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Oxidatively Damaged Erythrocytes Are Recognized by Membrane Proteins of Macrophages

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Human erythrocytes incubated with an iron catalyst ADP-chelated Fe^{3+} undergo oxidative damage of the membrane including lipid peroxidation, protein oxidation, and protein aggregation, and become susceptible to recognition by human macrophages. In order to clarify the membrane components of macrophages responsible for the recognition of the oxidized erythrocytes, binding of the oxidized cells to dot and Western blots of solubilized membrane of macrophages was investigated. The oxidized erythrocytes but not unoxidized cells bound to the dot blots. The binding was effectively inhibited by saccharide chains of band 3, a major glycoprotein of human erythrocytes, and lowered when the saccharide chains of band 3 were removed from the cell surface by pretreatment of the cells with endo-β-galactosidase which specifically cleaves the polylactosaminyl saccharide chains of band 3. The oxidized erythrocytes bound to the membrane proteins of macrophages with molecular mass of about 50, 80, and 120 kDa on Western blots depending on the saccharide chains of band 3 on their surface. The results suggest that the oxidatively damaged erythrocytes are specifically recognized by these proteins of macrophage membrane having saccharide binding ability.

Keywords: Oxidative stress, oxidatively damaged erythrocyte, macrophage recognition, polylactosamine, band 3, host defense

Abbreviations: BSA, bovine serum albumin; DPBS, Dulbecco's phosphate buffered saline; DPBS(–), DPBS without Ca^{2+} and Mg^{2+}; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene fluoride; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride

INTRODUCTION

Oxidative stress on cells results in oxidative modification and deterioration of cellular components,[1] and is considered to be damaging to cells and tissues causing their dysfunction.[2] For example, human erythrocytes incubated *in vitro* with an iron catalyst ADP-chelated Fe^{3+} (ADP/Fe^{3+}) undergo membrane lipid peroxidation,[3,4] membrane protein oxidation,[5] and membrane protein aggregation.[6]

Previously, we investigated response of host defense system to the oxidatively damaged cells, and found that natural IgG autoantibodies to band 3 glycoprotein of erythrocyte membrane (anti-band 3 IgG) specifically bind to the oxidized erythrocytes.[3] This suggested that oxidatively

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damaged erythrocytes are marked with the antibodies and destined to be removed by macrophages by Fc receptor-mediated phagocytosis. We have also found that similarly oxidized human erythrocytes are directly recognized by human macrophages in the absence of antibodies.[7] Thus, oxidative modifications of erythrocyte membrane render the cells susceptible to the recognition by macrophages through the antibody-dependent and independent mechanisms.

The band 3 determinants of the oxidized erythrocytes recognized by the antibodies are neither newly produced structures nor cryptic peptides exposed by oxidation, but are the poly-*N*-acetyllactosaminyl saccharide chains of band 3.[8–10] Interestingly, the determinants of the oxidized erythrocytes directly recognized by human macrophages were also found to be the saccharide chains of band 3.[10] It is therefore likely that membrane proteins which specifically recognize the oxidized erythrocytes through the saccharide chains of band 3 exist on the macrophages.

In this report, we investigated binding of the oxidized erythrocytes to the dot blots and Western blots of the solubilized membrane of human macrophages, and demonstrate that the proteins which specifically recognize oxidized erythrocytes through the saccharide chains of band 3 exist in the macrophage membrane.

