Evidence for Accumulation of Lipid Hydroperoxides during the Aging of Human Red Blood Cells in the Circulation

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Young and old human red blood cells (RBC) were separated from freshly collected human blood by Percoll density gradient centrifugation. The amounts of lipid peroxidation products in the lipid fractions of these RBC were determined by high performance liquid chromatography-chemiluminescence, thiobarbituric acid and LPO-586 methods. The levels of phosphatidylcholine and phosphatidylethanolamine hydroperoxides in old RBC were higher than in young RBC. The products reflecting the advanced stage of lipid peroxidation, malonaldehyde (free and bound forms), 4-hydroxyalkenals (free and bound forms) and other aldehydes, were also higher in old RBC than in young RBC. The levels of these lipid peroxidation products increased when whole RBC were mildly oxidized with ferric ion. These results indicate that oxidative damage of RBC in the circulation takes place during the aging process, and lipid hydroperoxides and other lipid peroxidation products accumulate in old RBC.

Key words old red blood cell; phosphatidylcholine hydroperoxide; phosphatidylethanolamine hydroperoxide; thiobarbituric acid reactive substance; malonaldehyde; 4-hydroxyalkenal

Originating in the bone marrow, human red blood cells (RBC) circulate for an average of 120 d and the aged RBC are then finally removed from the blood by macrophages of the reticuloendothelial system.^{1,2)} RBC aging in the circulation is associated with various changes³⁾: decreased cell deformability,⁴⁾ increased cell density,⁵⁾ decreased levels of ATP, phospholipids and cholesterol,⁵⁾ loss of activity of enzymes including acetylcholinesterase⁶⁾ and glucose-6-phosphate dehydrogenase,⁷⁾ and release of sugars from the cell membrane.^{6,8)} Because RBC carry oxygen, they may be susceptible to oxidative stress in the circulation as an initial event in the aging process. There are several lines of evidence suggesting that oxidative damage is involved in cell aging. For instance, membrane proteins are polymerized,^{9–12)} fluorescent phospholipids are produced^{9,10,13)} and carbonyl groups are produced in proteins¹⁴⁾ during cell aging.

In the present investigation, we measured and compared the levels of phospholipid hydroperoxides together with those of products of the advanced stage of lipid peroxidation in young and old RBC fractionated by Percoll density gradient centrifugation in order to investigate if lipid hydroperoxides accumulate in the cell membrane during aging.

MATERIALS AND METHODS

Materials Phosphatidylcholine (egg yolk, PC) and phosphatidylethanolamine (egg yolk, PE) were obtained from Nippon Fats and Oil Liposome Company (Tokyo, Japan) and Taiyo Kagaku Company (Tokyo, Japan), respectively. Thiobarbituric acid (TBA) was obtained from Merck. LPO-586 (*N*-methyl-2-phenylindole) was obtained from Bioxytech S.A. (Bonneuil sur Marne, France). Cytochrome c and luminol were purchased from Sigma Chemical Company (St. Louis, MO) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Standard phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) were prepared as previously described.¹⁵⁾ PCOOH

and PEOOH obtained showed an absorption maximum at 234 nm (methanol) and a peroxide value of 2230 and 820 neq/mg, respectively.

Human Blood and RBC Human venous blood was withdrawn from healthy donors into acid-citrate-dextrose tubes at a Japanese Red Cross blood transfusion center. Collected blood was stored at 4 °C and used within 4 d. RBC were collected from 40 ml of blood and washed using standard procedures.

Young and Old RBC RBC were then separated into young and old RBC by density gradient centrifugation on a Percoll gradient.^{16,17)} The cells with a density of 1.09 (about 5% of the total RBC) were collected as young RBC, and the cells with a density of 1.12 (about 5% of the total RBC) were collected as old RBC.

