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Biochemical and Biophysical Research Communications 363 (2007) 57–62
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Clearance of oxidized erythrocytes by macrophages: Involvement of caspases in the generation of clearance signal at band 3 glycoprotein

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Received 8 August 2007
Available online 27 August 2007

Abstract

Human erythrocytes exposed to appropriate concentrations of H_2O_2 for 1 h became susceptible to the binding and phagocytosis by macrophages. The binding was inhibited by anti-band 3 serum and prevented by pretreatment of erythrocytes with a polylactosamine-cleaving enzyme endo-β-galactosidase, indicating that polylactosaminyl sugar chains of band 3 are recognized by macrophages. The macrophage receptor involved was suggested to be nucleolin, a recently identified macrophage surface protein recognizing sialylpolylactosaminyl-chain clusters on early apoptotic cells, because anti-nucleolin antibody and a soluble form of recombinant nucleolin blocked the recognition. Treatment of erythrocytes with caspase inhibitors Z-VAD-fmk or Z-DQMD-fmk (caspase 3 selective) before the oxidation resulted in lowered binding of the oxidized erythrocytes to macrophages, suggesting that actions of caspases, particularly those of caspase 3, are prerequisite for the membrane changes leading to band 3 aggregation. Moreover, the cytosolic caspase 3 was found to be activated by H_2O_2, and the extent of the activation correlated well with the susceptibility of the oxidized erythrocytes to the macrophage recognition. These results suggest that oxidative stress renders the erythrocytes susceptible to clearance by macrophages through activation of caspases leading to band 3 aggregation.

Keywords: Oxidative stress; Caspase 3; Erythrocyte; Band 3; Sugar chain; Phagocytosis; Apoptosis; Cell clearance; Nucleolin

Oxygen carrying cells erythrocytes are continuously exposed to oxidative stress in the circulation, and undergo various oxidative damages. In vitro studies have demonstrated that oxidatively damaged erythrocytes are recognized and removed by phagocytes [1,2]. In previous studies, we showed that erythrocytes briefly modified by iron-catalyzed oxidation were recognized and phagocytosed by macrophages, and the major determinants recognized were the polylactosaminyl sugar chains of band 3 glycoprotein [3,4]. It was suggested that band 3 molecules on the membrane tend to form clusters upon the cell oxidation, providing the multivalent high-affinity sugar chain ligands on the cell-surface for the macrophage receptors [4,5].

In another series of recent works, we found that human T lymphatic Jurkat cells at an early stage of apoptosis are recognized and phagocytosed by macrophages through the polylactosaminyl sugar chains of CD43 undergoing capping depending on caspase activities [6,7]. We also identified the macrophage receptor for the early apoptotic cells as nucleolin [8], a multifunctional shuttling protein present in nucleus, cytoplasm and on the surface of some types of cells. These findings and another report that human erythrocytes express caspases such as caspases 3 and 8 [9] suggest the possibilities that erythrocyte caspases are involved in the generation of the cell-clearance signal at band 3 glycoprotein (i.e., clustering of band 3 glycoprotein), just like as the caspase-dependent CD43 capping on

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doi:10.1016/j.bbrc.2007.08.089

early apoptotic Jurkat cells [6], and that the macrophage receptor for the oxidized erythrocytes is also nucleolin.

In the present study, we investigated whether erythrocyte caspases are responsible for the band 3 glycoprotein-mediated macrophage recognition of human erythrocytes oxidized with H_2O_2, and whether the oxidized erythrocytes are recognized by nucleolin on macrophages.

