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Presence of membrane-bound proteinases that preferentially degrade oxidatively damaged erythrocyte membrane proteins as secondary antioxidant defense

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Abstract

Human erythrocytes were oxidized with xanthine/xanthine oxidase/ferric ion or ADP/ferric ion at 37°C for several hours. Band 3 protein and spectrin of the oxidized cells were found to be significantly modified as analyzed by radiolabeling with tritiated borohydride. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the xanthine/xanthine oxidase/ferric iron-oxidized cells and subsequent immunoblotting with anti band 3 protein showed that band 3 protein was fragmented into smaller molecular-weight fragments. When the cell membrane obtained from the oxidized cells were incubated at pH 7.4 and 37°C for several hours in the presence of α-tocopherol, extensive degradation of band 3 protein and spectrin was observed. Band 3 protein was found to be most susceptible to the degradation. Degradation of band 3 protein was also observed after similar incubation of the membrane from the ADP/ferric ion-oxidized cells. Membrane-bound serine- and metalloproteinases were responsible for the degradation of band 3 protein, because the degradation was remarkably inhibited by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, and partially by ethylenediaminetetraacetic acid. Hence, the membrane proteins became susceptible to membrane-bound proteinases by oxidative stress. This observation suggests that these membrane-bound proteinases exist to remove oxidatively damaged proteins from the cell membrane.

Keywords: Oxidative stress; Membrane protein degradation; Membrane-bound proteinase; Band 3 protein; Spectrin; (Erythrocyte)

1. Introduction

It is well-known that oxidative stress results in the damage of cells, which may lead to the loss of cell function. Most cells have primary antioxidant defense to prevent the oxidative damage of the cells: superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, tocopherol, ascorbic acid and so on [1]. However, if the oxidative stress in the cells is high enough or the primary antioxidant defense in the cells is insufficient, the cells can be oxidatively damaged. Recent studies have demonstrated that cells have lipolytic, DNA-repair and proteolytic systems that prevent the formation or accumulation of oxidatively damaged phospholipids, DNA and proteins, and they are referred to as secondary antioxidant defense [1]. The presence of proteinases in erythrocyte and reticulocyte extracts [2–9], mitochondria [10,11] and *Escherichia coli* extracts [6,12–14] that are specific to the oxidatively damaged intracellular proteins has been demonstrated. A multicatalytic proteolytic complex composed of several subunits has been shown to be responsible for the degradation of the oxidized intracellular proteins in erythrocytes and reticulocytes [7–9].

In the present study, it was found that oxidative stress to erythrocytes resulted in the increase in susceptibility of the membrane proteins to membrane-bound proteinases. This suggests the presence of the secondary antioxidant defense mechanisms in the erythrocyte membrane for the removal of the oxidatively damaged cell membrane proteins by proteinases.

Abbreviations: DFP, diisopropyl fluorophosphate; DPBS, Dulbecco's phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; X, xanthine; XO, xanthine oxidase.

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2. Materials and methods

2.1. Materials

ADP monopotassium salt was purchased from Oriental Yeast, Tokyo, Japan. Xanthine (X), xanthine oxidase (XO) (EC 1.1.3.22, from buttermilk, grade III), diisopropyl fluorophosphate (DFP), leupeptin, pepstatin A and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma, St. Louis, MO. α-Tocopherol was from Eisai, Tokyo, Japan. XO was purified as follows. The enzyme solution (50 units/ml) was diluted with a buffer composed of 25 mM potassium dihydrogen phosphate, 125 mM choline chloride, 0.01 mM ethylenediaminetetraacetic acid (EDTA) and 25 mM sodium azide (pH 7.4) to make the enzyme concentration at 1 unit/ml. To 1 ml of the solution 5 μl of 0.2 M DFP solution in ethanol and 2 μl of 0.2 M PMSF solution in ethanol were added, and the mixture was allowed to stand for 2 h to inactivate the contaminated proteinases [15]. After the mixture was dialyzed against the same buffer, it was stored at −80°C until use.