MATERIALS AND METHODS

Materials

ADP monopotassium salt was obtained from Oriental Yeast Company (Tokyo, Japan). Phorbol 12-myristate 13-acetate (PMA), diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), lactoferrin (human milk), bovine serum albumin (globulin free) (BSA), α_1-acid glycoprotein (human), bovine fetuin, and mannan (yeast) were obtained from Sigma Chemical Company (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. RPMI 1640 medium and penicillin-streptomycin were obtained from GIBCO Laboratories (Grand Island, NY). Fetal calf serum (FCS) was obtained from Bio Whittaker (Walkersville, MD). Polyvinylidene fluoride (PVDF) blotting membrane and Bio-Beads SM-2 were obtained from ATTO Corporation (Tokyo, Japan) and Bio-Rad Laboratories (Hercules, CA), respectively. Other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan). Glycophorin A was isolated from human erythrocytes as described previously.[7] Band 3 was isolated from human erythrocyte membrane as described previously.[7] Band 3 oligosaccharides and LF oligosaccharides were prepared by hydrazinolysis of the corresponding glycoprotein as described previously.[7]

THP-1 Cell Membrane

The human monocytic leukemia cell line THP-1 was donated by the Japanese Cancer Research Resources Bank, and maintained in a suspension culture. The cells were differentiated into macrophages by treatment with 100 nM PMA for 2 days as described previously.[7] The differentiated cells adherent to the surface of a culture flask were mechanically detached with a rubber scraper in 20 mM Tris-HCl (pH 8.0)-0.15 M NaCl-2 mM EDTA, and centrifuged at 500 × g for 10 min. The resulting cell pellet was resuspended in 20 mM Tris-HCl (pH7.6)-0.25 M sucrose-2 mM DFP-1 mM PMSF-25 mM KCl. The cells were broken by homogenizing with a Potter-type homogenizer in an ice bath. The homogenate was centrifuged at 500 × g for 10 min to remove cell debris and a nuclear fraction, and the supernatant was then centrifuged at 7,000 × g for 30 min. The resulting supernatant was centrifuged at 100,000 × g for 1 h to obtain a membrane pellet. The membrane pellets of THP-1 cells were dissolved in 20 mM Tris-HCl (pH 7.6)-0.15 M NaCl-2 mM DFP-1 mM PMSF-2% Triton

X-100, and the insoluble material was sedimented at 100,000 × g for 1 h. The supernatant was, then, subjected to hydrophobic chromatography using SM-2 beads to remove an excess amount of Triton X-100. The eluate was used as solubilized membrane of THP-1 cells.

Oxidation of Erythrocytes

Erythrocytes were separated by centrifugation from human venous blood withdrawn using citrate-phosphate-dextrose as an anticoagulant, washed four times with Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (DPBS(-)). An erythrocyte suspension in DPBS (–) (20% hematocrit) was mixed with an equal volume of a solution of an iron catalyst ADP/Fe^{3+} (a mixture of ADP and $FeCl_3$ in isotonic saline at a molar ratio of 17:1) and incubated under aerobic conditions at 37°C for 3 h. The cells were pelleted by centrifugation, washed four times with DPBS(–) at 4°C, and resuspended in DPBS to make a 2% cell suspension.

Erythrocyte-binding Assay by Dot Blotting and Western Blotting

In the case of dot blotting, aliquots of the solubilized membrane of THP-1 cells were dotted on a PVDF blotting membrane set in a blotting apparatus (ATTO Corporation) through a slot (3-mm diameter). In the case of Western blotting, the solubilized membrane of THP-1 cells was concentrated by ultrafiltration using Ultrafree C3LGC (Millipore Corporation, Bedford, MA) and then electrophoresed in the presence of SDS on 5–20% gradient polyacrylamide slab gels under nonreducing conditions by the method of Laemmli.[11] Separated proteins were electroblotted onto a PVDF membrane at 20 mA/cm^2 for 2 h with 0.025 M Tris-HCl (pH 8.3)-0.192 M glycine-0.1% SDS. The PVDF membrane absorbed with the solubilized membrane of THP-1 cells by dot and Western blotting was soaked with a solution of 2% BSA in 20 mM Tris-HCl (pH 7.6)-0.15 M NaCl-0.1% NaN_3 at 4°C overnight to coat the surface of the PVDF membrane where no protein was absorbed. Erythrocyte-binding to dot and Western blots was performed according to the procedure reported previously.[12] A 2% suspension of oxidized erythrocytes (200 µl) was loaded onto the PVDF membrane and incubated at 4°C for 1 h. After removal of unbound cells by gentle washing with DPBS(–), bound cells were fixed using a solution of 1.25% glutaraldehyde in DPBS (–). The bound cells on dot blots and Western blots were photographed using a blue filter. The number of the bound cells on each dot blot was counted using a standard transmission light microscope mounted with a grid in an ocular. Molecular-weight standards used in Western blotting are: β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), BSA (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa).