Materials and methods

Materials. Hydrogen peroxide, pepstatin A, leupeptin hemisulfate-monohydrate, phenylmethylsulfonyl fluoride (PMSF), and diisopropyl fluorophosphate (DFP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hanks' balanced salt solution (HBSS) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Bovine serum albumin (BSA), phorbol myristate acetate and rabbit serum were obtained from Sigma–Aldrich (St. Louis, MO). Endo-β-galactosidase (E.C.3.2.1.103, *Escherichia freundii*) was obtained from Seikagaku Fine Chemicals (Tokyo, Japan). Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk), benzyloxycarbonyl-Asp (OMe)-Gln-Met-Asp (OMe)-fluoromethylketone (Z-DQMD-fmk), and acetyl-Asp-Asn-Leu-Asp-methylcoumarylamide (Ac-DNLD-MCA) were the product of Peptide Institute (Osaka, Japan). HemogloBind™ was purchased from Biotech (North Brunswick, USA). Ultrafree-MC centrifugal filter units were purchased from Millipore (Bedford, USA). Anti-band 3 antibody (anti-band 3) was raised in rabbits using a human band 3 peptide corresponding to the C-terminal 78 amino acids beginning at amino acid H834. An antibody against a synthetic octapeptide corresponding to the residues 295–302 of nucleolin (anti-NUC295) [8] was raised in rabbits and affinity purified as described previously [8]. Normal rabbit serum was obtained from DAKO (Glostrup, Denmark). Recombinant human nucleolin composed of the C-terminal 427 amino acids beginning at amino acid M284 (rNUC284), and a control recombinant 42-kDa chloramphenicol acetyl transferase (rCAT) with the same tags were prepared as described [8].

Erythrocyte preparation and oxidation. Erythrocyte-rich fraction of healthy human blood containing acid citrate dextrose (ACD) as an anticoagulant, and mannitol-adenosine-phosphate (MAP) as a preservative was obtained from a local blood center of Japanese Red Cross, stored at 4 °C, and used within 2 weeks after the collection. Erythrocyte-rich fraction was centrifuged (320*g*, 10 min) twice at 4 °C to remove residual plasma and buffy coats. Erythrocytes were washed three times with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline, pH 7.3 (DPBS(−)) at 4 °C and resuspended in HBSS to make a 20% (v/v) cell suspension. The cell suspension was mixed with an equal volume of an appropriate concentration of H_2O_2, and incubated at 37 °C for appropriate hours with gentle shaking. The oxidized cells were washed three times with HBSS at 4 °C by centrifugation, and subjected to binding assays or analyses.

Binding and phagocytosis assays. THP-1 monocytes (Japanese Cancer Research Resources Bank, Osaka Japan) were differentiated into macrophages on glass coverslips (15-mm diameter) as described previously [4,6] except that the cell number plated was 8×10^4 cells/coverslip, and the cell monolayers were washed in DPBS(−) before use. The oxidized erythrocytes obtained as above were resuspended in RPMI 1640 medium buffered with 20 mM HEPES, pH 7.2 (RPMI 1640-HEPES) at 2%, and the suspension was loaded onto the macrophage monolayers (150 μl/coverslip). The cells were incubated at 37 °C without shaking in 5% CO_2 atmosphere for 1 h. After the incubation, unbound cells were removed by gentle washing in DPBS(−), and bound erythrocytes and macrophages were fixed with 1.25% glutaraldehyde. The number of bound erythrocytes and macrophages was counted under a light microscope (400× magnification). The data are expressed as the number of bound erythrocytes/100 macrophages (Cell binding) as counting more than 300 macrophages. For measurement of phagocytosis, unbound and surface-bound erythrocytes were lysed with 0.83% NH_4Cl in 10 mM Tris–HCl buffer, pH 7.6, and the remaining cells were stained with a 3% Giemsa solution for 20 min. Erythrocytes taken up by macrophages were identified under a light microscope (400× magnification).

Measurement of caspase-3 activity of oxidized erythrocytes. H_2O_2-oxidized erythrocytes (100 μl) were washed three times with HBSS at 4 °C, and dissolved in 900 μl of a lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 w/v % Triton-X 100, 5 mM EDTA, 1 mM PMSF, and 1 μg/ml leupeptin). Immediately after the dissolution, DFP was added at a final concentration of 1 mM. Protein concentration of the erythrocyte lysate was determined using MicroBCA™ protein assay kit (Pierce) and adjusted to 2 mg/ml with the lysis buffer. Caspase-3 activity of the lysate was measured using Ac-DNLD-MCA as a substrate that releases fluorescent 7-amino-4-methylcoumarin (AMC) when hydrolyzed by caspase 3. Equal volumes of the cell lysate and 0.1 mM Ac-DNLD-MCA in a substrate buffer (20 mM HEPES, pH 7.4, 20 w/v % sucrose, 0.2 w/v % CHAPS, 0.1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 4 mM dithiothreitol) were mixed and incubated at 37 °C in the dark for 4 h. To remove hemoglobin that interferes with the fluorescence measurement, the reaction mixture was subjected to ultrafiltration using Ultrafree-MC centrifugal filter units with an exclusion limit of 10,000 Da, and a small amount of leaked hemoglobin was then removed using a hemoglobin adsorbent HemogloBind™. The fluorescence intensity of AMC of the hemoglobin-free reaction mixture was measured using a microspectrometer (SAFIRE, Tecan) at excitation and emission wavelengths of 380 and 460 nm, respectively.