Human venous blood withdrawn from a healthy donor using acid-citrate-dextrose as an anticoagulant was stored at 4°C for a couple of days. The blood was centrifuged ($320 \times g$, 10 min) at 4°C to remove plasma and buffy coats. Erythrocytes were washed four times by centrifugation ($320 \times g$, 10 min) at 4°C with ice-cold Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline (pH 7.3) (DPBS(−)). By this procedure, usually erythrocyte preparations with only about 0.006% of leukocyte contamination, as determined by crystal violet staining, were obtained. Passing the erythrocyte preparation through a leukocyte-adsorbing filter for blood transfusion (Sepacell RS-200, Asahi Medical, Tokyo, Japan) did not reduce the number of leukocytes significantly. Hence, erythrocyte preparations obtained by the centrifugation procedure were used as purified erythrocytes. The purified cells were resuspended in DPBS(−) at 40% suspension and used for the modification. Band 3 protein [15] and spectrin [16] were prepared as described.

Rabbit was immunized with a mixture of spectrin and Freund's complete adjuvant. The precipitate obtained from serum of rabbit by 40% ammonium sulfate saturation was passed through a column of DE 52 equilibrated with 17.5 mM phosphate buffer (pH 6.3), and the IgG fraction that was not adsorbed to the column was dialyzed against DPBS(−) containing 0.02% sodium azide to obtain rabbit anti human spectrin. The preparation was stored in a refrigerator until use. Rabbit was immunized with band 3 protein, and the IgG fraction obtained as above was adsorbed to a band 3-Sepharose 4B affinity column [15] equilibrated with DPBS(−) containing 0.02% sodium azide. Rabbit anti-human band 3 adsorbed to the column was eluted with 0.1 M glycine-HCl (pH 2.6) and the eluate was dialyzed against DPBS (−) containing 0.02% sodium azide after neutralization by addition of an equal volume of 1 M Tris-HCl (pH 7.4). The preparation was stored in a refrigerator until use.

2.2. Analyses

Protein was determined according to the method of Lowry et al. [17] using bovine serum albumin as a reference standard.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the discontinuous buffer system of Laemmli [18], with a 10% separating gel and a 4% stacking gel under reducing conditions. Protein bands were visualized by staining with Coomassie brilliant blue R-250 (CBB) or silver (silver staining kit, Daiichi Chemicals, Tokyo, Japan). Major protein bands are numbered according to Steck [19]. Densitometric measurement of CBB stained SDS-PAGE gels was performed with a Shimadzu CS9000 double beam flying spot scanner. Immunoblotting detection of band 3 protein and spectrin was carried out according to the method of Towbin et al. [20] with slight modifications. Briefly, protein bands on the SDS-PAGE gel were transferred to a Western blotting membrane (Clear Blot Membrane-P, ATTO, Tokyo, Japan), and the transfer of the protein was confirmed by Amido black staining of the membrane. The transferred proteins were incubated with rabbit anti-human band 3 or spectrin and then with protein A-horseradish peroxidase conjugate (Bio-Rad, Richmond, CA, USA). The peroxidase activity was detected with hydrogen peroxide and 4-chloro-1-naphthol. No proteins were detected when a control IgG obtained from a nonimmunized rabbit was used.

2.3. Oxidation of erythrocytes

Oxidation of erythrocytes was performed as described previously [15,21]. Oxidation with X/XO/$FeCl_3$: A 20% erythrocyte suspension in DPBS was incubated with 1 mM X/10 or 40 mU XO per ml/0.1 mM $FeCl_3$ at 37°C for 3 or 6 h. Oxidation with ADP/ferric ion: A 20% erythrocyte suspension in DPBS was incubated with ADP/$FeCl_3$ (1.7 mM/0.1 mM or 8.5 mM/0.5 mM) at 37°C for 3 or 6 h. Control erythrocytes were obtained by incubation in the absence of the oxidants. The cell pellet was washed twice with DPBS at 4°C and resuspended in DPBS. The cell suspension was mixed with 1 mM DFP, 0.2 mM PMSF and 1 mM EDTA and incubated at room temperature for 1 h before dissolution into the sample buffer for SDS-PAGE. The number of erythrocytes loaded per lane was $8.3 \cdot 10^6$ cells.

