Macrophage Recognition of Thiol-Group Oxidized Cells: Recognition of Carbohydrate Chains by Macrophage Surface Nucleolin as Apoptotic Cells

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Received May 29, 2012; Accepted August 20, 2012; Online Publication, November 7, 2012 [doi:10.1271/bbb.120413]

The mechanism was investigated for macrophage recognition of cells oxidized by diamide, a thiol group-specific oxidizing reagent. Jurkat cells exposed to various concentrations of diamide were recognized by macrophages, the cells exposed to 25 μM diamide being best recognized. CD43, a major glycoprotein on the Jurkat cell surface, tended to form clusters upon diamide oxidation, and pretreating Jurkat cells with the anti-CD43 antibody inhibited macrophage binding. This indicates that macrophages appeared to recognize CD43. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and a Western blot analysis of CD43 of the diamide-oxidized cells showed no increase in the amount of cross-linked CD43 compared with control cells, indicating that cross-linking of CD43 by a disulphide bond was not involved in the clustering. Both CD43 clustering and binding of the oxidized cells to macrophages was prevented by the caspase inhibitor, benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk), suggesting that the oxidized and macrophage-bound cells were undergoing apoptosis. A closer examination revealed that the caspase-3 activity, chromatin condensation, and DNA fragmentation in Jurkat cells were all increased by oxidation. The macrophage receptor involved in the binding appeared to be the cell-surface protein, nucleolin; an anti-nucleolin antibody treatment inhibited the binding. These results suggest that thiol-group-oxidized cells underwent early apoptosis and were recognized by nucleolin on macrophages as early apoptotic cells.

Key words: apoptosis; CD43; macrophage; nucleolin; thiol group

Oxidative stress is usually induced to maintain the homeostasis of an organism, for example, to prevent bacterial infection by mobilizing defensive phagocytes.¹ However, excessive oxidative stress can injure cells and tissue, and is involved in the etiology of various diseases, including cancer.²–⁴ Oxidative stress is known to cause damage to cellular components including nucleic acids, lipids and proteins.²,³,⁵ Damage to cellular components induced by oxidation includes degradation, adduct formation, cross-linking, and bond-breaking reactions.²,³,⁵ Oxidatively stressed cells are therefore thought to undergo cell-membrane damage, including membrane protein denaturation and cross-linking by oxidation.

Thiol-groups (SH) are readily oxidized, and in vivo SH oxidation of proteins has been suggested to occur in pathologic lymphocytes and erythrocytes associated with cancer,⁶ glucose-6-phosphate dehydrogenase deficiency,⁷ β-thalassemia,⁸ and sickled erythrocytes.⁹ We have previously shown that human T-lymphocytes and mouse erythrocytes that underwent oxidation of free SH in vitro by diazene dicarboxylic acid bis (dimethylamido) (diamide) or periodate were susceptible to binding to macrophages.¹⁰–¹² These findings suggest that SH-oxidized cells occurring in vivo were recognized by macrophages as targets to be eliminated from the host.¹⁰–¹²

SH-oxidized cells are recognized by macrophages, not through a chemically modified or denatured membrane structure, but rather via preexisting carbohydrate chains containing poly-N-acetyllactosaminyl on the cell membrane.¹⁰–¹² It has been suggested that membrane glycoproteins (erythrocytes, band 3; T lymphocytes, unknown) that contain poly-N-acetyllactosaminyl chains on the cell membrane form clusters upon cell oxidation, providing multivalent high-affinity carbohydrate-chain ligands on the cell surface for the macrophage receptors to bind.¹⁰–¹¹ It has been assumed that aggregation of poly-N-acetyllactosaminyl chains occurs by a cross-linking reaction such as the formation of disulphide bonds between these glycoproteins. However, while a specific SH-oxidizing agent (diamide oxidation) can cross-link membrane proteins, cross-linking of proteins may not be involved in the enhanced adhesion to macrophages, since a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the membrane proteins of diamide-oxidized cells showed no significant increase in the amount of cross-linked proteins.¹² The mechanism for glycoprotein clustering of SH-oxidized cells therefore remains unclear.