In Vitro Oxidation of RBC RBC were mildly oxidized as described previously.¹⁸⁾ A suspension of whole RBC in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (40% hematocrit) was mixed with an equal volume of 3.4/0.2 mM ADP/ferric ion in isotonic saline and the cells were incubated under aerobic conditions at 37 °C for 3 h. RBC were incubated similarly in saline as control RBC.

Isolation of the Lipid Fraction of RBC To 1.0 ml of the RBC pellet 1.0 ml water was added. A 20- μ l aliquot of the lysate was diluted with 2.0 ml water and the mixture centrifuged at 9600 $\times g$ for 20 min. Absorbance of the supernatant at 523 nm was measured, and the hemoglobin concentration was calculated using a molecular extinction coefficient of 7880 and a molecular weight of 64000.¹⁹⁾ RBC volume was calculated using a mean hemoglobin concentration of 339 g/l RBC.²⁰⁾ The lysate was extracted with a mixture of 11 ml isopropanol and 7 ml chloroform.²¹⁾ The organic layer collected (18 ml) was filtered through a glass wool column and a 16-ml aliquot was evaporated to dryness at below 30 °C *in vacuo*. It was found that more than 70% of a large amount of PCOOH and PEOOH (290 μ eq) added to 0.4 ml RBC was recovered by this extraction procedure.

High Performance Liquid Chromatography (HPLC)-

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Chemiluminescence Assay PCOOH and PEOOH were determined by the HPLC-chemiluminescence method developed by Miyazawa *et al.*^{22,23} The lipid fraction of RBC was dissolved in 5.0 ml chloroform-methanol (2:1, v/v) and washed twice with 1 ml 0.05 M KCl to obtain 3.5 ml of the organic layer. A 3.0-ml portion of the organic layer was evaporated to dryness at below 30 °C and redissolved in 50 μ l chloroform-methanol (1:9, v/v). A 40- μ l portion of this extract was subjected to HPLC using a Hitachi L-6200 Intelligent Pump equipped with a column (4.6 $\times$ 250 mm) of YMC A-012 S-5 120 A SIL (Yamamura Chemical Laboratories, Ltd., Kyoto, Japan) and a mobile phase composed of chloroform-methanol (1:9, v/v) at a flow rate of 1.1 ml/min. A solution composed of 10 μ g/ml cytochrome c and 1 μ g/ml luminol in 50 mM borate buffer (pH 9.3) was mixed with the eluate at a flow rate of 1.0 ml/min and the chemiluminescence generated was detected using a JASCO 825-CL Intelligent CL detector. The chemiluminescent peaks due to PEOOH and PCOOH exhibited retention times of 3.0 and 6.8 min, respectively. The amounts (neq) of PCOOH and PEOOH were determined by comparing their peak areas with a calibration curve of the peak areas of standard amount of PCOOH and PEOOH (0–1000 neq).

TBA Assay The TBA assay was performed in the presence and absence of ethylenediaminetetraacetic acid (EDTA).²⁴ The lipid fraction of RBC was suspended in 900 μ l water. To 200 μ l of this suspension, 650 μ l of a mixture of 0.20 ml 5.2% sodium dodecylsulfate in water, 50 μ l 0.8% butylated hydroxytoluene in glacial acetic acid, 1.50 ml 0.8% TBA solution in water, 1.70 ml water (or 2 mM EDTA solution) and 150 μ l 3.3 M acetate buffer (pH 3.5) were added. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 60 min. The red pigment produced was extracted into 1.0 ml of a mixture of 1-butanol-pyridine (15:1, v/v) by shaking vigorously and centrifuging at 320 $\times g$ for 10 min. The absorbance of the organic layer at 532 nm was measured. A solution of 20 μ M tetramethoxypropane was similarly treated as a reference standard.