Flow cytometry. Binding of rabbit anti-band 3 antibody to erythrocyte surface was measured by flow cytometry using Alexa Fluor 488 goat anti-rabbit IgG as a secondary antibody. Amount of phosphatidylserine (PS) on erythrocyte surface was also measured by flow cytometry using fluorescein isothiocyanate-labeled annexin V (FITC-annexin V) as described previously [7].

Statistical analysis. The data are presented as the mean ± SD of at least triplicate experiments. The data were compared using a Student's *t* test, and statistical significance was determined. *, $p < 0.05$ and **, $p < 0.01$.

Results

Susceptibility of H_2O_2-oxidized erythrocytes to phagocytic recognition depending on H_2O_2 concentrations and time of the oxidation

Susceptibility of oxidized human erythrocytes to macrophage recognition was assessed as the functions of H_2O_2 concentration and the time of the oxidation using human monocytic THP-1 cells differentiated into adherent cells as macrophages. As shown in Fig. 1A, erythrocytes oxidized with various concentrations of H_2O_2 for 1 h became susceptible to the macrophage binding. The susceptibility of the cells to the macrophage binding maximized at 0.1 mM H_2O_2, and decreased at higher concentrations. Time course measurement of the susceptibility of 0.1 mM H_2O_2-oxidized cells to the macrophage binding showed that the cells become most susceptible to the macrophage binding at 1 h oxidation (Fig. 1B). Further oxidation at this concentration resulted in lesser recognition by macrophages. Cells incubated in the absence of H_2O_2 only slightly became susceptible to the recognition. These results indicate that there exist optimal time and concentrations of H_2O_2-treatment in the generation of cell-surface changes of erythrocytes recognized by macrophages. The cells oxidized under the optimal binding condition (i.e., 0.1 mM H_2O_2 for 1 h) were confirmed to be phagocytosed when incubated with macrophages for 3 h (Fig. 1C, left and right). Thus, we hereafter oxidized

