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Increased Release of Free Fe Ions in Human Erythrocytes During Aging in the Circulation

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We investigated whether free Fe ions were released in erythrocytes during aging process in the circulation. Young and senescent erythrocytes were separated from freshly drawn human blood by Percoll density gradient centrifugation. Two different methods were employed for determination of free Fe ions in erythrocytes, desferrioxamine (DFO) method and bleomycin method. DFO-chelatable Fe ions were detected in whole erythrocytes from 2 donors, and the DFO-chelatable free Fe ion levels in senescent erythrocytes were higher than those in young erythrocytes. Bleomycin-sensitive Fe ions, which was rather lower than DFO-chelatable Fe ions, were also detected in whole erythrocytes from 5 donors, and the free Fe ion levels in senescent erythrocytes were also higher than those in young erythrocytes. Free Fe ions may be derived from oxidative damage of hemoglobin, because treatment of whole erythrocytes or purified oxyhemoglobin with hydrogen peroxide gave increased free Fe ions. The results indicated that free Fe ions were released from erythrocytes during aging process in the circulation. Released free Fe ions would promote oxidative damages of the cells during aging process.

Keywords: Aging; Bleomycin; Desferrioxamine; Erythrocyte; Fe ion; Hemoglobin; Oxidative damage

INTRODUCTION

Human erythrocytes undergo various oxidative modifications during aging process in the circulation. The oxidative modifications occurring during *in vivo* erythrocyte aging include formation of hemichrome and Heinz body,[1,2] fluorescent lipids[3,4] or lipid peroxidation products in membranes,[5] high-molecular weight cross-linked proteins in membranes,[6,7] borohydride-reducing structures in membranes,[8] aggregates of glycoproteins in membranes[9] and glycooxidation products in membranes.[10] Among them a physiologically important oxidative event may be aggregation of glycoproteins of membranes,[9] because to the aggregated sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3 protein on the membrane surface anti-band 3 autoantibody is effectively bound.[11] The aggregates of erythrocyte membrane glycoproteins to which anti-band 3 binds[9,12] and human macrophage directly binds[13] are formed by the *in vitro* mild oxidation of erythrocytes with Fe catalysis. Another important event is the modification of erythrocyte membrane proteins by advanced glycation end products during aging in the circulation,[10] in which process participation of Fe ions has been suggested.

To our knowledge there are no lines of evidence showing the presence of free Fe ions in human normal erythrocytes. If free Fe ions are participating in the *in vivo* oxidation of erythrocytes in the circulation, free Fe ion levels in senescent erythrocytes must be higher than those in young erythrocytes. In the present study, free Fe ion levels in the isolated young and senescent erythrocytes were determined by two different methods. It was found that the free Fe ion levels in senescent erythrocytes were always higher than those in young erythrocytes. Free Fe ions may be originated from oxidation of hemoglobin. Free Fe ions thus

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generated may promote oxidative modifications of erythrocyte membranes during aging process in the circulation.

MATERIALS AND METHODS

Materials

Desferrioxamine monomethanesulfonate (Desferal®, DFO) was obtained from Ciba-Geigy Ltd. (Tokyo, Japan). Calf-thymus DNA (type I), glutathione (reduced form, GSH), glutathione peroxidase (from rat erythrocytes) (EC 1.11.1.9), L(+)-ascorbic acid and sodium ascorbate were obtained from Sigma Chemical Company, (St. Louis, MO, USA). Thiobarbituric acid and Kieselgel 60 were obtained from Merck (Darmstadt, Germany). Chelex 100 resin was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Ultrafree-MC filter unit was obtained from Millipore Company, (Bedford, MA, USA). CM-Sephadex C-50 gel and Percoll were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Bleomycin chloride and other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Human Erythrocytes and Purified Oxyhemoglobin

Human venous blood withdrawn from several healthy donors (including males, females and different ages) and using citrate–phosphate–dextrose as an anticoagulant was used within three days. During the storage no considerable amounts of free Fe ions may be released, because the amount of free Fe ions in erythrocytes stored for three days was same as that in erythrocytes just after collections. Erythrocytes were collected from blood by centrifugation and washed using standard procedures as described elsewhere,[9–13] and resuspended in an appropriate buffer before use. Human normal oxyhemoglobin was purified from the hemolysate of erythrocytes by DEAE-Sephadex column chromatography as described previously.[14] The concentration of hemoglobin in hemolysate was determined by measurement of the absorbance at 523 nm, a molecular extinction coefficient of 7880 and a molecular weight of 64,000.[12] Erythrocyte volume was calculated using mean hemoglobin concentration of 339 g/l erythrocyte.[15]