RESULTS AND DISCUSSION

Binding of Oxidized Erythrocytes to Dot Blots

Cells of human monocytic leukemia cell line THP-1 were treated with 100 nM PMA for 2 days, and used as human macrophages. A membrane fraction was prepared from the PMA-treated THP-1 cells, solubilized with Triton X-100, and the solution was clarified by centrifugation. After removal of an excess of Triton X-100 by hydrophobic chromatography using SM-2 beads, the solubilized membrane of THP-1 cells was dotted onto PVDF membrane. Erythrocytes were incubated in a solution of an iron catalyst ADP/Fe^{3+} at the concentrations of 0/0, 1.7/0.1, 3.4/0.2, 5.1/0.3, and 6.8/0.4 mM at 37°C for 3 h. This treatment with ADP/Fe^{3+} had been shown to induce oxidative modification of the cells including lipid peroxidation, protein oxidation, and membrane protein aggregation.[3–6] The cells were washed with DPBS(–) and loaded onto the PVDF

membrane dotted with the solubilized membrane of THP-1 cells. After incubation at 4°C for 1 h, unbound cells were removed by gentle washing. The adherent cells on the PVDF membrane were fixed and counted under a microscope. The ADP/Fe^{3+}-oxidized erythrocytes but not unoxidized control cells bound to the dot blots of the solubilized membrane of THP-1 cells (Fig. 1). The binding of oxidized erythrocytes to the dot blots was increased with an increase in the concentration of ADP/Fe^{3+}, possibly depending on the extent of the membrane oxidation, and reached a plateau at 5.1/0.3 mM of ADP/Fe^{3+} (Fig. 1). To obtain maximum binding, 5.1/0.3 mM of ADP/Fe^{3+} was used for oxidation of erythrocytes in the following assay. The observed plateau was not due to saturation of the area of the dot blots with the oxidized erythrocytes since the space unoccupied by the cells was still left as observed under the microscope. It appears that susceptibility of the cells to adherence to the blots of the solubilized membrane of THP-1 cells maximized by oxidation at this concentration of ADP/Fe^{3+}. The result suggests that membrane components, possibly proteins, which recognize the oxidized erythrocytes are present in the solubilized membrane of THP-1 cells.

It is likely that proteins in the solubilized membrane of THP-1 cells that are responsible for recognition of the oxidized erythrocytes recognize the erythrocyte-surface components altered by ADP/Fe^{3+}-induced oxidation. Incubation of human erythrocytes with ADP/Fe^{3+} *in vitro* as been shown to cause membrane phospholipid peroxidation,[3,4] membrane protein oxidation,[5] and aggregation of membrane proteins including band 3 glycoprotein.[6] In the previous study, we have observed that binding of ADP/Fe^{3+}-oxidized