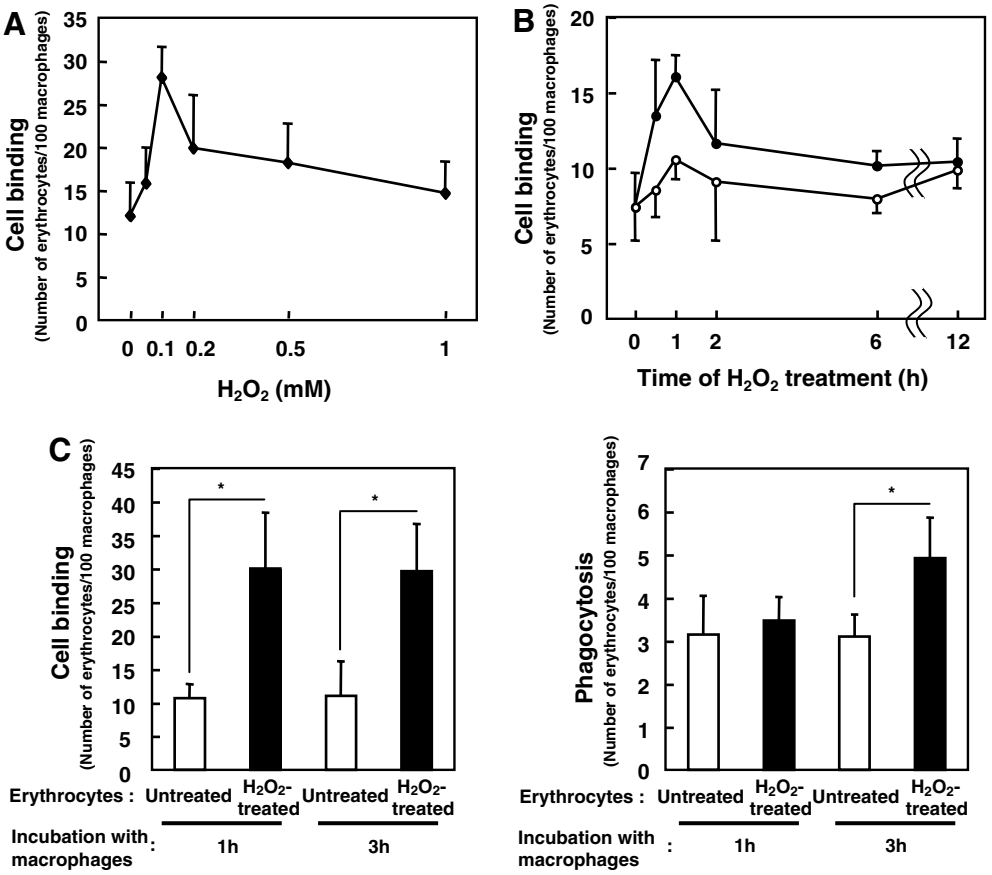

Fig. 1. Conditions for phagocytic recognition of oxidized erythrocytes by macrophages. (A and B) Changes in the susceptibility of erythrocytes to macrophage binding as the functions of concentration (A) and time (B) of H_2O_2-treatment. Erythrocytes were incubated with the indicated concentrations of H_2O_2 for 1 h (A, filled circles) at 37 °C, or with (B, filled circles) or without (B, open circles) 0.1 mM H_2O_2 for the indicated hours at 37 °C, and subjected to the macrophage binding assay as described in Materials and methods. Data are expressed as ''cell binding,'' which is defined under Materials and methods. Each point represents the mean ± SD of triplicate experiments. (C) Time dependency in phagocytosis. Erythrocytes were incubated with or without 0.1 mM H_2O_2 for 1 h, and subjected to binding and phagocytosis assays as described in Materials and methods. Each column represents the mean ± SD of triplicate experiments. *, $P < 0.05$.

erythrocytes with 0.1 mM H_2O_2 for 1 h, and used for the binding experiments.

Involvement of poly-N-acetyllactosaminyl sugar chains of band 3 glycoprotein in the recognition of the oxidized erythrocytes by macrophages

The ligands on the H_2O_2-oxidized erythrocytes responsible for the macrophage recognition were then examined. The most possible candidate for the ligands appeared to be poly-*N*-acetyllactosaminyl sugar chains of band 3 glycoprotein since iron-oxidized erythrocytes were recognized by THP-1 macrophages through this type of chains [4], contained in band 3 glycoprotein [10]. As shown in Fig. 2A, when H_2O_2-oxidized erythrocytes were preincubated with anti-band 3 antiserum, the macrophage recognition of the oxidized cells was inhibited, while normal serum did not. In addition, when erythrocytes were treated with endo-β-galactosidase, an enzyme specifically cleaving polylactosaminyl chains [11], they did not become susceptible to the macrophage recognition after H_2O_2-oxidation (Fig. 2B). These results suggest that the ligands on the H_2O_2-oxidized erythrocytes recognized by macrophages are poly-*N*-acetyllactosaminyl sugar chains of band 3 glycoprotein.

When the amount of the cell-surface band 3 glycoprotein was compared by flow cytometry using the anti-band 3 antibody, there was no difference between the oxidized and unoxidized erythrocytes (data not shown). It is thus likely that the band 3 change on the oxidized cells is topological change such as clustering and capping that would increase the binding avidity of the poly-*N*-acetyllactosaminyl sugar chains to the macrophage receptor.

To see whether PS is exposed on the H_2O_2-oxidized erythrocyte surface, PS on the cell surface was measured by flow cytometry using FITC-annexin V. Under the oxidation conditions carried out (0.1–1.0 mM H_2O_2, 0–12 h oxidation), PS exposure was not detected while treatment with a calcium ionophore A23187 at 4 μM for 1 h, performed as a positive control, resulted in significant amount of PS exposure (data not shown). It is therefore likely that much higher concentrations of H_2O_2 and longer oxidation is necessary for PS exposure in human erythrocytes.