2.4. Determination of the degree of the oxidative damage of band 3 protein and spectrin

Membrane of oxidized or control erythrocytes was obtained by hemolysis of the cells by addition of 40-fold volume of 5 mM phosphate buffer (pH 8.0) and washing

several times with the same buffer by centrifugation (18 000 × *g*, 20 min) under ice-cooling until the washing buffer became colorless. The cell membrane was suspended in 10 mM phosphate buffer (pH 7.0) containing 0.1 mM α-tocopherol to prevent further membrane oxidation, and stored at −80°C. Band 3 protein and spectrin were isolated from the cell membrane as described. Amino acid analysis of band 3 protein and spectrin was performed according to the method previously described [22] using a Hitachi L-8500 high performance amino acid analyzer. The degree of oxidative damage of the membrane proteins was determined by tritium labeling using tritiated borohydride [23,24]. To a 160-μl solution of band 3 protein (2–3 mg/ml) or spectrin (300–900 μg/ml), 40 μl of 10 mM Tris-HCl (pH 7.4) and 25 μl of 0.01 N NaOH solution containing 19 MBq (0.5 mCi) of NaB^3H_4 (355 GBq (9.6 Ci)/mmol, Amersham) were added, and the mixture was allowed to stand at room temperature for 2 h. To the mixture an equal volume of 20% trichloroacetic acid solution was added, and the mixture was centrifuged (18 000 × *g*, 10 min). The sediment was washed with 10% trichloroacetic acid solution four times and dissolved into 1 ml of 5% SDS/0.2 M Tris-HCl (pH 7.4). The solution was dialyzed extensively against 10 mM Tris-HCl (pH 7.4) to obtain tritiated band 3 protein or spectrin. The radioactivity was determined by a liquid scintillation counter.

2.5. *Incubation (2nd incubation) of membrane from oxidized erythrocytes in the presence and absence of proteinase inhibitors*

To 2 volumes of the cell membrane suspension (2.5 mg protein/ml) containing 0.1 mM α-tocopherol, 1 volume of DPBS with or without proteinase inhibitors was added, and the mixture was incubated at 37°C for 3 or 6 h. After the 2nd incubation the mixture was subjected to SDS-PAGE. Samples containing 30 μg protein was loaded per lane.

3. Results

Human erythrocytes were treated with $X/XO/Fe^{3+}$ or ADP/Fe^{3+} at 37°C for 3 or 6 h. As has been shown previously [15,21], the thiobarbituric acid-reactive substances in the medium were increased by the treatment, whereas the cell deformability and osmotic fragility were unchanged. Two major membrane proteins, band 3 protein and spectrin, were isolated from the oxidized cells to determine whether the proteins were modified. Amino acid analysis showed that the amino acid composition of the proteins was changed little by the oxidation. In contrast,

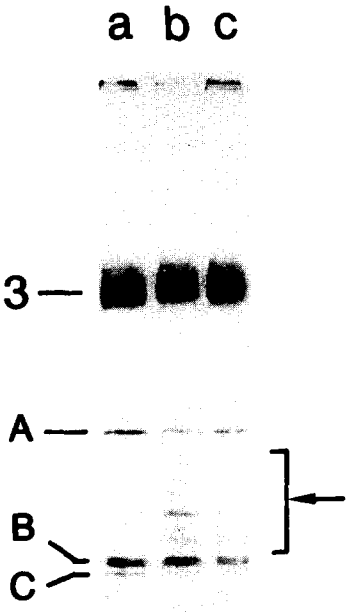

Fig. 1. SDS-PAGE of oxidized erythrocytes and subsequent immunoblotting using rabbit anti human band 3. Erythrocytes treated with none (lane a), $X/XO/Fe^{3+}$ at 1 mM/10 mU ml^{-1}/0.1 mM (lane b) and at 1 mM/40 mU ml^{-1}/0.1 mM (lane c) at 37°C for 3 h were washed and dissolved into the sample buffer for SDS-PAGE. After SDS-PAGE under reduced conditions, immunoblotting detection of band 3 and its fragments using rabbit anti-human band 3 was performed. Bands A, B and C indicate band 3 fragments present in unoxidized erythrocytes, and an arrow indicates band 3 fragments newly appeared by the oxidation.