We found in a series of recent studies on apoptotic cells that CD43, a membrane glycoprotein of hematopoietic cells containing poly-N-acetyllactosaminyl chains, of Jurkat cells aggregated into clusters at an

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Abbreviations: anti-NUC295, antibody against amino acid residues 295–302 of nucleolin; BSA, bovine serum albumin; diamide, diazene dicarboxylic acid bis (dimethylamide); DPBS(−)-, Ca²⁺- and Mg²⁺-free Dulbecco’s phosphate-buffered saline; dUTP-FITC, deoxyuridine triphosphate-fluorescein isothiocyanate; HRBS, Hank’s balanced salt solution; Hoechst 33258, bisbenzimide; HRP, horseradish peroxidase; RPMI 1640-HEPES, RPMI 1640 medium buffered with 20 mM HEPES at pH 7.2; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SH, thiol group; Z-VAD-fmk, benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone
early stage of apoptosis, and that the early apoptotic Jurkat cells were recognized and taken up by macrophages through the CD43 clusters.\textsuperscript{13} The antigenic determinants on the CD43 glycoprotein of early apoptotic cells were suggested to be poly-N-acetyllactosaminyl chains.\textsuperscript{13} We identified the macrophage receptor for poly-N-acetyllactosaminyl chains on apoptotic cells as nucleolin,\textsuperscript{14} a multifunctional shuttle protein present in the nucleus and cytoplasm, and on the surface of some cell types including macrophages.\textsuperscript{15,16}

Considering the similarity between the carbohydrate chain ligands on SH-oxidatively damaged cells and those on early apoptotic cells in terms of macrophage recognition, it seems likely that SH-oxidative stress would be one of the factors that can induce apoptosis.\textsuperscript{17,18} It is conceivable that the SH-oxidatively damaged cells that we have so far investigated were also apoptotic cells, and that they would thus be recognized by macrophages as apoptotic cells. To test this hypothesis, we investigated whether the ligands on SH-oxidized Jurkat cells could be recognized by macrophages and that apoptosis could be involved in that recognition.

Materials and Methods

Materials. Bisbenzimide (Hoechst 33258) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Hank’s balanced salt solution (HBSS) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan), and diaminobenzidine (Sigma-Aldrich Co. (St. Louis, MO, USA). Benzylxoxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk) was from Peptide Institute (Osaka, Japan), and endo-β-galactosidase (E.C.3.2.1.103, Escherichia freundii) was obtained from Seikagaku Fine Chemicals (Tokyo, Japan). The anti-CD43 mouse monoclonal antibody (clone DF-T1) was from Dako (Glostrup, Denmark), and the Alexa Fluor-488 goat anti-mouse IgG1 (H+L) conjugate was from Molecular Probes (Eugene, OR, USA). The NuView™ 488 caspase-3 assay kit for live cells was from Biotium (Hayward, CA, USA) and the Mebstain direct apoptosis kit was purchased from Medical and Biological Laboratories (Nagoya, Japan). The anti-nucleolin antibody (anti-NUC2935) was raised in rabbits and affinity purified as previously described.\textsuperscript{19}

Diamide treatment of Jurkat cells. Jurkat cells were treated with diamide (Riken Cell Bank, Tsukuba, Japan) by incubating 1 x 10^6 cells/mL of Jurkat cells in HBSS with 0–100 μM diamide at 37 °C for 1 h while gently shaking.

Binding assay for Jurkat cells. THP-1 cells (Japanese Cancer Research Resources Bank, Osaka, Japan) were differentiated into macrophages by phorbol myristate acetate as previously described.\textsuperscript{13} Macrophages on coverslips were washed with HBSS, resuspended in an RPMI 1640 medium buffered with 20 mM HEPES at pH 7.2 (RPMI 1640-HEPES) at 4 x 10^6 cells/mL, and co-incubated with macrophage monolayers on coverslips at 37 °C for 2 h while gently shaking. The unbound cells were removed by gentle washing, and then the 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 for counts of more than 200 macrophages.

Measurement of cell-surface CD43. Jurkat cells were treated with 10 μg/mL of the anti-CD43 mouse monoclonal antibody (clone DF-T1)\textsuperscript{20} in RPMI 1640-HEPES with 0.2% bovine serum albumin (BSA) at 4 °C for 30 min and washed with DPBS(−) at 0 °C. The bound antibody was detected by treating the cells with 10 μg/mL of the Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugate in RPMI 1640-HEPES with 0.2% BSA at 0 °C for 30 min and washing with DPBS(−) at 0 °C. The cells were resuspended in RPMI 1640-HEPES without phenol red at 0 °C and immediately subjected to a microscopic observation with a μ-Radiance confocal laser scanning fluorescence microscope (Bio-Rad, Hercules, CA, USA). The cell suspension was kept at 0 °C throughout the immunofluorescence staining process to prevent any antibody-induced antigen redistribution that may occur at higher temperatures.

