Participation of caspase-3-like protease in oxidation-induced impairment of erythrocyte membrane properties

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Abstract. Erythrocytes are very susceptible to oxidative stress, having a high content of intracellular oxygen and hemoglobin. In the present study, exposure to oxidative stress resulted in a significant impairment of erythrocyte membrane functions, such as deformability and anion exchange. Band 3 protein, also known as anion exchanger-1, plays an important role in these two functions. We show that oxidative stress activated caspase-3 inside the erythrocytes, which resulted in band 3 protein cleavage. Interestingly, inhibition of the caspase-3 with its specific inhibitor not only suppressed the digestion of band 3 protein, but also blunted the functional damage to erythrocytes, such as deformability and anion exchange, without changing the level of peroxidation of membrane lipids. These results provide experimental evidence that activation of caspase-3 plays an important role in the oxidative stress-induced impairment of membrane functions of erythrocytes.

Keywords: Band 3, oxidative stress, erythrocyte deformation, anion exchange

1. Introduction

Circulating erythrocytes are constantly exposed to oxidative stress as a result of the high cellular concentration of oxygen and hemoglobin, which is a source of the reactive oxygen species. Autooxidation of oxyhemoglobin to methemoglobin results in the release of superoxide anion $O_2^-$. Hydrogen peroxide is a secondary product of one-electron oxidation of $O_2^-$ via spontaneous or enzymatically catalyzed dismutation: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. H$_2$O$_2$ reacts with ferrous ion to produce ·OH in the Fenton reaction: Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + ·OH + OH$^-$. A reducing agent such as ascorbic acid reduces Fe$^{3+}$ to Fe$^{2+}$, which leads to a redox cycle and increases the production of ·OH [17].

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Exposure to oxidative stress causes several types of damage in erythrocytes, such as peroxidation of lipids, degradation of proteins, hemoglobin-spectrin cross-linking, glycosylation of proteins, inactivation of enzymes, and perturbation of membrane lipid bilayer. In parallel with these molecular damages, the function of erythrocyte’s membrane, such as viscosity, deformability and rouleaux formation alter dramatically upon exposure to oxidative stress [7,8,12]. However, the mechanisms underlying these functional damages of erythrocytes are still poorly understood.

Recent studies demonstrated that oxidative stress induces activation of caspases-3 [13,16], leading to apoptosis of erythrocytes, characterized by cell shrinkage, membrane blebbing, activation of proteases, phosphatidylserine (PS) externalization, and increased phagocytosis of erythrocytes by macrophages [5,10,11,14–16].

Caspase-3 is widely believed to be the most important mediator of apoptosis in mammalian cells. It cleaves a distinct range of substrates that share a common DEVD motif. These include proteins related to cell structure (such as gelsolin, focal adhesion kinase, and p21-activated kinase 2), and DNA repair (such as DNA-dependent protein kinase and poly (ADP-ribose) polymerase), and protein synthesis (such as eukaryotic translation initiation factor 4GI (eIF4GI) and 4GII (eIF4GII)) [19,20]. Mandal et al. reported that activated caspase-3 also cleaves band 3 protein in the erythrocyte’s membrane [13].

In light of the above information, we examined whether the inhibition of caspase-3 ameliorates the oxidative stress-induced impairment of the functions of erythrocyte membrane, such as deformability and anion exchange.

2. Materials and methods

2.1. Reagents

FeSO₄, ascorbic acid, and tert-butylhydroperoxide (t-BHP) were purchased from Wako pure chemicals (Osaka, Japan). Dextran T-40 was from Pharmacia (Uppsala, Sweden), recombinant human active caspase-3 from Alexis Biochemicals (San Diego, CA, USA), and zDEVD-fmk (caspase-3 inhibitor) from Peptide Institute (Osaka, Japan).

2.2. Preparation of erythrocytes

Heparinized venous blood was obtained, after written informed consent, from healthy donors, and immediately centrifuged. After a careful removal of plasma and buffy coat, erythrocytes were purified by three cycles of resuspension and washing with isotonic HEPES-buffered saline (HBS) containing 100 mg/dl glucose at 4°C, and resuspended at 40% hematocrit in HBS for further analysis.