Table 1
Tritium incorporation into band 3 protein and spectrin isolated from oxidized erythrocytes

Erythrocyte treatment	^3H incorporation (cpm/μg protein)	
	Band 3 protein	Spectrin
None	229 ± 18	499 ± 17
$X/XO/Fe^{3+}$ (1 mM/10 mU ml^{-1}/0.1 mM)	1750 ± 916	726 ± 35
None	253 ± 8	594 ± 40
ADP/Fe^{3+} (1.7 mM/0.1 mM)	519 ± 15	1032 ± 331
None	862 ± 38	813 ± 59
ADP/Fe^{3+} (8.5 mM/0.5 mM)	3197 ± 1603	2172 ± 204

Erythrocytes were treated with $X/XO/Fe^{3+}$ or ADP/Fe^{3+} at the indicated concentrations at 37°C for 3 h. Band 3 protein and spectrin were isolated from the oxidized cells, and treated with NaB^3H_4 as described in Materials and methods. Data shown are the mean values ± errors of duplicate labeling.

tritium incorporation into the proteins upon reduction with tritiated borohydride, a sensitive measure for oxidative modification of proteins [23,24], was increased remarkably by the oxidation (Table 1), indicating that these membrane proteins were modified by the oxidation. The results indicate that the cells and the membrane proteins were mildly but not extensively damaged upon the treatment with the oxidizing agents.

Erythrocytes treated with the $X/XO/Fe^{3+}$ were directly solubilized with SDS and subjected to SDS-PAGE. Protein bands of the oxidized cells appeared unchanged when visualized by CBB or silver staining. Highly sensitive immunoblotting technique by use of rabbit anti-human band 3 revealed that band 3 protein of the control cells had been fragmented into three fragments A, B and C (Fig. 1, lane a), and band 3 of the oxidized cells had been fragmented into many other fragments (Fig. 1, lanes b and c, arrow) in addition to the fragments A, B and C. It is likely that band 3 protein was fragmented upon treatment of the cells with the oxidizing agent. Spectrin was not fragmented upon oxidation of the cells with the agent as detected by immunoblotting using rabbit anti-human spectrin (data not shown).

The cell membrane isolated from the $X/XO/Fe^{3+}$-oxidized erythrocytes was incubated at 37°C for additional 6 h in the presence of α-tocopherol which prevents further oxidation of the membrane (2nd incubation) and subjected to SDS-PAGE. CBB staining of the gel showed that band 3 protein and spectrin (bands 1 and 2 in the figure) from the oxidized cells were significantly decreased as compared with those from unoxidized cells (Fig. 2, lanes a, b and c), and a new fragmented protein band appeared. Immunoblotting using rabbit anti-human band 3 showed that band 3 protein from the oxidized cells was significantly decreased as compared with that from unoxidized cells (Fig. 2, lanes d, e and f), and new fragmented band 3 protein bands appeared. Immunoblotting using rabbit anti-human spectrin showed that spectrin from the oxidized cells was extensively decreased (Fig. 2, lanes g, h and i), and new fragmented spectrin bands appeared.