LPO-586 Assay The LPO-586 assay was carried out according to the reagent manual (Bioxytech S.A.). This involved the lipid fraction of RBC being suspended in 900 μ l water. To 200 μ l of the suspension, 650 μ l of a solution of *N*-methyl-2-phenylindole, and 150 μ l of a solution of methane sulfonic acid (or concentrated HCl solution) were added. The mixture was heated at 45 °C for 40 min (or 60 min) and the absorbance of the mixture at 586 nm was measured immediately. Standard solutions of 10 mM tetramethoxypropane in Tris buffer or 10 mM 4-hydroxynonenal in acetonitrile were similarly treated.

RESULTS

Young and old RBC were separated from the RBC of three healthy donors by Percoll density gradient centrifugation.^{16,17} The lipid fraction of the RBC was extracted with a mixture of isopropanol-chloroform (11:7, v/v).²¹ By this procedure more than 70% of added PCOOH and PEOOH was recovered. The amounts of PCOOH and PEOOH in the lipid fractions of RBC were

Fig. 1. HPLC Chromatogram of the Lipid Fractions of Whole, Young and Old RBC from Donor 1 (A) and Whole, Control and Oxidized RBC from Donor 4 (B) Detected by Chemiluminescence Generated in Contact with Cytochrome c and Luminol

determined by the HPLC-chemiluminescence method developed by Miyazawa *et al.*^{22,23} The representative chromatograms of the lipid fractions of whole, young and old RBC revealed peaks corresponding to PCOOH and/or PEOOH (Fig. 1A). The levels, nmol/g hemoglobin (or nmol/l RBC), of PCOOH and PEOOH in whole, young and old RBC of three different donors (Table 1, 1st column) indicated that the levels of both PCOOH and PEOOH in old RBC were higher than in young RBC. These results indicate that phospholipid hydroperoxides accumulate during cell aging in the circulation.

The TBA assay of the lipid fractions of RBC in the presence and absence of EDTA was performed.²⁴ In both assays, BHT was added in order to avoid lipid peroxidation during the assay. During the assay, acid-labile bound forms of aldehydes may generate free forms of the aldehydes to be determined. In the assay without EDTA, thiobarbituric acid reactive substances (TBARS) consisting of malonaldehyde, alkenals and alkadienals (free and bound forms) are determined while in the assay with EDTA, TBARS consisting of malonaldehyde (free and bound forms) alone are determined.²⁴ Absorption spectra of the assay mixtures in the presence and absence of EDTA (Fig. 2A) showed that the spectra were exclusively due to a red pigment with a characteristic absorption maximum at 532 nm. The levels, nmol/g hemoglobin (or nmol/l RBC), of TBARS reflecting the relative levels of total aldehydes, the absolute level of malonaldehyde (free and bound forms) and the relative levels of other aldehydes including alkenals, alkadienals and 4-hydroxyalkenals (free

Table 1. Levels, nmol/g Hemoglobin (or nmol/l RBC), of Lipid Peroxidation Products in RBC