SDS–PAGE and Western blot analysis. Twenty-five μμm diamide-treated or untreated Jurkat cells were washed with DPBS(−), and the cell pellets (4 x 10^6 cells) were treated with a lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 1% NP–40, 10% glycerol, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin, and 1 mM phenylmethylsulfonylfluoride). The cell lysate that contained 2.5 μg of protein was analyzed by SDS–PAGE under non-reducing (without dithiothreitol) or reducing conditions (with dithiothreitol) by the method of Laemmli.\textsuperscript{21} The gel was transblotted to a polyvinylidene difluoride membrane and incubated with 5% skim milk in DPBS(−) for blocking, after which the membrane was washed and treated with the primary antibody for CD43 (clone L10; Southern Biotech, Birmingham, USA) or β-actin (Novus Biologicals, Littleton, Colorado, USA) and then with a secondary antibody for the horseradish peroxidase (HRP)-conjugated anti-mouse IgG sheep polyclonal antibody (CD43; GE Healthcare, Tokyo, Japan) or HRP-conjugated anti-rabbit IgG donkey (β-actin; GE Healthcare). The immunoreactive bands were visualized with an ECL detection system (Amersham Pharmacia Biotech, New Jersey, USA).

Measurement of apoptosis. Apoptosis of the diamide-treated cells was evaluated by caspase-3 activation, chromatin condensation and DNA fragmentation. Caspase-3 activity was measured by the fluorescence intensity of the fluorogenic DNA dye released from the dye-DENV substrate by activated caspase-3, using a commercial assay kit (NuView™ 488 caspase-3 assay kit for live cells). Jurkat cells (2 x 10^6 cells) were suspended in RPMI 1640-HEPES (without phenol red) containing appropriate concentrations of the substrate, and incubated for 30 min at room temperature in the dark according to the manufacturer’s instructions. The cell suspension was diluted with RPMI1640-HEPES and immediately analyzed by flow cytometry (FACSCalibur, Becton-Dickinson), using Cellquest software to measure the forward scatter region (correlates with the cell volume) and side scatter region (dependent on the inner complexity of the particle) of the intact Jurkat cells. Chromatin condensation was measured by fixing the diamide-treated Jurkat cells with 1.25% glutaraldehyde in DPBS(−) at room temperature for 30 min and then staining overnight with 0.1 mM Hoechst 33258 in DPBS(−). The morphology of nuclei of the Jurkat cells was observed under a fluorescence microscope. DNA fragmentation was measured by quantifying the binding of deoxyuridine triphosphate-fluorescein isothiocyanate (dUTP-FITC) to nicked DNA of the cells, using a Mebstain direct apoptosis commercial assay kit (the TdT-mediated dUTP-biotin nick end labeling method) in accordance with the manufacturer’s instructions. The cells were subjected to a flow cytometric analysis as already described, after treating with the commercial kit.

Detection of caspase activated cells after the binding assay. The unbond Jurkat cells were removed as already described, after co-incubating the diamide-treated Jurkat cells with macrophages. The macrophages and bound Jurkat cells were then treated for 30 min with a NuView™ 488 caspase-3 assay kit for live cells at room temperature in the dark according to the manufacturer’s instructions, and immediately subjected to a microscopic observation with the μ-Radiance confocal laser scanning fluorescence microscope.

Pretreatment of the anti-nucleolin antibody to macrophages. Macrophages were preincubated at 4 °C for 30 min with 2 μg/mL of anti-NUC295 or control rabbit IgG (Dako) in RPMI1640-HEPES containing 0.2% BSA, washed and then subjected to a binding assay using oxidized cells as already described.

Statistical analysis. All data are presented as the mean ± SD of at least three experiments. The data were analyzed by Student’s t-test.
Results

Recognition of diamide-oxidized Jurkat cells by macrophages

We first determined the extent of Jurkat cell oxidation required for suitable recognition by macrophages. Human monocytic THP-1 cells differentiated into adherent cells were used as macrophages. We have previously revealed that human erythrocytes treated with diamide at 37°C for 1 h were susceptible to form clusters of band 3, and thus macrophage binding. 22–24 We therefore investigated the susceptibility of Jurkat cells to macrophage binding as a function of the concentration of diamide for 1 h of treatment. Jurkat cells were treated with diamide at 0–100 μM at 37°C for 1 h while gently shaking and subjected to the macrophage binding assay. Figure 1 shows that the maximal recognition was observed when Jurkat cells were oxidized by 25 μM diamide. We therefore subsequently used Jurkat cells oxidized with 25 μM diamide for 1 h, unless otherwise indicated.