The effect of oxidation time of erythrocytes with $X/XO/Fe^{3+}$ on the band 3 and spectrin degradation in the 2nd incubation of the cell membrane was investigated. Both band 3 and spectrin from the cells oxidized for 3 h were more susceptible to degaradation than those from the cells oxidized for 6 h (data not shown). The milder oxida-

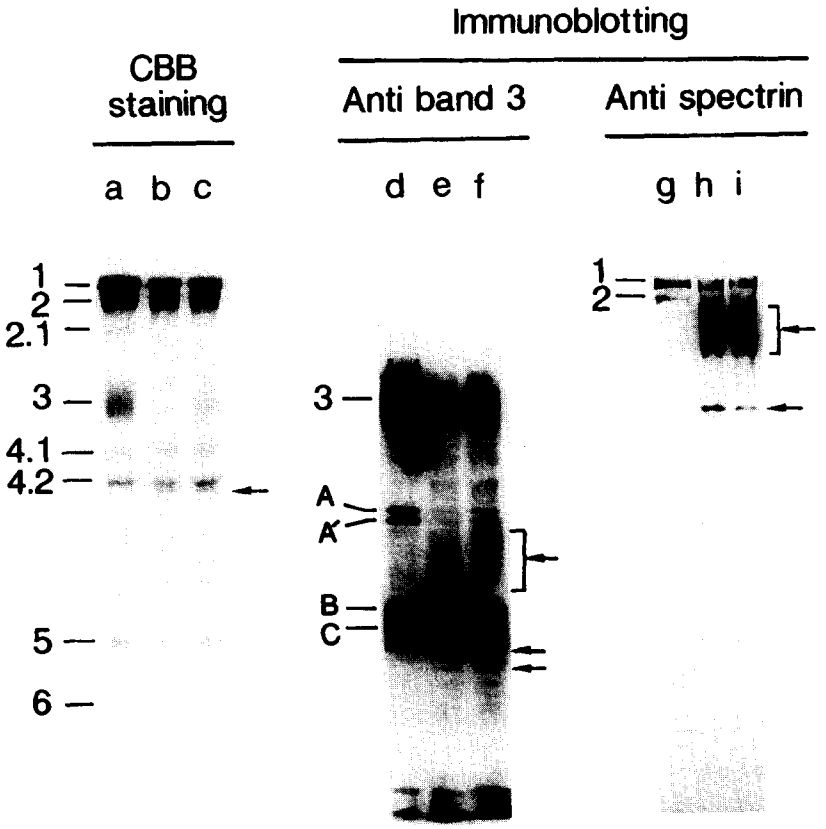

Fig. 2. SDS-PAGE of membrane isolated from the $X/XO/Fe^{3+}$-oxidized erythrocytes after the 2nd incubation. Cell membrane was isolated from erythrocytes treated with none (lanes a, d and g), and with $X/XO/Fe^{3+}$ at 1 mM/10 mU ml^{-1}/0.1 mM (lanes b, e and h) and at 1 mM/40 mU ml^{-1}/0.1 mM (lanes c, f and i) at 37°C for 3 h, and incubated in the presence of α-tocopherol at 37°C for 6 h (2nd incubation). The mixture was subjected to SDS-PAGE under reducing conditions and stained by CBB and immunoblotting using rabbit anti-human band 3 and anti-human spectrin. Bands indicated by arrows are newly formed fragments by the oxidation.

tion of the cells was found to give the higher susceptibility of the membrane proteins to the fragmentation in the 2nd incubation.

It was found that band 3 protein was most susceptible to the degradation, because the densitometric measurement of the protein bands in CBB stained gel of the incubated membrane of the $X/XO/Fe^{3+}$-oxidized cells indicated that the staining intensity of band 3 protein was reduced extensively as compared to those of other membrane proteins (Fig. 3). The decrease of band 3 protein of the $X/XO/Fe^{3+}$-oxidized cells in the 2nd incubation of the isolated membrane during 6 and 12 h was determined by the densitometry of the CBB stained gels (Fig. 4). It is apparent that band 3 protein of the oxidized cells is susceptible to degradation in the 2nd incubation of the cell membrane.