Assay method	HPLC-chemiluminescence		TBA assay ^{a)}			LPO-586 assay ^{b)}		
Product determined	PCOOH	PEOOH	TBARS due to			Malonaldehyde + 4-hydroxy-alkenals	Malonaldehyde	4-Hydroxy-alkenals
			Total aldehydes	Malonaldehyde	Other aldehydes			
Donor 1 (Blood group O)								
RBC Whole	1.16 (394)	0.33 (113)	29.7 (10080)	6.4 (2160)	23.4 (7920)	12.9 (4380)	7.5 (2540)	5.4 (1840)
Young	1.26 (428)	0.26 (87)	3.5 (1180)	1.5 (510)	2.0 (670)	4.9 (1660)	3.3 (1100)	1.6 (560)
Old	1.46 (494)	0.54 (164)	39.1 (13270)	11.4 (3870)	27.7 (9400)	24.7 (8390)	17.3 (5870)	7.4 (2520)
Donor 2 (Blood group O)								
RBC Whole	0.30 (100)	0.32 (107)						
Young	0.33 (111)	0 (0)						
Old	0.41 (139)	0.62 (209)						
Donor 3 (Blood group O)								
RBC Whole	0.15 (50)	0.27 (93)	33.6 (11390)	16.6 (5640)	17.0 (5750)	24.4 (8260)	16.1 (5470)	8.3 (2790)
Young	0 (0)	0.20 (69)	30.7 (10400)	14.6 (4930)	16.1 (5470)	20.0 (6770)	12.5 (4230)	7.5 (2540)
Old	0.19 (65)	0.36 (121)	43.1 (14620)	26.5 (8990)	16.6 (5630)	33.1 (11230)	20.4 (6930)	12.7 (4300)
Donor 4 (Blood group O)								
RBC Whole	0.04 (13)	0.13 (44)	13.1 (4450)	2.01 (680)	11.1 (3770)	9.11 (3090)	5.94 (2010)	3.17 (1080)
Control	0.19 (65)	0.67 (227)	14.1 (4780)	1.85 (630)	12.3 (4150)	8.72 (2960)	5.07 (1720)	3.65 (1240)
Oxidized	2.27 (768)	5.07 (1717)	44.5 (15090)	6.09 (2070)	38.4 (13020)	18.2 (6180)	9.24 (3130)	8.98 (3050)

a) The levels of TBARS obtained from the absorbance with EDTA reflect the relative levels of total aldehydes. The levels of TBARS obtained from the absorbance without EDTA reflect the absolute level of malonaldehyde (free and bound forms). The levels of TBARS obtained by subtracting the absorbance with EDTA from the absorbance without EDTA reflect the relative but not absolute levels of other aldehydes including alkenals, alkadienals and 4-hydroxyalkenals (ref. 24). b) The sum of the levels of malonaldehyde (free and bound forms) and 4-hydroxyalkenals (free and bound forms) was obtained from the absorbance in the methane sulfonic acid reaction. The level of malonaldehyde (free and bound forms) was obtained from the absorbance in the concentrated HCl reaction. The level of 4-hydroxyalkenals (free and bound forms) was obtained by subtracting the absorbance in concentrated HCl from the absorbance in methane sulfonic acid.

Fig. 2. Absorption Spectra of the Reaction Mixtures of Whole, Young and Old RBC from Donor 1 (A) and Whole, Control and Oxidized RBC from Donor 4 (B) in the TBA Assay with and without EDTA

and bound forms) in whole, young and old RBC of two different donors are listed in Table 1, 2nd column. The levels of these three kinds of aldehyde in old RBC were much higher than in young RBC. Furthermore, the levels of these aldehydes in old RBC were much higher than those of PCOOH and PEOOH.

The LPO-586 assay of the lipid fractions of RBC was

performed. Since this assay with *N*-methyl-2-phenylindole has not been generally employed in lipid peroxidation studies, before applying it to RBC lipids, we investigated its usefulness for measuring lipid peroxidation products of advanced stage. While this assay gave almost no color with alkanals, alkenals and alkadienals, a violet pigment absorbing at 586 nm was produced from

Fig. 3. Absorption Spectra of the Reaction Mixtures of Whole, Young and Old RBC from Donor 1 (A) and Whole, Control and Oxidized RBC from Donor 4 (B) in the LPO-586 Assay in Methane Sulfonic Acid and Concentrated HCl

malonaldehyde (free and bound forms) and 4-hydroxyalkenals (free and bound forms) in the methane sulfonic acid reaction and from malonaldehyde (free and bound forms) alone in the concentrated HCl reaction. Calibration curves of both positive aldehydes were linear which showed that the efficiency of color development from both aldehydes as similar. When this assay was applied to RBC lipids, a characteristic absorption maximum at 586 nm was observed in the methane sulfonic acid and the concentrated HCl reactions of whole, young and old RBC (Fig. 3A). The levels, nmol/g hemoglobin (or nmol/l RBC), of malonaldehyde (free and bound forms) and 4-hydroxyalkenals (free and bound forms) in whole, young and old RBC of two different donors are listed in Table 1, 3rd column. The levels of these aldehydes in old RBC were much higher than in young RBC. The levels of these aldehydes in old RBC were much higher than PCOOH and PEOOH.