2.3. Exposure of erythrocytes to oxygen radicals

The cells were treated with varying concentration of FeSO₄ (0.5–2 mM) in the presence of 10 mM ascorbate, or t-BHP (0.1 or 0.25 mM) as described previously [8,15], and incubated at 37°C for 1 h at room temperature with gentle shaking.
2.4. Deformability analysis

Deformability of erythrocytes was measured at 0.3% hematocrit in isotonic HBS containing 25% Dextran T-40, at 25°C with a high-shear rheoscope. The degree of ellipsoidal deformation of erythrocytes was expressed as the deformation index $DI = (L - S)/(L + S)$, where $L$ and $S$ were the long and short radii of the deformed cells, respectively [8].

2.5. Evaluation of anion exchange function

Hematocrit was determined with a microhematocrit centrifuge, and number of erythrocytes with an automatic counter (Toa, Model CC-110, Kobe, Japan). On the basis of these values, mean corpuscular volume (MCV) was calculated.

The anion exchange function of erythrocytes was evaluated by measuring the decrease of MCV of erythrocytes equilibrated in phosphate-buffered saline (PBS) as previously described [3]. Briefly, erythrocytes were washed twice, equilibrated in isotonic PBS, containing 70 mM Na-phosphate and 84 mM NaCl at 37°C for 20 min, and MCV was measured. The same sample was equilibrated in isotonic HBS, containing 10 mM HEPES, and MCV was measured as a control. When equilibrated in PBS, the volumes of normal erythrocytes decrease, because two HCO$_3^-$ anion and one H$^+$ cation are exchanged with one H$_2$PO$_4^-$ anion through the erythrocyte membrane. When equilibrated in HBS, the volumes of normal erythrocytes do not change, because one HCO$_3^-$ anion is exchanged with one Cl$^-$ anion. So, $\Delta$MCV = (MCV)$_{HBS} - (MCV)_{PBS}$ was calculated and used as index for the anion exchange function.

2.6. Detection of caspase-like activity in erythrocytes

Caspase-3, -8, -9-like activities in erythrocytes were measured by using Caspase-Glo 3/7, 8, 9 assay kits (Promega, Madison, WI, USA) according to the manufacturer’s protocol, respectively. Each assay provides a luminogenic caspase-3/7, 8, 9 substrate, which contains the tetrapeptide sequence DEVD, LETD, LEHD, respectively, in a reagent optimized for caspase activity, luciferase activity and cell lysis.

Additionally, caspase-3-like activity in erythrocytes was assayed by using FAM-DEVD-fmk reagent (Biocarta, San Diego, CA, USA) and FACS Calibur (Becton Dickinson, San Jose, CA, USA) according to the manufacturer’s protocol. Briefly, FAM-DEVD-fmk reagent was dissolved in dimethylsulfoxide to obtain a 150× concentrated stock solution. Prior to use, a 30× working solution of FAM-DEVD-fmk was prepared by diluting the stock solution in PBS. Erythrocytes were suspended in HBS at 10$^6$ cells/ml. Ten l FAM-DEVD-fmk was added to the 300 l suspension to obtain 10 M final concentration, and incubated at 37°C for 1 h. Following incubation, the cells were pelleted, rinsed with HBS, and resuspended in 1 ml HBS. Fluorescence was measured by FACS Calibur flow cytometer using the standard emission filter for green fluorescence (FL1) and analysis was done with a Cell quest software.

2.7. Detection of PS externalization

The externalized PS on the outer surface of erythrocytes was detected by labeling the cells with Annexin-fluos kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. Data acquisition and analysis were performed as described above.
2.8. Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation products formed from the oxidative breakdown of polyunsaturated fatty acids were measured in the erythrocyte’s ghost membrane using TBARS assay, as described previously [8].

2.9. Membrane ghosts preparation

Erythrocytes in 250 l suspension (40% hematocrit) were lysed on ice with hypotonic PBS (20 mOsm), pH 7.4, and centrifuged for 10 min at 4°C at $10^4 \times g$. After removal of clear supernatant, the sediment was resuspended in ice-cold hypotonic PBS and centrifuged again. The washing procedure was repeated three times to obtain the membrane ghosts.