Association with macrophage recognition of the clustering of CD43 on diamide-oxidized Jurkat cells

We investigated whether the poly-N-acetyllactosaminyl chains of CD43 were involved in the recognition of SH-oxidized Jurkat cells. Jurkat cells that had been pretreated with endo-β-galactosidase, which specifically cleaved the poly-N-acetyllactosaminyl structure (i.e., (Galβ1→4GlcNAcβ1-3) repeats) at the β-galactosidic bond, 25 did not bind to macrophages before diamide oxidation (Fig. 2A), indicating that the macrophages recognized Jurkat cells through the poly-N-acetyllactosaminyl chains on the SH-oxidized Jurkat cells. Figure 2B shows that CD43 on the SH-oxidized Jurkat cells tended to be clustered (arrow). This clustering of CD43 was increased by the 25 μM diamide treatment and declined at higher doses (Fig. 2C). In addition, binding of the SH-oxidized Jurkat cells was inhibited by an anti-CD43 monoclonal antibody, an antibody directed against a sialic acid-dependent unidentified epitope of CD43. 26 (Fig. 2D). We had previously reported that the clustering of CD43 on the cell surface was a suitable condition for removal by macrophages. 26 It is therefore likely that the SH-oxidized Jurkat cells were recognized by macrophages through the poly-N-acetyllactosaminyl chains of clustered CD43.

We investigated whether or not the cross-linking of CD43 was initiated by the diamide-induced disulfide bond formation. Diamide-treated or untreated Jurkat cells were lysed and SDS-PAGE performed under non-reducing or reducing conditions, and then CD43 (130 kDa for the whole molecule) was detected by a Western blot analysis. The left panel in Fig. 2E shows no difference in the CD43 bands over 130 kDa, particularly in multiples of 130 kDa, between the untreated cells and diamide-treated cells under non-reducing conditions, indicating that cross-linking of CD43 by disulfide bonds had not been formed by the diamide treatment, and therefore that cross-linking of CD43 was not involved in the clustering of CD43. Several thin bands were apparent over 130 kDa in both the untreated cells and diamide-treated cells under non-reducing conditions (left panel). These bands were also apparent under reducing conditions (right panel). These bands were therefore unrelated to disulfide bonds, and may have been CD43 and physiologically associated proteins that were bound by covalent bonds, rather than disulphide bonds. These bands could be visualized by non-specific binding of the primary antibody and/or secondary antibody during the Western blot analysis.

Involvement of apoptosis in macrophage recognition

We have reported in our previous study that early-stage apoptotic cells were recognized by macrophages through clustered CD43. 13,27 Figure 2 shows that SH-oxidized cells were also recognized by macrophages through clustering of CD43. This similarity shows that SH-oxidized cells might be recognized as early apoptotic cells. We therefore examined whether apoptosis was involved in the recognition of SH-oxidized cells. Figure 3A shows that the caspase-3 activity increased dose dependently with the diamide treatment. We also examined the chromatin condensation, the number of chromatin-condensed cells also increasing dose dependently with the diamide treatment (Fig. 3B). DNA fragmentation also increased dose dependently with the diamide treatment (Fig. 3C). Figure 3C shows that the 25 μM diamide treatment slightly but significantly induced DNA fragmentation (p value of 0.0246). Pretreating the Jurkat cells with pan-caspase inhibitor Z-VAD-fmk suppressed the clustering of CD43 on the SH-oxidized Jurkat cells (Fig. 3D). Pretreating with Z-VAD-fmk also inhibited recognition of the SH-oxidized cells by macrophages (Fig. 3E), indicating that caspase-activated apoptotic cells were recognized by macrophages. The activation of caspases was clearly observed in macrophage-bound cells (Fig. 3F). These results indicate that the SH-oxidized cells were recognized via intracellular apoptotic signaling, rather than through chemical modification on the cell membrane.