Separation of Young and Senescent Erythrocytes

Young and senescent erythrocyte were separated by density gradient centrifugation on the Percoll density gradient according to the method described previously.[9,11] The whole erythrocyte suspension in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline [DPBS(−)] (80% hematocrit) was loaded on the top of the Percoll density gradient (density: 1.02–1.14) and centrifuged at 1500*g* for 30 min. The top layer with a density of 1.09 (about 5% of whole erythrocytes) was collected as young erythrocytes, and the bottom layer with a density of 1.12 (about 5% of whole erythrocytes) was collected as senescent erythrocytes. These cells were washed with DPBS(−) to remove Percoll. The cell pellets of whole, young and senescent erythrocytes were used in the following experiments.

In Vitro Oxidation of Whole Erythrocytes and Oxyhemoglobin with Hydrogen Peroxide

For oxidation of erythrocytes, a 10-ml suspension of 10% whole erythrocytes in DPBS(−) was incubated with 1 or 5 mM hydrogen peroxide at 37°C for 1 h. The reaction mixture was centrifuged at 650*g* for 5 min to remove the supernatant, and cell pellet was washed once with DPBS(−) and twice with 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl. For oxidation of oxyhemoglobin, a 1-ml solution of 8.8 mg/ml (0.14 mM) purified oxyhemoglobin in DPBS(−) was incubated with various concentrations of hydrogen peroxide at 37°C for the indicated periods, and the reaction mixture was ultrafiltrated immediately by centrifugation using a Ultrafree-MC tube (Millipore) to remove hemoglobin. The filtrate (80 μl) was mixed with a 140-μl solution of 60 mM GSH and 70 units/ml glutathione peroxidase and incubated at 37°C for 15 min to destroy any remaining hydrogen peroxide. The reaction mixture was stored at −20°C until use as a sample for bleomycin method.

Desferrioxamine (DFO) Method

The assay was performed according to the method described elsewhere.[16,17] Water and buffers were passed through a Chelex-100 resin column to remove contaminating Fe ions. A 0.5-ml suspension of 50% erythrocytes in 0.05 M Tris–maleate buffer containing 0.1 M KCl (pH 7.4) was incubated with 200 μM DFO for 30 min at 37°C. The cells were washed and lysed by addition of 0.5-ml water, and the mixture was centrifuged at 9600*g* for 20 min to obtain hemolysate. The hemolysate (0.2 ml) was passed through a CM-Sephadex C-50 column (7.5 mm × 40 mm) equilibrated with pH 6 to remove hemoglobin. The eluate was applied on a silica gel column (Kieselgel 60) (5 mm × 10 mm), and the column was eluted with water to remove the excess amount of DFO and the buffer salts. The column was made free of water by introduction of methanol. DFO chelatable Fe ions were eluted with a solvent composed of methanol/acetonitrile/acetic acid/25% ammonia (49:49:0.35:0.2, by vol.). The

eluate was evaporated to dryness and redissolved in 0.5 ml of the HPLC solvent, and the amount of the DFO-chelatable Fe ions was measured by HPLC. HPLC was performed on an equipped with a column (4 mm × 250 mm, 5 μm) of Lichrosorb® Si 60 (Merck). The column was eluted with a mobile phase composed of methanol/acetonitrile/acetic acid/10 M NaOH (49:49:0.2:0.1, by vol.) at a flow rate of 1.5 ml/min. The peak of DFO-chelatable Fe ions (ferrioxamine) was detected at 420 nm. The retention time of ferrioxamine was 9 min. The standard samples composed to 0–20 μM $FeSO_4$ and 100 μM DFO in 0.05 M Tris–maleate buffer containing 0.1 M KCl (pH 7.4) were similarly processed. Free Fe ion concentration in erythrocytes was determined by comparing the peak area with that of the calibration curve of the standard.

