in disappearance of the CD43 cap cannot be excluded, it is very likely that decrease in or disappearance of the CD43 level caused disappearance of CD43 cap. Disappearance of the CD43 cap from the apoptotic cell surface is very likely due to proteolytic degradation of the protein, since CD43 is known to be proteolytically downregulated upon stimulation [23–25] or spontaneously [25]. It is also known that extracellular fragment of CD43 is released into plasma [26,27].

The time course of PS exposure coincided very well with that of CD43 degradation and therefore these two events may be mechanistically related. Because a CD43 molecule has a long and extended mucin-like extracellular domain [17] that is disturbing for interaction of the cell surface components with other cells [17]. Thus, CD43 degradation on apoptotic cells may facilitate the externalized PS molecules to interact with their receptors on macrophages. Whether or not other cell membrane proteins degrade at this stage of apoptosis is an interesting question to be investigated.

The macrophage-apoptotic cell binding assays in the present study as well as in the previous one [15] were performed in the absence of serum to avoid any effect of serum such as opsonic or opsonin-like activity of complements, natural antibodies, and other factors. Hence, in the presence of serum, the binding and phagocytosis observed in this or in the previous assays would be reinforced. It is also another study to be carried out.

Finally, the present results obtained in vitro study need to be confirmed in vivo. Using mouse primary cells in vitro, we have obtained similar results to those presented here. Thus, it is possible that the successive recognition through carbohydrates and PS as observed here is operating in the body.

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