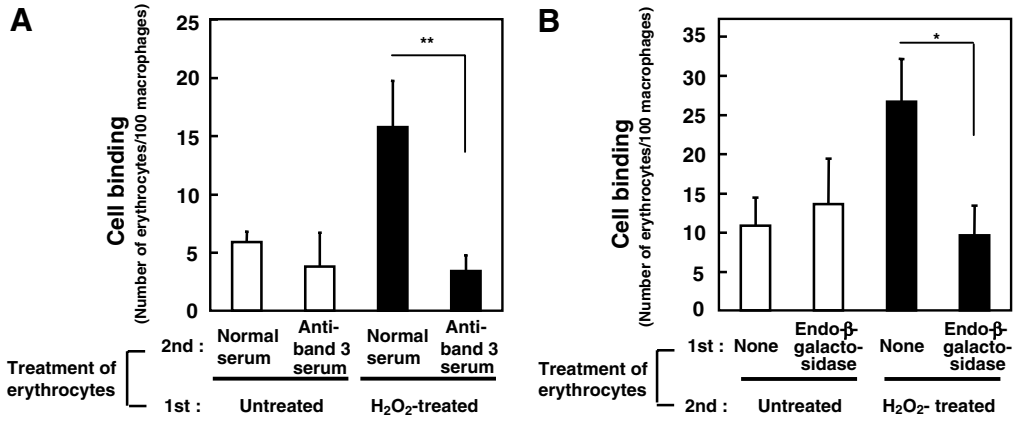

Fig. 2. Involvement of sugar chains of band 3 glycoprotein in the macrophage recognition of oxidized erythrocytes. Each column represents the mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$. (A) Inhibition of the binding of oxidized erythrocytes to macrophages by anti-band 3 antiserum. Erythrocytes treated with or without 0.1 mM H_2O_2 for 1 h were washed with HBSS, and incubated with anti-band 3 antiserum containing 2 μg/ml of antibody or with normal rabbit serum containing 2 μg/ml of IgG in RPMI 1640-HEPES-0.2% BSA at 4 °C for 30 min. The cells were washed and subjected to the binding assay with macrophages. (B) Loss of binding of oxidized erythrocytes to macrophages by pretreatment of erythrocytes with endo-β-galactosidase. Erythrocytes (20 v/v %) were incubated with endo-β-galactosidase (0.1 U/ml) in HBSS for 1 h at 37 °C, washed with HBSS, and treated with or without 0.1 mM H_2O_2 for 1 h. The cells were subjected to the binding assay with macrophages.

Involvement of macrophage surface nucleolin in the recognition of the oxidized erythrocytes by macrophages

To see whether the macrophage surface nucleolin is responsible for the recognition of the H_2O_2-oxidized erythrocytes by macrophages, inhibition studies were carried out. When THP-1 macrophages were preincubated with the antibody against nucleolin (anti-NUC295), the macrophage binding to the H_2O_2-oxidized erythrocytes was inhibited, while control IgG had no effect (Fig. 3A). Moreover, a soluble form of recombinant nucleolin (rNUC284) composed of the C-terminal 427 amino acids beginning at amino acid M284, inhibited the macrophage binding of the H_2O_2-oxidized erythrocytes, while a control recombinant protein rCAT did not (Fig. 3B). These results suggest that the macrophage surface nucleolin is responsible for the recognition of the H_2O_2-oxidized erythrocytes.

Involvement of caspases in the changes of erythrocyte membrane inducing the macrophage recognition

To see whether or not erythrocyte caspases are involved in the erythrocyte membrane changes inducing the sugar chain-mediated macrophage recognition, effect of caspase inhibitors was tested. When erythrocytes were preincubated with Z-VAD-fmk, a caspase inhibitor with broad specificity, and a caspase 3-specific inhibitor Z-DQMD-fmk (Fig. 4A and B, respectively), the cells did not become susceptible to the macrophage recognition by the H_2O_2 oxidation. This suggests that caspases, including casapase 3,