Bleomycin Method

This assay was performed according to the method described.[18] Water, buffers, the bleomycin solution, the DNA solution and the ascorbate solution used were shaken with Chelex-100 resin at 4°C overnight and then spun at 2000*g* to remove the resin. Erythrocyte pellet (0.5 ml) was lysed by addition of 1-ml water and centrifuged, and the supernatant was ultrafiltered using a Ultrafree-MC tube (Millipore) to remove hemoglobin. To a 5 ml-content plastic tube pretreated with Chelex-100 resin, 500 μl of a calf thymus DNA solution (0.5 mg/ml), 50 μl of a bleomycin chloride solution (50 μg/ml), 100 μl of a $MgCl_2$ solution (5 mM), 130 μl of water, 20 μl of a Tris–HCl buffer (pH 7.4) (20 mM), 100 μl of a sample filtrate or a standard $FeSO_4$ solution (0–20 μM) and finally 100 μl of an ascorbate solution (0.7 mg/ml) were added in order. The mixture was incubated at 37°C for 120 min with continuous shaking. One milliliter of 0.1 M EDTA was added to stop reaction. To 1 ml of the reaction mixture, a 500-μl solution of 1% thiobarbituric acid dissolved in 50 mM NaOH was added, and the mixture was acidified by addition of a 500-μl solution of 3 M HCl. The mixture was heated at 100°C for 15 min. The amount of the produced red pigment was determined by measurement of the absorbance at 532 nm.

Statistical Analysis

Data were analyzed by Student's *t*-test.

RESULTS

Young and senescent human erythrocytes in the circulation were separated from freshly drawn human blood by the Percoll density gradient centrifugation. The amount of free Fe ions in erythrocytes was determined by two methods. DFO method[16,17] including HPLC analysis of ferrioxamine was employed for determination of DFO-chelatable Fe ions. Bleomycin method[18] including thiobarbituric assay for malonaldehyde produced by bleomycin/Fe(II)-dependent degradation of DNA was employed after reduction of free Fe ions into Fe (II) ion.

DFO-chelatable free Fe ions in erythrocytes from 2 healthy donors were measured (Table I). DFO-chelatable Fe ion levels in whole erythrocytes were 8–9 μM, and those in senescent erythrocytes (12–15 μM) were higher than those in young erythrocytes (8–9 μM). Mol% of DFO-chelatable free Fe ions against hemoglobin in whole, young and senescent erythrocytes were estimated to be 0.1–0.3%. Similar free Fe ion levels were obtained when erythrocytes were treated with ascorbate, because Fe(II) ion similarly produces ferrioxamine generating hydroxyl radical.[19,20]

Free Fe ion levels in erythrocytes from 5 healthy donors after reduction into Fe(II) were measured by bleomycin method (Table II). Free Fe ion levels in whole erythrocytes were ranged from 0.2 to 5.2 μM, and those in senescent erythrocytes (6–14 μM) were always higher than those in young erythrocytes (0.3–4.5 μM). Mol% of free Fe ions against hemoglobin in whole, young and senescent erythrocytes were estimated to be 0.1–0.26%. The amounts of free Fe ions obtained by bleomycin method were rather lower than those of DFO-chelatable Fe ions. Free Fe

TABLE I Level of DFO-chelatable free Fe ions in whole, young and senescent erythrocytes

	Fe ions					
	Whole		Young		Senescent	
Donors*	μM in erythrocyte	Mol%† against hemoglobin	μM in erythrocyte	Mol%† against hemoglobin	μM in erythrocyte	Mol%† against hemoglobin
1	8.42	0.16	8.07	0.15	14.99	0.28
2	8.59	0.16	8.76	0.17	12.77	0.24

*Fresh bloods were withdrawn from healthy donor 1 (male aged 23) and donor 2 (male aged 38). †Mol% of DFO-chelatable Fe ions against hemoglobin was calculated.