2.10. Preparation of inside-out vesicles from erythrocytes

Inside-out vesicles were produced from erythrocyte ghosts as described previously [9]. Briefly, erythrocyte ghosts prepared from 250 l erythrocyte suspension (40% hematocrit) were incubated in 30 volume of 0.1 mM EGTA, pH 8.5, at 37°C for 30 min. Subsequently, they were centrifuged, washed once, and the volume was adjusted to 200 l, prior to use.

2.11. Membrane protein digestion by recombinant caspase-3

The 1 l inside-out vesicles were digested with 1 l recombinant human active caspase-3 (1 unit/l) in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol at 37°C for 2 h.

2.12. SDS-Polyacrylamide gel electrophoresis

The erythrocyte ghost membranes were subjected to SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gel and stained in 0.025% Coomassie brilliant blue.

2.13. Statistical analysis

Group differences were analyzed with one-way analysis of variance using SPSS6.1J software. Critical significance level were set at $p < 0.05$. Post-test analyses using two tailed Student’s t-test were carried out.

3. Results

3.1. Effects of oxidative stress on erythrocyte membrane lipids

1 h incubation of erythrocytes with FeSO$_4$/ascorbate resulted in a dose-dependent lipid oxidation reflected by the formation of TBARS (Fig. 1). Similarly, the incubation with t-BHP resulted in a dose-dependent lipid oxidation. It should be noted that in both cases after pretreatment with 100 M zDEVD-fmk, the caspase-3 inhibitor, did not change TBARS value. No hemolysis was observed in any of the experiments.
3.2. Effects of oxidative stress on activity of caspases

Erythrocytes were exposed to oxidative stress by incubating with FeSO_4/ascorbate or t-BHP at different concentrations for 1 h. Caspase-3-like activity, caspase-8-like activity and caspase-9-like activity were measured by using Caspase-Glo 3/7, 8, 9 assay kits (Figs 2 and 3). Both FeSO_4 and t-BHP increased caspase-3-like activity in a dose-dependent manner, but did not increase caspase-8-like activity or caspase-9-like activity. To confirm the increase of caspase-3-like-activity in each cell, we employed flow cytometric analysis with FAM-DEVD-fmk reagent (Fig. 4). This reagent enters into each erythrocyte and covalently binds to active caspase-3. By using flow cytometry and detecting the green fluorescence of FAM, the caspase-3-like activity in each cell could be measured. It was observed that oxidative stress significantly increased the geometric mean of caspase-3-like activity in the cells. These results suggest that oxidative stress increases caspase-3-like activity in erythrocytes, but does not modify caspase-8- or caspase-9-like activity.

3.3. Effects of oxidative stress on PS externalization

When erythrocytes were exposed to weak oxidative stress induced by 1.0 or 2.0 mM FeSO_4/ascorbate, or 0.3 mM t-BHP, the cell populations showed single peaks in mean fluorescence intensity from annexin-binding cells (Fig. 5B–D). When they were exposed to stronger oxidative stress induced by 3.0 or 10 mM t-BHP, the cell populations showed bimodal peaks (Fig. 5E, F). The geometric mean of annexin-binding (Fig. 5G) and the percentage of Annexin-fluos positive cells in the total population (Fig. 5H) were significantly higher than saline-treated control under strong oxidative stress, while they were not significant under weak oxidative stress. The results both from weak and strong oxidative stress agree with the previous report [11]. But the results from strong oxidative stress may not represent the increase
Fig. 2. Effects of oxidative stress on activity of caspase-3. Erythrocytes were incubated with FeSO$_4$/ascorbate (A) or t-BHP (B) at indicated concentrations for 1 h in the absence or presence of the caspase-3 inhibitor, and then caspase-3-like activity was measured, as described in Section 2. Data are expressed as relative units, mean ± SD of 5 independent experiments. *$p<0.05$, significant difference versus untreated control (c). Note that FeSO$_4$ and t-BHP activated caspase-3-like protease in dose-dependent manner.