The cell membrane was isolated from the ADP/Fe^{3+}-oxidized cells, and the isolated membrane was similarly incubated for 6 and 12 h in the presence of α-tocopherol, and subjected to SDS-PAGE followed by CBB staining. As was the case for the membrane from the $X/XO/Fe^{3+}$-oxidized cells, the amount of band 3 protein was reduced upon the 2nd incubation as measured by densitometry of the stained gels (Fig. 4). It is interesting to note that the decrease of band 3 protein was greater at the lower concentrations of the oxidizing agents than at the higher concentrations. Mildly oxidized band 3 protein was more susceptible to the degradation.

Contribution of the membrane bound proteinases to the degradation of band 3 protein in the 2nd incubation of the membrane isolated from the $X/XO/Fe^{3+}$-oxidized cells was investigated. For this purpose, the 2nd incubation of

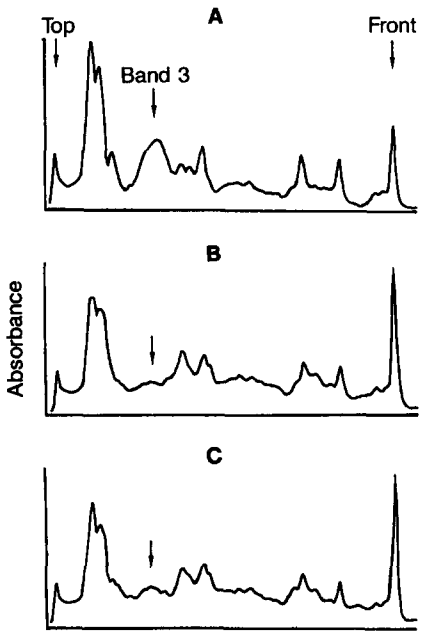

Fig. 3. Densitometric measurement of the CBB stained gels of the incubated membrane of the $X/XO/Fe^{3+}$-oxidized erythrocytes. (Panel A) Fig. 2, lane a. (Panel B) Fig. 2, lane b. (Panel C) Fig. 2, lane c.

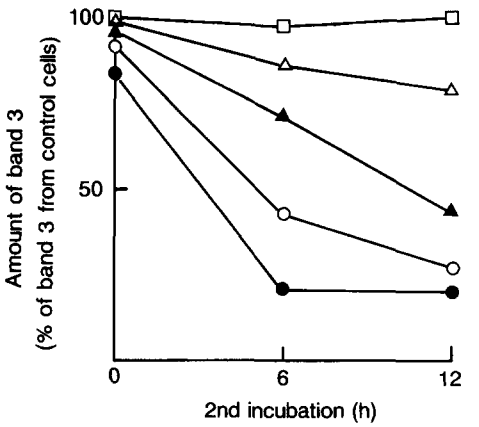

Fig. 4. Decrease of band 3 protein of the membrane isolated from the $X/XO/Fe^{3+}$- and the ADP/Fe^{3+}-oxidized erythrocytes after the 2nd incubation. Cell membrane was isolated from erythrocytes treated with none (□), with $X/XO/Fe^{3+}$ at 1 mM/10 mU ml^{-1}/0.1 mM (●) and at 1 mM/40 mU ml^{-1}/0.1 mM (○), and with ADP/Fe^{3+} at 1.7 mM/0.1 mM (▲) and at 8.5 mM/0.5 mM (△) at 37°C for 3 h was incubated in the presence of α-tocopherol at 37°C for 6 and 12 h (2nd incubation). The mixture was subjected to SDS-PAGE under reducing conditions and stained with CBB. The amount of band 3 protein was determined by densitometry. Data shown are the representative results of three determinations.

the isolated membrane was performed in the presence of proteinase inhibitors. Serine proteinase inhibitors DFP and PMSF, a metalloproteinase inhibitor EDTA, a calpain inhibitor leupeptin, and an acid proteinase inhibitor pepstatin A were used. SDS-PAGE and subsequent CBB staining of the incubation mixture of the membrane is shown in Fig. 5, and the amount of band 3 protein determined by the densitometry is shown in Table 2. The increased degradation of band 3 protein during the incubation of the membrane was inhibited efficiently by DFP (Fig. 5, lane c) and PMSF (lane d), partially by EDTA (lane e), little by leupeptin (lane f) and papstatin A (lane g), and almost completely by the combination of all of these inhibitors (lane h). The results indicate that band 3 protein in the membrane from the oxidized erythrocytes was susceptible to the membrane-bound serine- and metalloproteinases.