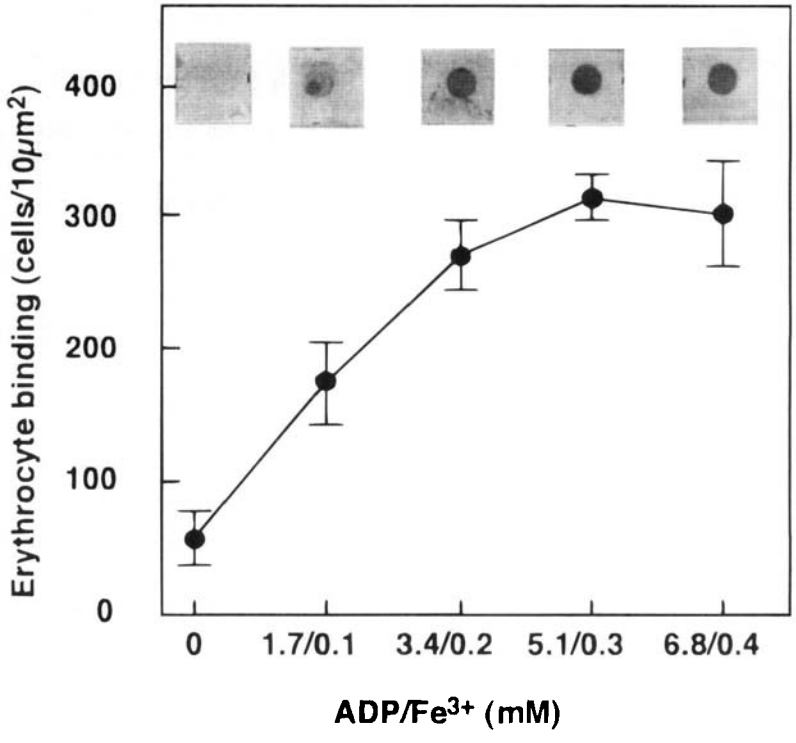

FIGURE 1 Binding of erythrocytes to the dot blots of solubilized membrane of THP-1 cells as a function of the concentration of ADP/Fe^{3+} used for oxidation of erythrocytes. Membrane preparations of PMA-stimulated THP-1 cells were solubilized and dotted onto PVDF membrane, and binding of human erythrocytes oxidized with the indicated concentrations of ADP/Fe^{3+} at 37°C for 3 h to each dot blot was measured as described in Materials and Methods. The photograghs shown are the cells bound to one of triplicate dot blots of the solubilized membrane of THP-1 cells. Each point represents the mean ± S.D. of triplicate blots.

human erythrocytes to the monolayer of PMA-stimulated THP-1 cells was effectively inhibited by saccharide chains of band 3, and suggested that the THP-1 cells bind the oxidized erythrocytes by recognizing the clusters of cell-surface saccharide chains of band 3 that are formed as a result of the oxidation-induced band 3 aggregation in the membrane.[7] It is, thus, likely that recognition of the saccharide chains of band 3 is involved in the recognition of the ADP/Fe^{3+}-oxidized erythrocytes by proteins in the solubilized membrane of THP-1 cells. To examine this possibility, effect of glycoproteins and saccharide chains on the oxidized-erythrocyte binding to the dot blots of the solubilized membrane of THP-1 cells was tested. Effect of various kinds of glycoproteins including glycophorin A and band 3 isolated from human erythrocyte membrane and mannan on the binding of oxidized erythrocytes is shown in Table I (section A). Human lactoferrin, a glycoprotein containing poly-*N*-acetyllactosamine-type saccharide chains (i.e., saccharide chains containing Galβ1→4GlcNAcβ1→3 repeats) like band 3, completely inhibited the binding at 0.2 mg/ml, and band 3, glycophorin A and fetuin partly inhibited the binding at 0.2 mg/ml. Saccharide chains of band 3 and lactoferrin were prepared by hydrazinolysis of the parent glycoproteins and subjected to the inhibition assay. Band 3 oligosaccharides effectively inhibited the binding and lactoferrin oligosaccharides partially inhibited the binding (Table I, section B). The observed inhibition of oxidized-erythrocyte binding by some of the glycoproteins and oligosaccharides suggests that saccharide chains of erythrocyte surface are involved in the binding of oxidized erythrocytes to the blots of the solubilized membrane of THP-1 cells. Particularly, inhibition by band 3, lactoferrin, and their oligosaccharides suggests involvement of saccharide chains of band 3, possibly poly-*N*-acetyllactosaminyl chains, in the binding. Since lactoferrin completely inhibited the binding at 0.2 mg/ml while lactoferrin oligosaccharides only partially inhibited the binding at 0.5 mg/ml, possibility of contribution of lactoferrin polypeptide to the inhibitory activity can not be ruled out. Since saccharide chains of glycophorin A and fetuin do not contain poly-*N*-acetyllactosaminyl structure, partial inhibition by these glycoproteins may suggest involvement of other types of saccharide chains of erythrocyte membrane in the binding to the blots.