TABLE II Level of free Fe ions in whole, young and senescent erythrocytes measured by bleomycin method

	Fe ions					
	Whole		Young		Senescent	
Donors*	μM† in erythrocyte	Mol%‡ against† hemoglobin	μM† in erythrocyte	Mol%‡ against hemoglobin	μM† in erythrocyte	Mol%‡ against hemoglobin
1	4.09	0.08	4.13	0.08	11.96	0.23
2	0.24	0.01	0.37	0.01	6.72	0.13
3	2.99	0.06	4.38	0.08	8.43	0.16
4	5.14	0.10	0.38	0.01	13.51	0.26
5	4.40	0.09	1.50	0.03	10.00	0.19

*Fresh bloods were withdrawn from five healthy donors [donor 1 (male aged 55), donor 2 (female aged 46), donor 3 (male aged 39), donor 4 (male and 20), donor 5 (female aged 31)]. †Amounts of Fe ions were determined after reduction into Fe(II) ion by the ascorbate treatment. ‡Mol% of Fe ions against hemoglobin was calculated.

ion levels in erythrocytes were found to be increased in aging process in the circulation as estimated by two different methods. There were no significant differences in the levels of free Fe ions between the erythrocytes obtained from old subjects and those from young subjects, and between males and females.

In order to know whether free Fe ions were originated from hemoglobin, release of free Fe ions from whole erythrocytes and purified oxyhemoglobin by oxidation was investigated. When whole erythrocytes from 2 donors were incubated with 5 mM hydrogen peroxide *in vitro*, the levels of DFO-detectable free Fe ions (Fig. 1a) and bleomycin-sensitive free Fe ions (Fig. 1b) increased about 2-fold. When purified oxyhemoglobin was incubated with hydrogen peroxide *in vitro*, the levels of bleomycin-sensitive free Fe ions increased time- and dose-dependently (Fig. 2). These results demonstrated

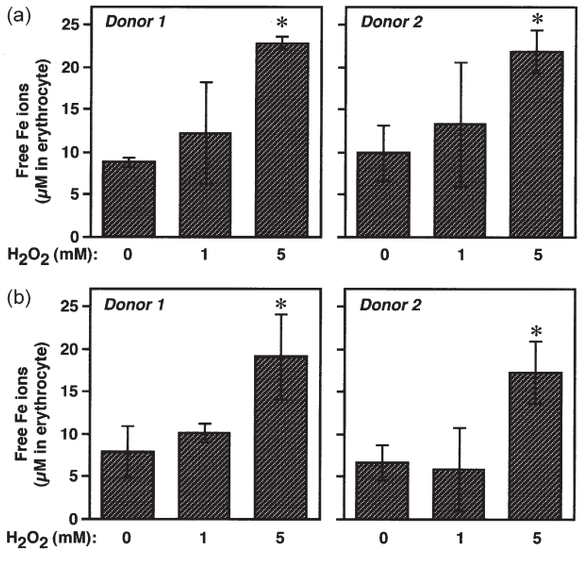

FIGURE 1 Free Fe ion levels in erythrocytes oxidized with hydrogen peroxide *in vitro*. Fresh bloods were withdrawn from healthy donor 1 (male aged 20) and donor 2 (male aged 39). A 10-ml suspension of 10% whole erythrocytes in DPBS(−) was incubated with 1 or 5 mM hydrogen peroxide at 37°C for 1 h. After washing the cells, the amount of the free Fe ions in the cells was measured by DFO method (a) and bleomycin method (b) as described under "Materials and Methods" section. The results are expressed as the mean ± SD of triplicate determinations. *Significantly different from the controls at $P < 0.001$.