Macrophage nucleolin as a receptor for diamide-oxidized Jurkat cells

The receptor on macrophages that recognizes SH-oxidized cells had not previously been detected. We have identified the macrophage receptor for CD43-clustered apoptotic cells as nucleolin under such differ-
ent apoptotic stimuli as etoposide, the anti-Fas antibody, and a hydrogen peroxide treatment. SH-oxidized cells were recognized by macrophages as apoptotic cells (Fig. 3). We therefore examined whether nucleolin might be the receptor for SH-oxidized Jurkat cells. Figure 4 shows that macrophage binding to SH-oxidized Jurkat cells was inhibited by the anti-nucleolin antibody (anti-NUC295, an antibody against amino acid residues 295–302 of nucleolin14), while control rabbit IgG was ineffective, indicating that macrophage surface nucleolin was indeed involved in the binding of SH-oxidized Jurkat cells.

**Discussion**

The present study has revealed that the macrophage recognition of cells was not due to recognition of the denatured cell-surface components, but rather due to recognition of the membrane poly-N-acetyllactosaminyl chains that undergo clustering of CD43 by SH oxidation-induced apoptosis. In addition, we have shown that the macrophage receptor for SH-oxidized cells was nucleolin. Clustering of CD43 on SH-oxidized cells was not induced from CD43 tying by disulphide bonds formed by the SH-coupling reaction, but by
A previous SDS–PAGE analysis of membrane proteins of the diamide-oxidized cells has shown no significant increase in the amount of cross-linked proteins. Present study also investigated whether or not cross-linking of CD43 was initiated and involved in redistribution through SH oxidation-induced apoptotic signaling.

A previous SDS–PAGE analysis of membrane proteins of the diamide-oxidized cells has shown no significant increase in the amount of cross-linked proteins. Present study also investigated whether or not cross-linking of CD43 was initiated and involved in the clustering by using an SDS–PAGE Western blot analysis under non-reducing and reducing conditions. If cross-linking of CD43 were formed by disulfide bonding, multiples of the 130 kDa bands from diamide-treated cells would be greater than with untreated control cells under non-reducing conditions. Moreover, the band formed by disulfide bonding would not be present under reducing conditions. These bands were not apparent in this study, indicated that cross-linking of CD43 by disulphide bonding did not occur and was not involved in the clustering of CD43. Figure 3D shows that caspase inhibitor Z-VAD-fmk inhibited clustering of CD43 on SH-oxidized cells. These results indicate that clustering of CD43 was not induced from cross-linking of CD43 by disulfide bonding, but was induced by intracellular caspase-dependent apoptotic signaling.

This study indicated that caspase-3 activation and chromatin condensation were dose-dependently increased by diamide (Fig. 3A and B). In addition, DNA fragmentation was also slightly increased and significantly so (Fig. 3C). Our previous study has shown that chromatin condensation was apparent earlier than DNA fragmentation in the apoptotic process. SH oxidation therefore induced clustering of CD43 at an early stage of apoptosis, and SH-oxidized apoptotic cells were therefore rapidly recognized by macrophages.

The target of diamide remains to be elucidated. Ueda et al. have reported that the activation of caspase-3-like protease by diamide was mediated, at least partly, by the...
release of cytochrome c from mitochondria into the cytosol, which is sensitive to oxidative stress, and that endogenous reducing factors, including thioredoxin, were required for caspase activity to induce apoptosis.\textsuperscript{18} It is also well known that mitochondrial apoptosis is regulated by the release of cytochrome c from mitochondria, leading to the activation of initiator caspase-9 which then cleaves and activates downstream executioner caspase-3, leading to apoptosis.\textsuperscript{29} Diamide may therefore induce mitochondrial apoptosis by SH oxidation-induced intracellular imbalance of the oxidation-reduction status, and activated caspase-9 or caspase-3 may be involved in the clustering of CD43.

It is known that CD43 binds to cytoskeleton proteins,\textsuperscript{30,31} and that the bridge formed by moesin, a cross-linking protein that links the actin cytoskeleton and plasma membrane proteins, between CD43 and actin is abrogated by active caspases during the early stages of apoptosis.\textsuperscript{32} We have previously shown that the actin polymerization inhibitor, cytochalasin B, induced the capping of CD43 when used to treat the capping of CD43 induced by Jurkat cells.\textsuperscript{29} Seveau \textit{et al.} have also reported that treating with colchicine, a drug that inhibits microtubule polymerization, induced clustering of CD43 in cell uropods.\textsuperscript{33} One possible explanation for the clustering of CD43 is that CD43 was conjugated to several cytoskeleton proteins, and disrupting the actin bond by diame-activated caspases caused active movement of other cytoskeleton proteins that then induced the clustering of CD43.