TABLE I Effect of glycoproteins, saccharides, EDTA and Ca^{2+} on the oxidized-erythrocyte binding to the dot blots of solubilized membrane of THP-1 cells

	Oxidized-erythrocyte binding (% of control assay)		
A. Glycoproteins and a polysaccharide (0.2 mg/ml)			
Glycophorin A	523	±	5.0
Band 3	74.4	±	5.3
Lactoferrin	2.3	±	0.5
α_1-Acid glycoprotein	91.4	±	7.9
Fetuin	71.9	±	6.6
Mannan	96.9	±	6.7
B. Oligosaccharides (0.5 mg/ml)			
Band 3 oligosaccharides	16.5	±	4.7
Lactoferrin oligosaccharides	65.8	±	13.3
C. EDTA and Ca^{2+} (1 mM)			
EDTA	84.4	±	9.7
Ca^{2+}	99.5	±	15.5

Human erythrocytes oxidized with ADP/Fe^{3+} (5.1/0.3 mM) at 37°C for 3 h were assayed for binding to the dot blots of solubilized membrane of THP-1 cells in the absence (control assay) or presence of the indicated concentrations of the tested materials. Each value represents the mean ± S. D. of triplicate blots.

On macrophages, there are a variety of carbohydrate-binding proteins (lectins), which are classified into C-type (Ca^{2+}-dependent) and S-type (SH-dependent) lectins.[13] To examine involvement of C-type lectins in the oxidized-erythrocyte binding to the dot blots, effect of addition of Ca^{2+} and EDTA on the binding was tested. As shown in Table I (section C), 1 mM of EDTA and Ca^{2+} and did not affect the binding, indicating that C-type lectins on THP-1 cells are unlikely to be involved in the binding.

In order to know directly whether saccharide chains of band 3 and sialic acid residues are involved in the oxidized-erythrocyte binding, effect of removal of poly-*N*-acetyllactosaminyl chains and sialic acid residues from the erythrocyte surface on the binding was examined. Erythrocytes were pretreated with endo-β-galactosidase, which specifically cleaves poly-*N*-acetyllactosaminyl chains, or neuraminidase, which removes sialic acid residues from the nonreducing terminal of the saccharide chains, and then oxidized with ADP/Fe^{3+}. The pretreatment of erythrocytes with endo-β-galactosidase reduced the oxidized-cell binding to the dot blots by about 50% (Fig. 2). Since band 3 is a major carrier of polylactosaminoglycans among membrane glycoconjugates of erythrocytes,[14] the result indicates that at least a half of the binding was mediated by the saccharide chains of band 3. The pretreatment of erythrocytes with neuraminidase did not affect the binding of subsequently oxidized cells (Fig. 2), indicating that sialic acid residues at the nonreducing termini of saccharide chains on erythrocytes are not necessary for the cell binding. The results of the inhibi-