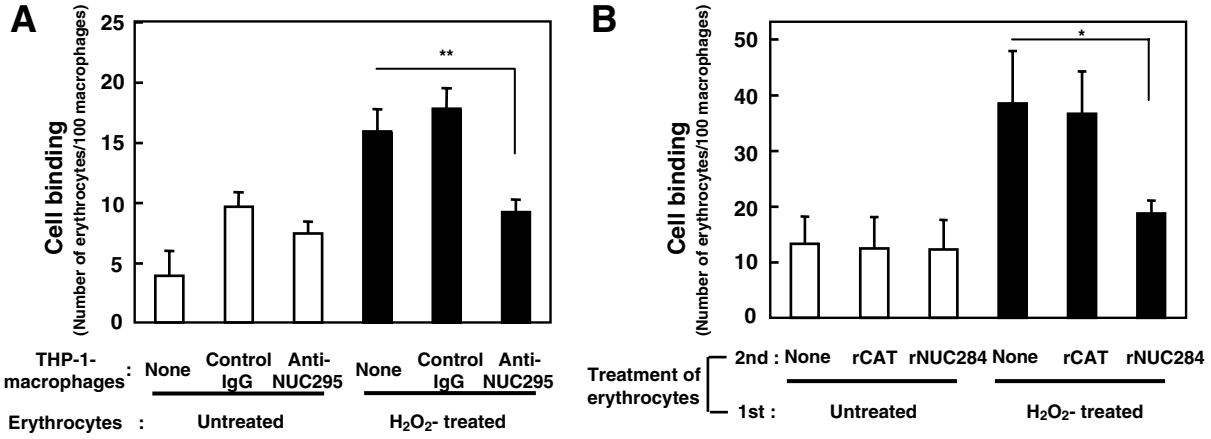

Fig. 3. Involvement of cell-surface nucleolin of macrophages in the recognition of oxidized erythrocytes. Each column represents the mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$. (A) Inhibition of the binding of oxidized erythrocytes to macrophages by antibody against nucleolin. Macrophages were preincubated with 2 μg/ml of anti-NUC295 or control IgG in RPMI 1640-HEPES at 4 °C for 30 min, washed, and subjected to the binding assay using erythrocytes treated with or without 0.1 mM H_2O_2 for 1 h. (B) Inhibition of the binding of oxidized erythrocytes to macrophages by a soluble form of recombinant nucleolin rNUC284. Erythrocytes treated with or without 0.1 mM H_2O_2 for 1 h were incubated with 20 nM rNUC284 or control rCAT at 4 °C for 30 min, washed, and subjected to the binding assay with macrophages.