Although such apoptotic features as caspase-3 activity, chromatin condensation, and DNA fragmentation were increased dose-dependently by diame, clustering of CD43 and the recognition by macrophages declined when treating at high doses of diame. Considering that such high doses could induce apoptotic signaling, an excessive SH-coupled reaction at high doses may have disturbed the redistribution of CD43 on the cell surface (i.e., denaturation or malfolding of membrane proteins or a mobility change in the cell membrane) or the signaling between activated caspases and the clustering of CD43. Further investigation is therefore needed to elucidate the precise details of the clustering mechanism.

This study has revealed the receptor of diame-oxidized cells to be nucleolin. The macrophage receptor for the removal of oxidized cells is poorly understood. Ramprasad \textit{et al.} have reported macroisalin, the mouse homologue of human CD68, as a candidate for the receptor of oxidatively damaged erythrocytes, oxidized low-density lipoprotein, and apoptotic thymocytes.\textsuperscript{34,35} Terpata \textit{et al.} have reported that receptors other than scavenger receptor A participated in the clearance of oxidatively damaged cells.\textsuperscript{36,37} The macrophage receptor for the removal of oxidized cells has therefore remained to be elucidated. Our present finding contributes to classification of the mechanism for oxidized cell removal.

Phosphatidylserine on oxidized cells is a possible candidate for the target of macrophage recognition.\textsuperscript{38,39} However, our previous studies on Jurkat cells and erythrocytes have shown that these cells, when mildly oxidized with such oxidants as diame,\textsuperscript{40} hydrogen peroxide,\textsuperscript{27} and an iron-catalyst,\textsuperscript{41} were recognized by mouse or human macrophages without expressing phosphatidylserine on the outer membrane, indicating that phosphatidylserine was not involved in macrophage recognition in this case.

This study has shown that SH-oxidized cells were recognized by macrophages as apoptotic cells. Apoptotic cells are recognized and ingested by phagocytes, preventing their lysis and subsequent release of harmful and immunogenic intracellular contents. These contents, if not removed, injure the surrounding tissue and induce abnormal autoimmune responses. The phagocytosis of apoptotic cells is therefore crucial for maintaining tissue homeostasis and turnover.\textsuperscript{42–45} Phagocytosing macrophages suppress inflammation by inhibiting pro-inflammatory cytokine production and releasing anti-inflammatory cytokines.\textsuperscript{43–46} It follows that, if SH-oxidized cells are not removed, apoptotic SH-oxidized cells would injure the surrounding tissue and host. The mechanism for macrophage recognition that has been shown in this study would contribute to maintaining tissue homeostasis and preventing these critical problems.

Diamide induces thioul group-specific oxidation without radical species.\textsuperscript{17,18} In contrast, such oxidative stressors as superoxide radicals, hydrogen peroxide, and lipid peroxides induce non-specific degeneration in cell membrane components by radical species.\textsuperscript{11} Hydrogen peroxide is a well-known oxidant and is associated with various diseases, including Alzheimer’s disease, Parkinson’s disease, vascular disease, and cancer.\textsuperscript{47–50} Our previous studies have revealed that hydrogen peroxide-oxidized Jurkat cells and erythrocytes initiated CD43 or band 3 clustering.\textsuperscript{27,51} Hydrogen peroxide can induce hydroxyl radicals that also add readily to carbon-carbon double bonds that induce protein aggregation;\textsuperscript{48} it was expected that this double-bond formation would be involved in the clustering of CD43 in hydrogen peroxide-oxidized cells, but the activation of caspases was in fact the predominant factor.\textsuperscript{27,51} Although diame and hydrogen peroxide showed different oxidative reactions, oxidatively damaged cells were recognized by macrophages through our caspase-dependent carbohydrate-clustering mechanism, regardless of the nature of the oxidant or the consequences of the oxidative reaction.

In conclusion, the results of the present study suggest that macrophage recognition of SH-oxidized cells was not due to the recognition of oxidatively damaged or denatured cell-surface components, but instead to recognition of the membrane glycoprotein molecules that undergo clustering by SH oxidation-induced apoptotic signaling. We have previously suggested that apoptosis-stimulating agents, including etoposide, the anti-Fas antibody, and hydrogen peroxide, would also induce clustering of CD43 at an early stage of apoptosis.\textsuperscript{13,27} Clustering of CD43 is therefore a general observation regardless of the apoptotic stimulus.

References