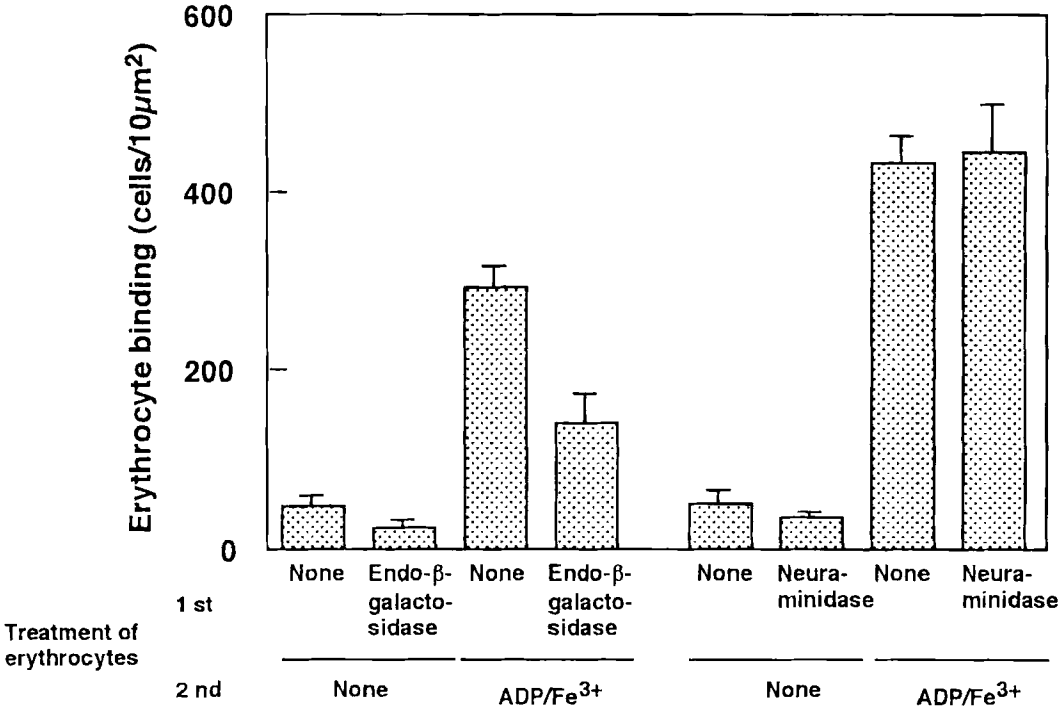

FIGURE 2 Effect of glycosidase treatment of erythrocytes on their binding to the dot blots of solubilized membrane of THP-1 cells. Human erythrocytes (40% suspension) were incubated with endo-β-galactosidase (30 mU/ml) in 0.1 M sodium acetate buffer (pH 5.8) containing 0.07 M NaCl or the buffer alone at 37°C for 2 h, or with neuraminidase (50 mU/ml) in DPBS or DPBS alone at 37°C for 2 h. After being washed 4 times with DPBS (–), the cells were treated with or without ADP/Fe^{3+} (5.1/0.3 mM) at 37°C for 3 h, and subjected to the assay for binding to the dot blots of solubilized membrane of THP-1 cells on PVDF membrane. Each value represents the mean ± S.D. of triplicate blots.

tion studies using various glycoproteins and saccharide chains, and the glycosidase pretreatment study shown above are consistent with the previously obtained results in the binding of oxidized erythrocytes to the monolayer of THP-1 cells,[7] except significant inhibitory effect of glycophorin A. This indicates that the membrane proteins of macrophages responsible for the interaction with the oxidized erythrocytes do exist on the blots of the solubilized membrane of THP-1 cells.

Detection of Oxidized Erythrocyte-Binding Proteins by Western Blotting

To visualize the proteins responsible for the oxidized-erythrocyte binding through the saccharide chains of band 3, the solubilized membrane of THP-1 cells was subjected to SDS-PAGE followed by Western blotting, and binding of oxidized erythrocytes to the Western blots was examined. As shown in Fig. 3, the proteins with molecular mass of about 50, 80, and 120 kDa were visualized by oxidized-erythrocyte binding (lane 2), while control erythrocytes did not bind to the proteins (lane 1). The binding was significantly inhibited by 0.2 mg/ml of band 3 oligosaccharides and lactoferrin oligosaccharides (Fig. 3, lanes 3 and 4). Furthermore, decreased binding of oxidized erythrocytes to these protein bands was observed when the erythrocytes were pretreated with endo-β-galactosidase prior to oxidation (Fig. 3, lanes 5 and 6). These results indicate that the detected proteins specifically bind oxidized erythrocytes through the saccharide chains of band 3. Hence, it is likely that the 50, 80, and 120 kDa proteins are the macrophage proteins responsible for the recognition of oxidized erythrocytes. Since the molecular weights of the detected proteins are different from those of so far reported macrophage non-C-type lectins, these proteins may be novel macrophage lectins.