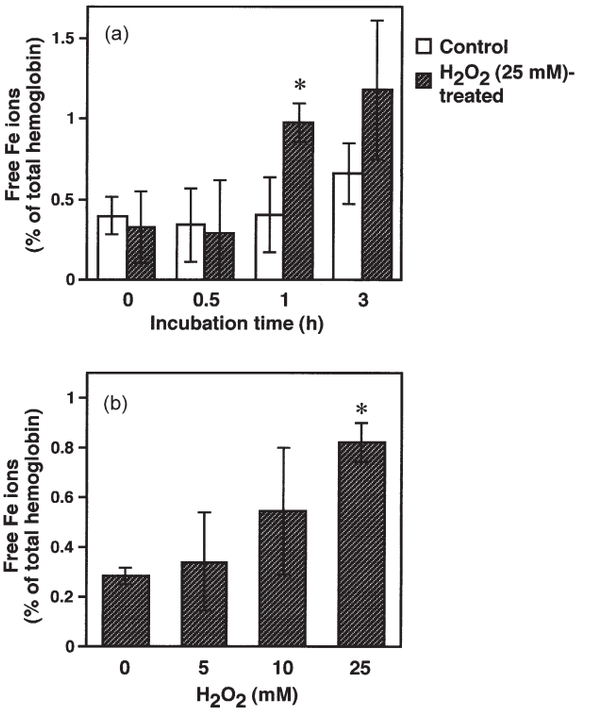

FIGURE 2 Free Fe ions from hemoglobin treated with hydrogen peroxide *in vitro*. A 1-ml solution of 0.14 mM purified oxyhemoglobin in DPBS(−) was incubated with various concentrations of hydrogen peroxide at 37°C. (a) Time course of the release at 25 mM hydrogen peroxide. (b) Dose-dependence of hydrogen peroxide in incubation for 1 h. The reaction mixture was ultrafiltrated to remove hemoglobin. The filtrate (80 μl) was mixed with a 140-μl solution of 60 mM GSH and 70 units/ml glutathione peroxidase and incubated at 37°C for 15 min to decompose any remaining hydrogen peroxide. The amount of free Fe ions was measured by bleomycin Method as described under "Materials and Methods" section. The results are expressed as the mean ± SD of triplicate determinations. *Significantly different from the controls at $P < 0.001$.

that free Fe ions could be released from hemoglobin in erythrocytes by oxidative stress.

DISCUSSION

In the present study, we determined free Fe ions in young and senescent erythrocytes by two different methods. In the measurement of DFO-chelatable free Fe ions, Fe(III) ion is trapped as ferrioxamine, and Fe(II) ion is also trapped as ferrioxamine generating hydroxyl radical.[19,20] Free Fe(II) ion levels after reduction of free Fe ions on ascorbate treatment were similar. In bleomycin assay, because free Fe(II) ion can be measured selectively by binding to bleomycin to generate hydroxyl radical and produces malonaldehyde from DNA molecules,[17] free Fe ions must have been reduced into Fe(II) ion prior to the determination. The free Fe ion levels determined by bleomycin method seemed rather lower than those determined by DFO method. The results obtained by bleomycin method are more reliable because DFO method may measure some artificial free Fe ions owing to complex and tedious procedures. The levels of free Fe ions in whole erythrocytes thus determined were less than 10 μM depending on the donors of erythrocytes. The levels were lower than about 0.2 mol% of Fe in hemoglobin.

It was found that free Fe ion levels in senescent erythrocytes were always higher than those of young erythrocytes of each of the donors. Increased free Fe ion levels in senescent erythrocytes may promote further oxidative damages in senescent erythrocytes. Participation of free Fe ions in oxidative damages in the *in vivo* circulation can be readily supposed based on our earlier observations showing that lipid peroxidation in membranes,[5] and aggregates of erythrocyte membrane glycoproteins to which anti-band 3 bind[9,12] and human macrophage direct bind[13] are induced by the *in vitro* mild oxidation of erythrocytes with Fe catalysis, and also on the observation showing that *in vitro* incubation of human erythrocytes in phosphate buffer under aerobic conditions released free Fe ions accompanying membrane protein oxidation.[21]

Increased free Fe ion levels have been observed in human erythrocytes with abnormal hemoglobins, sickle cell[22] and β-thalassemia cells.[23] High affinity of Fe ions to membrane aminophospholipids to promote oxidative damages in the membranes is demonstrated.[22] Moreover, animal studies have shown the free Fe ion release from mouse erythrocytes in allyl alcohol intoxication.[24] *In vitro* exposure of mouse erythrocytes to oxidants such as phenylhydrazine, divicine and isouramil releases DFO-chelatable free Fe ions.[25]

It is known that ferritin releases free Fe(II) ion by reaction with superoxide,[26] and ferritin is associated with membranes of sickle erythrocytes[22] that have unusual properties suggestive of oxidative modification of cellular components.[27–30] Hence, it is possible that free Fe ions in senescent erythrocytes are released from ferritin under oxidative stress. However, because treatment of whole erythrocytes and purified oxyhemoglobin with hydrogen peroxide increased free Fe ion levels, oxidative damage of hemoglobin may have occurred in senescent erythrocytes to increase free Fe ion levels.

In conclusion, free Fe ion levels in senescent erythrocytes were found higher than those in young erythrocytes. Free Fe ions may be originated from oxidative damage of hemoglobin, and would promote oxidative damages of erythrocyte membranes in the circulation.

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