Fig. 3. Effects of oxidative stress on activities of caspase-8 and caspase-9. Erythrocytes were incubated with FeSO$_4$/ascorbate at indicated concentrations for 1 h, and then caspase-8-like activity (A) and caspase-9-like activity (B) were measured, as described in Materials and methods. Data are expressed as relative units, mean ± SD of 5 independent experiments. Note that FeSO$_4$ treatment did not significantly activated caspase-8 or caspase-9.

of PS exposure to the outer membrane. The presence of a bimodal peak under strong oxidative stress suggests that holes, through which Annexin-fluos can pass, were generated on erythrocytes’ membrane.

In the following experiments, we employed a relatively low level of oxidative stress (2.0 mM FeSO$_4$/ascorbate), because such weak oxidative stress seems to more closely mimic physiological con-
Fig. 4. Oxidative stress-induced activation of caspase-3 detected by flow cytometry. Erythrocytes were incubated with saline alone (A) or saline containing 2.0 mM FeSO₄/ascorbate (B) for 1 h. Subsequently, caspase-3-like activity in erythrocytes was detected by using FAM-DEVD-fmk reagent and FACS Calibur as described in Materials and methods. The horizontal axis indicates the fluorescence intensity from FAM-DEVD-fmk in arbitrary units, which represents caspase-3 activity in each cell. (C) Geometric means ± SD (n = 5) of the fluorescence intensity from FAM-DEVD-fmk in arbitrary units after exposure to saline alone and saline containing 2.0 mM FeSO₄/ascorbate. *p < 0.05.

3.4. Effects of oxidative stress on membrane properties of erythrocytes

The membrane properties of erythrocytes were evaluated by their deformability and anion exchange function. The former was measured with the high shear-rheoscope under various levels of shear stress (from 15 to 135 dyn/cm², Fig. 6). The treatment with 2.0 mM FeSO₄/ascorbate resulted in a significant decrease of deformability at all values of the shear stress measured. Surprisingly, pretreatment with caspase-3 inhibitor zDEVD-fmk blunted this decrease of deformability over the whole range of the shear stress used.
Fig. 5. Effects of oxidative stress on erythrocyte PS externalization. Erythrocytes were incubated with saline alone (A), saline containing FeSO₄/ascorbate (B, C) or t-BHP (D–F) at indicated concentrations for 1 h, and then erythrocyte PS externalization was detected by using Annexin-fluos kit and FACS Calibur as described in Materials and methods. The horizontal axis shows the fluorescence intensity from FITC-Annexin V in arbitrary units, which represents the amount of PS exposed to outer membrane of each cell. The relative number of cells found in the indicated region was defined as the percentage of Annexin-fluos–positive erythrocytes. (G): Means ± SD (n = 5) of the fluorescence intensity from FAM-DEVD-fmk in arbitrary units. *p < 0.05, significant difference versus saline-treated control A. (H): Means ± SD (n = 5) of Annexin-fluos–positive cells in % of the total population. *p < 0.05, significant difference versus saline-treated control A.
Fig. 6. Effect of oxidative stress on erythrocyte deformability in the absence or presence of caspase-3 inhibitor. (A): Erythrocytes were incubated with saline alone or 100 M caspase-3 inhibitor zDEVD-fmk for 1 h. (B): Erythrocytes were incubated with saline alone, 2 mM FeSO₄/ascorbate or 2 mM FeSO₄/ascorbate in the presence of 100 M caspase-3 inhibitor zDEVD-fmk for 1 h. Deformability of erythrocytes was measured as described in Materials and methods. Values are mean ± SD (n = 10) of the deformation index. *p < 0.05, significant difference versus saline-treated control. #p < 0.05, significant difference versus 0.5 mM FeSO₄/ascorbate-treated erythrocytes.

The anion exchange function was evaluated by ∆MCV as described in Section 2 (Fig. 7). The treatment with 2.0 mM FeSO₄/ascorbate significantly impaired anion exchange function of erythrocytes. Interestingly, pretreatment with caspase-3 inhibitor zDEVD-fmk blunted the impairment, too.

Taken together, these results suggest that oxidative stress may impair these membrane properties of erythrocytes, at least partially, through activation of caspase-3.