The present study indicated that oxidatively damaged erythrocytes are recognized by macrophages through lectin-like membrane proteins, and the determinants on the oxidized erythrocytes recognized by these proteins are saccharide chains of band 3. Since band 3 molecules are aggregated in ADP/Fe^{3+}-oxidized cell mem-

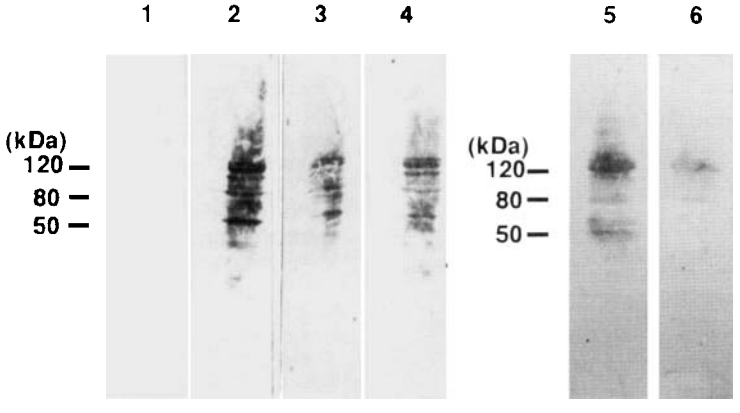

FIGURE 3 Detection of oxidized-erythrocyte-binding proteins of solubilized membrane of THP-1 cells on Western blots. Solubilized membrane of THP-1 cells was electrophoresed on 5–20% gradient slab gels in the presence of SDS and transferred to PVDF membrane. The PVDF membrane was coated with 2% BSA, and subjected to the erythrocyte-binding assay as described in Materials and Methods. Lanes 1 and 2, binding of control erythrocytes (cells incubated in the absence of ADP/Fe^{3+} at 37°C for 3 h) and oxidized erythrocytes (cells incubated in 5.1/0.3 mM of ADP/Fe^{3+} at 37°C for 3 h), respectively; lanes 3 and 4, binding of oxidized erythrocytes in the presence of 200 μg/ml of band 3 oligosaccharides and lactoferrin oligosaccharides, respectively; lane 5 and 6, binding of control oxidized erythrocytes (oxidized cells preincubated in the absence of endo-β-galactosidase as described in the legend to Fig. 2) and oxidized erythrocytes pretreated with endo-β-galactosidase as described in the legend to Fig. 2, respectively. The molecular-weights of the visualized bands are indicated at the left side of the membranes.

brane,[6] resultant clusters of saccharide chains of band 3 on the cell surface have been suggested to play a role as high-affinity antigens for anti-band 3 IgG,[6,10] allowing divalent high avidity binding of the antibodies. It is thus likely that such clusters of band 3 saccharide chains on oxidized erythrocytes are effective ligands for the lectin-like proteins of THP-1 cell membrane detected here. The present finding indicates that host defence system recognizes the oxidatively damaged cells through the specific membrane proteins to remove them.

Sambrano et al.[15] reported that human erythrocytes oxidized with Cu^{2+}/ascorbic acid or H_2O_2 are recognized by mouse peritoneal macrophages through the scavenger receptors for oxidized LDL. They suggested that the determinants of the recognition on the oxidized cell surface are lipid-protein conjugates generated by reaction of membrane proteins with the products of lipid peroxidation, although they did not characterize the oxidized cell membrane. There may be various mechanisms of macrophage recognition of oxidatively damaged erythrocytes, depending on extent of oxidation or how the cells are oxidized.

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