4. Discussion

It has been demonstrated that ATP-independent proteolytic system selective to oxidatively damaged proteins exists in the extracts of matured erythrocytes [3–6]. The system may prevent the formation and accumulation of the cytoplasmic proteins damaged by oxidative stress, and this system has been referred to as secondary antioxidant defense system [1]. In addition to the cytoplasmic secondary antioxidant defense system, it was found in the present study that the oxidation of erythrocytes resulted in the increased susceptibility of membrane proteins, i.e., band 3 protein and spectrin, to the membrane bound serine- and

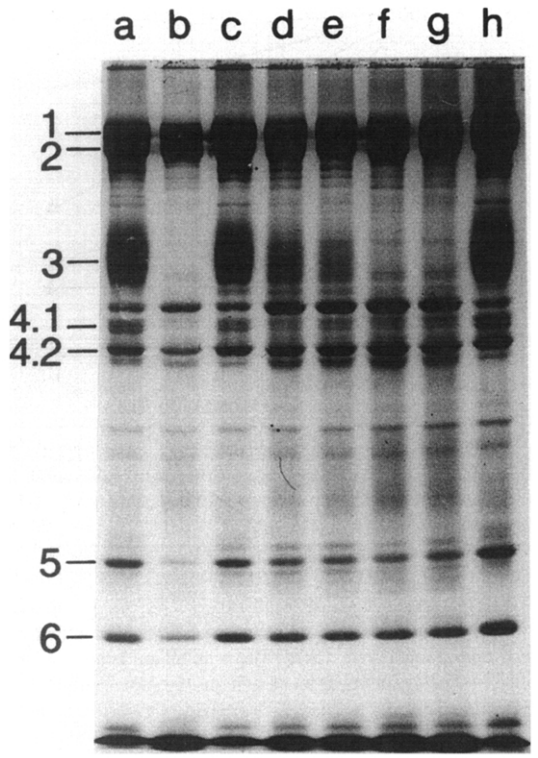

Fig. 5. SDS-PAGE of membrane from the X/XO/Fe^{3+}-oxidized erythrocytes after the 2nd incubation in the presence of proteinase inhibitors. Cell membrane isolated from erythrocytes treated with X/XO/Fe^{3+} (1 mM/10 mUml^{-1}/0.1 mM) at 37°C for 3 h (lane a) was incubated at 37°C for 6 h in the presence of α-tocopherol and no proteinase inhibitor (lane b), 1 mM DFP (lane c), 0.2 mM PMSF (lane d), 1 mM EDTA (lane e), 10 μg/ml leupeptin (lane f), 50 μM pepstatin A (lane g) and all together (lane h). The mixture was subjected to SDS-PAGE and stained with CBB.

metalloproteinases. This was shown by the direct SDS-PAGE of the oxidized erythrocytes, and more clearly by the electrophoresis after the 2nd incubation of the isolated cell membrane. The conditions for the cell oxidation were mild enough, because deformability and osmotic fragility of the cells and the amino acid composition of the membrane proteins were unchanged. However, it was evident that the cells were oxidatively damaged, because the thiobarbituric acid-reactive substances in the incubation medium of the cells as a marker of lipid peroxidation were slightly increased and tritium was significantly incorporated into the membrane proteins upon reduction with tritiated borohydride.

Table 2
The effect of proteinase inhibitors on the band 3 degradation in the 2nd incubation of the membrane isolated from X/XO/Fe^{3+}-oxidized erythrocytes

Inhibitor	Amount (%) of band 3 protein
Before the 2nd incubation	100
After the 2nd incubation	
None	24.8
DFP 1 mM	73.3
PMSF 0.2 mM	53.9
EDTA 1 mM	38.8
Leupeptin 10 μg/ml	27.4
Pepstatin A 50 μM	24.1
All together	89.9

The amount of band 3 protein on the SDS-PAGE gel (Fig. 5) was determined by densitometry, and amount (in %) of band 3 protein in the 2nd incubation was calculated against the amount before the 2nd incubation. Values shown are the representative results of three determinations.