3.5. Effects of oxidative stress on erythrocyte membrane proteins

Figure 8A presents a typical densitograms of SDS-PAGE analysis of erythrocyte membrane proteins. In contrast to the control cells, the SDS-PAGE patterns of erythrocytes treated with recombinant human caspase-3 and FeSO₄/ascorbate-treated erythrocytes are similar in that bands 3 and 4.1ab are markedly degraded, and a new 80 kDa band appears (Figs. 8B and 8C). Interestingly, the presence of caspase-3 inhibitor zDEVD-fmk suppressed the FeSO₄/ascorbate-induced degradation of band 3 and band 4.1ab and abolished the appearance of the new 80 kDa band (Fig. 8D).

4. Discussion

We demonstrate here that oxidative stress activates caspase-3 in erythrocytes and that inhibition of the caspase-3 ameliorates the oxidation-induced degradation of erythrocyte membrane proteins. These results agree with previous reports [13,16]. Interestingly, the inhibition of the caspase-3 ameliorated not only the degradation of proteins but also the oxidative stress-induced dysfunction of the erythrocyte’s...
membrane without changing the levels of membrane lipid peroxidation. This result implies that oxidative stress impairs the membrane’s functions, partially through digestion of membrane proteins by caspase-3. Membrane deformability and anion exchange participate in major functions of erythrocytes, the former playing an important role in the microcirculation, and the latter being crucial for O₂/CO₂ exchange. Exposure of erythrocytes to oxidative stress leads to the serious impairment of these functions. The deformation of erythrocytes is influenced by membrane stiffness, cellular viscosity and cell shape. Since the shape of the biconcave disc is maintained under oxidative stress (data not shown), we assume that the main cause of the decreased deformability by oxidative stress results from the alteration of viscoelastic properties of cell membranes and possible changes in cellular viscosity.

Some studies have reported that the increase in lipid peroxide rigidifies the erythrocyte membrane by changing the amino-phospholipid organization [18]. The malondialdehyde is one of the lipid peroxidation products and may play a role as an oxidative trigger that contributes to the hemorheological alterations [12]. Another important factor affecting deformability is protein organization of the membrane cytoskeleton. In the present study, following the exposure to oxidative stress, decrease in bands 3 and 4.1ab content, and appearance of new peptides at approximately 80 kDa were demonstrated. In addition, specific-caspase-3 inhibitor suppressed the digestion of bands 3 and 4.1ab, providing the experimental evidence that caspase-3 participates in oxidative stress-induced digestion of these proteins. Unlike caspase-3, increase in activation of caspase-8 or -9 was not observed upon oxidative stress, although mature erythrocytes are reported to contain considerable amounts of procaspase-8 [2].

Band 3 is a 95-kDa glycoprotein, comprising approximately 25% w/w of the total protein of the erythrocyte membrane. It is a member of the erythrocyte membrane cytoskeletal network, which anchors the cytoskeleton to the lipid bilayer. Its N-terminal cytoplasmic domain binds to cytoskeletal protein
spectrin via ankyrin [6]. It plays an important role in maintaining of erythrocyte’s biconcave shape and resistance to shear force. The oxidative scission of the band 3 protein can disturb membrane-cytoskeletal interactions and thereby alter rheological properties of cells. Furthermore, its C-terminal transmembrane domain mediates the $\text{HCO}_3^-/\text{Cl}^-$ anion exchange [21], leading to the $\text{O}_2/\text{CO}_2$ exchange in erythrocytes.

Erythrocyte aging leads to binding of analogous IgG followed by recognition and removal through phagocytosis, mainly by Kupffer cells in the liver. This process is triggered by the appearance of a senescent erythrocyte-specific antigen. The functional and structural characteristics of senescent erythrocytes suggest that this antigen originates from band 3 protein [1,4]. It is obvious that changes in band 3 occurring during the erythrocyte lifespan must precede this appearance of the antigen. Our data may thus imply not only the role of activated caspase-3 in erythrocyte dysfunction, but also suggest a mechanism by which oxidative stress accelerates the aging of erythrocytes.
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