The levels of the lipid peroxidation products in RBC oxidized *in vitro* were determined for comparison. Whole RBC were incubated with ADP/ferric ion (oxidized RBC)¹⁸⁾ or saline alone (control RBC). The levels of PCOOH and PEOOH, TBARS in the TBA assay, malonaldehyde and 4-hydroxyalkenals in the LPO-586 assay in oxidized RBC were much higher than in the control RBC (Figs. 1B, 2B and 3B, Table 1). While the levels of aldehydes in oxidized RBC were comparable with those in old RBC, the levels of hydroperoxides were much higher than in old RBC (Table 1).

DISCUSSION

Using three assay methods based on different principles, the accumulation of advanced stage phospholipid hydroperoxides and lipid peroxidation products in *in vivo* aged RBC was demonstrated. The HPLC-chemiluminescence method^{22,23)} can determine phospholipid hydroperoxides generated in the early stage of lipid peroxidation, and the TBA²⁴⁾ and the LPO-586 assays can determine free and bound forms of malonaldehyde, 4-hydroxyalkenals and other aldehydes generated in the advanced stage

of lipid peroxidation. Bound forms of these aldehydes may consist of various types of compounds including hydroperoxides. Accumulation of lipid peroxidation products in the aged RBC strongly suggests that the aged RBC have been exposed to oxidative stress in the circulation.

The presence of PCOOH (51–196 nmol/l RBC) and PEOOH (81–224 nmol/l RBC) in human RBC has been demonstrated by Miyazawa *et al.*²⁵⁾ The levels of hydroperoxides in whole RBC obtained in the present study were not very different from those of previous observations. It is interesting to note that these hydroperoxides remain for a long time in RBC during their circulation in the blood. However, a substantial amount of hydroperoxides may be converted into aldehydic compounds in the aging process because the levels of free and bound forms of these aldehydes in aged RBC were much higher than those of hydroperoxides.

While Hochstein and Jain^{9,10,13)} observed the accumulation of fluorescent chromolipids and high-molecular-weight protein polymers in the membrane of aged RBC, which was suggested to be due to malonaldehyde generated during lipid peroxidation, Jain¹³⁾ did not observe TBARS accumulation during cell aging. Our present study demonstrated that TBARS, both in the presence and absence of EDTA, increased during cell aging. Our recent studies have demonstrated that a wide variety of aldehydes other than malonaldehyde generated by lipid peroxidation can produce fluorescent and high-molecular-weight protein polymers in the membrane of RBC *in vitro*.^{26,27)} Aldehydic compounds generated by lipid peroxidation in the aged RBC may cause damage of structural proteins and enzymes and thus may change the physicochemical properties of the cells.

With regard to the final event of RBC aging, it is believed that autologous IgG binds to the aged RBC to cause Fc receptor-mediated phagocytosis by macrophages.^{28–30)} Our recent studies have demonstrated that anti-band 3 IgG binds to band 3 glycoprotein on the surface of both aged cells¹⁷⁾ and cells exposed to oxidative stress *in vitro*.^{18,19)} Furthermore, the binding sites of band 3 glycoprotein on the aged and oxidized cells are the same

sialylated poly-*N*-acetyllactosaminyl sugar chains. It is suggested that molecules of band 3 glycoprotein are forced to form clusters, which permit high affinity binding of anti-band 3 IgG to the sugar chains, by a series of reactions induced by oxidative stress.¹⁷⁾ Oxidative stress may be regarded as an initial event in the RBC aging process and may be of direct relevance to the topological alteration of band 3 glycoprotein and the increased affinity for the antibody.

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