It was found that band 3 protein became most susceptible to degradation by the oxidative stress. As shown in Fig. 1, lane a, unoxidized erythrocytes contained fragments A, B and C of band 3 protein. These fragments may be generated in intact erythrocytes, because presence of band 3 fragments in scenescent erythrocytes has been demonstrated [25,26]. Fragmentation of band 3 protein in erythrocytes in circulation may be caused by oxidative stress and ageing as a result of proteolysis by the membrane-bound proteinases.

Two possible mechanisms for the oxidation-induced increase in susceptibility of erythrocyte membrane proteins can be offered. It has been shown that the oxidation of various proteins makes them susceptible to degradation by proteinases [6,27–29]. Although it is not entirely clear by what mechanism proteins become better substrates for proteolytic degradation, an excellent correlation between increases in denaturation/hydrophobicity and proteolytic degradation has been shown [27,29,30]. Hydrophobicity may hold important clues for the mechanisms by which oxidative damage can increase proteolytic susceptibility. The membrane proteins in the oxidatively damaged erythrocytes may be denatured and become hydrophobic at their cytoplasmic surface, and thus they may become susceptible to the intrinsic membrane proteinases.

Alternative mechanism can be proposed. It has been known that oxidative stress to erythrocytes can cause attachment of cytoplasmic proteins hemoglobin or methemoglobin to the cytoplasmic surface of the membrane [15,31]. By oxidative stress it is possible that cytoplasmic proteinases becomes adherent to the cytoplasmic surface of the membrane. Hence, the increased susceptibility can be derived from the increased adherence of the cytoplasmic proteinase to the membrane.

In order to elucidate whether the oxidation-induced increase in the susceptibility of the membrane proteins to membrane-bound proteinases is due to the increased membrane protein denaturation/hydrophobicity or increased adherence of cytoplasmic proteinases to the membrane, it is necessary to purify and identify the proteinases involved in the degradation. So far, it has been shown that erythrocyte cytoplasmic proteinases responsible for the degradation of cytoplasmic oxidized proteins may be multicatalytic proteolytic complex composed of eight distinct polypeptide subunits with molecular masses of 21–34 kDa whose activity is totally inhibited by EDTA and partially by DFP [8]. Our membrane-bound proteinase activity responsible for membrane protein degradation appears to be

different from the activity of such cytoplasmic multicatalytic proteinases since membrane protein degradation was strongly inhibited by DFP and partially by EDTA. Furthermore, our recent study by radiolabeling of the DFP-reactive membrane proteinases with [^{3}H]DFP suggested that 72 and/or 80 kDa membrane proteins were responsible for the serine proteinase activity (Beppu, M., Ishikawa, T. and Kikugawa, K., unpublished results). Purification of the membrane proteinases is now under way.

The increase in the proteinase-susceptibility of the membrane proteins was greater by the milder oxidation: the susceptibility was reduced by the oxidation with the higher concentrations of the oxidizing agents or by the prolonged oxidation. Three explanations could be made for the observation. Firstly, excessive modification of the membrane proteins may render the proteins less susceptible to the proteolytic degradation. Secondly, excessive damage of the membrane may reduce the ability of cytoplasmic proteinases responsible for the membrane protein degradation to bind. Thirdly, excessive oxidative stress may inactivate proteinases responsible for membrane protein degradation. Measurement of the enzyme activity of the differently oxidized membrane using isolated membrane proteins as substrates would help to resolve this question.

In conclusion, mild oxidation of erythroyctes resulted in the increase of the susceptibility of the membrane proteins, i.e., band 3 protein and spectrin, to the membrane-bound serine- and metalloproteinases. This proteolytic system may protect the cells against the accumulation of oxidatively damaged proteins in the membrane.

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