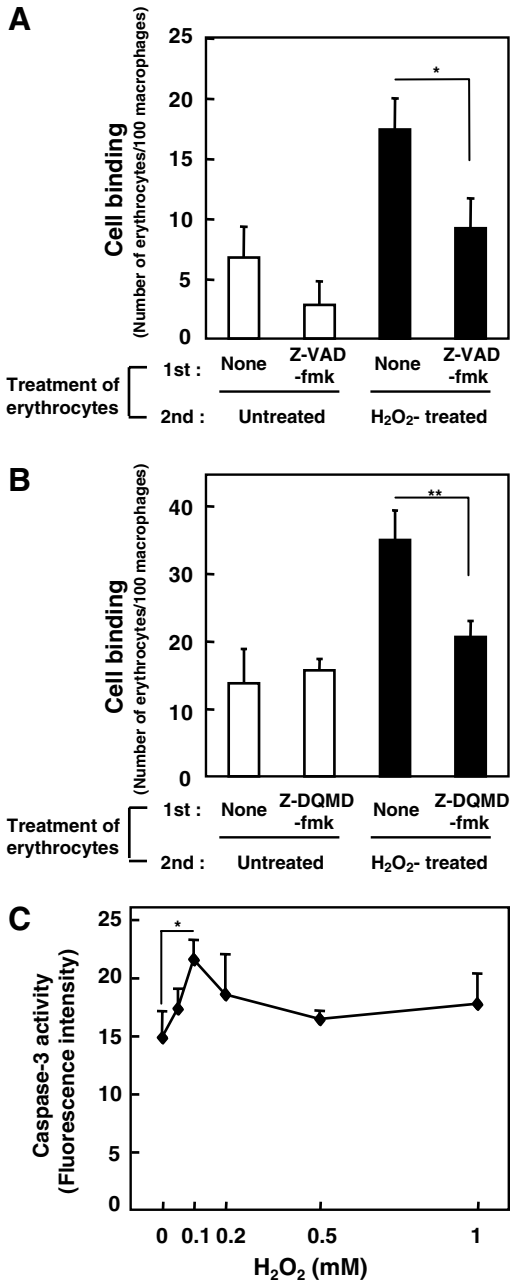

Fig. 4. Involvement of caspases in the changes of erythrocyte membrane leading to macrophage recognition. Each column or each point represents the mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$. (A and B) Prevention of oxidized-erythrocyte binding to macrophages by pretreatment of erythrocytes with Z-VAD-fmk (A) or Z-DQMD-fmk (B). Erythrocytes (20 v/v %) were incubated with 10 μM Z-VAD-fmk (A) or Z-DQMD-fmk (B) in HBSS for 1 h at 37 °C, and treated with or without 0.1 mM H_2O_2 for 1 h. The cells were washed and subjected to the binding assay with macrophages. (C) Activation of caspase 3 by H_2O_2 as a function of H_2O_2 concentrations. Erythrocytes were incubated with the indicated concentrations of H_2O_2 for 1 h at 37 °C, and intracellular caspase 3 activity was measured as described in Materials and methods.

participate in the changes of erythrocyte membrane leading to the macrophage recognition. To see whether erythrocyte procaspase 3 becomes activated by H_2O_2 oxidation, activity of cytosolic caspase 3 in the oxidized erythrocytes was measured using a caspase 3-specific fluorescent substrate Ac-DNLD-MCA. As shown in Fig. 4C, the activity of caspase 3 increased by H_2O_2 oxidation at 0.1 mM. However, it decreased at the higher concentrations. It is interesting to note that the profile of the H_2O_2-concentration dependent changes in casapase 3 activity coincided very well with that of the H_2O_2-concentration dependent binding of the oxidized erythrocytes to macrophages (Fig. 1A). This coincidence also suggests that active caspase 3 causes the band 3 changes, possibly its aggregation by cleaving its peptide chains, and leads the erythrocytes to the recognition and clearance by macrophages.

Discussion

In the present study, we demonstrated that caspases including caspase 3 are involved in the cell-surface changes of oxidized erythrocytes inducing the macrophage recognition and phagocytosis. In addition, the macrophage receptor for the oxidized erythrocytes was demonstrated to be nucleolin, a recently identified macrophage receptor for early apoptotic cells [8].

Considering the similarity in the mechanism of the macrophage recognition of oxidized erythrocytes [3,4] and that of early apoptotic cells [6], we expected involvement of common mechanisms between the two cellular systems. The present results indicate the common mechanisms are (1) activation of caspase 3 (i.e., processing of procaspase 3 to caspase 3), and (2) the following clustering of membrane glycoproteins (band 3 and CD43). It is also shown that even enucleate cells, like human erythrocytes, undergo apoptosis-like cellular changes and clearance by macrophages.

Mechanism of caspase 3 activation by H_2O_2 and by other oxidants is not known. Whether the oxidants activate initiator procaspases that will then activate procaspase 3 into caspase 3, or activate some cellular molecules at the upstream of the caspase signaling cascade remains to be clarified.

Clustering of band 3 glycoprotein appears to be dependent on caspase 3 activity (Fig. 4B), and thus caspase 3 may cleave band 3 glycoprotein directly, which may facilitate band 3 clustering. Recently, Mandal et al. [12] reported that human erythrocyte caspase 3 cleaves the peptide chain of the N-terminal cytoplasmic domain of band 3 protein at Asp^{45} and Asp^{205}, and thereby the band 3 interaction with the N-terminal domain of protein 4.2 is diminished, resulting in the loss of band 3-anchoring to the spectrin-based cytoskeletal network. Such processes may be involved in the band 3 clustering and macrophage recognition in the present study.

In the present study, we oxidized erythrocytes using 0 to 1 mM H_2O_2, and the maximal binding by macrophages was attained at 0.1 mM H_2O_2. Since this concentration of H_2O_2 is occurring in blood vessels in inflammatory tissues and in atherosclerotic lesions [13], the present experiments carried out using 0.1 mM H_2O_2 was suitable in view of in vivo situation.

Under the moderate and some excessive conditions, we measured PS exposure on the surface of H_2O_2-oxidized erythrocytes, but there was no exposure. In contrast, oxidation of human erythrocytes using 1 to 3 mM *t*-butylhydroperoxide was reported to cause caspase 3 activation and PS exposure [14]. It is conceivable that PS exposure on erythrocytes may require intense or/and long-term exposure to the oxidants.

Acknowledgments

This work was supported in part by a grant for a "High-tech research center" project for private universities: matching fund subsidy from MEXT, Japan, 2006.

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