Biochimica et Biophysica Acta 937 (1988) 205–210
Elsevier

BBA 73829

Evidence for membrane lipid peroxidation during the in vivo aging of human erythrocytes

Sushil K. Jain

Department of Pediatrics, Louisiana State University School of Medicine, Shreveport, LA (U.S.A.)

(Received 7 October 1987)

Key words: Lipid peroxidation; Aging; (Human erythrocyte membrane)

This study has examined the occurrence of lipid peroxidation in in vivo aged human erythrocyte membranes. Erythrocytes of various ages were separated on discontinuous stractan density gradients. Three erythrocyte fractions were analyzed: (I) Light — erythrocytes staying between stractan densities 1.053 and 1.043 g/ml, (II) predensest — erythrocytes staying between stractan densities 1.081 and 1.111, and (III) densest — erythrocytes passing stractan density 1.111. Peroxidative lipid damage of erythrocytes was assessed by measuring lipid extract fluorescence, by lipid thin-layer chromatography for the presence of adduct of phosphatidylserine (PS), phosphatidylethanolamine (PE) and malondialdehyde, and by thiobarbituric acid-reactivity. Fractions I, II and III contained, respectively, 0.2 ± 0.1 (S.E.), 1.1 ± 0.1 and 1.5 ± 0.1 of phospholipid-malondialdehyde adduct (percent of total phospholipids), and relative lipid fluorescence 22.5 ± 0.8, 29.3 ± 0.5, and 33.4 ± 0.8 per ml packed cells, respectively. Thiobarbituric acid-reactivity of erythrocytes in various fractions was similar. Untreated densest erythrocytes contained significantly reduced PS ($12.9 \pm 0.5\%$), in contrast to light erythrocytes ($16.1 \pm 0.1\%$) and increased PC (31.2 ± 0.3 versus $27.8 \pm 0.8\%$ of the total phospholipid). This study provides evidence for significant lipid peroxidative damage in the erythrocyte membrane during aging in vivo.

Introduction

A human erythrocyte survives in the circulation for nearly 120 days, after which it is removed by the narrow splenic sinusoids or the reticuloendothelial system. During the life span of the erythrocyte, its membrane undergoes changes in lipid and protein content, enzyme activity, ion permeability, size, and deformability [1–3]. The present study has examined the occurrence of membrane peroxidative lipid damage in human erythrocytes of varying ages (density) separated on stractan density gradients.

Materials and Methods

1 unit of blood freshly drawn in acid/citrate/dextrose as anti-coagulant was obtained each time from the local blood bank for every single experiment. The blood was screened for the absence of sickle cell trait and for Rh^+ ve. The blood was processed within 18 h of collection from the donors, during which time it was stored at 4°C. Blood was filtered through cotton wool to remove leukocytes. Erythrocytes were suspended to about 30% hematocrit in buffered saline containing glucose and potassium (7.808 g NaCl + 0.373 g KCl + 2.302 g Na_2HPO_4 + 0.194 g NaH_2PO_4 + 1.0 g

Abbreviations: MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; MDA, malondialdehyde; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.

Correspondence: S.K. Jain, Department of Pediatrics, LSU Medical Center, 1501 Kings Highway, Shreveport, LA 71130, U.S.A.

glucose dissolved in 1000 ml distilled water, pH adjusted to 7.4).

Separation of erythrocytes into varying ages (density)

Isolation of varying aged normal erythrocytes using density as a criterion has been a widely used method in the study of erythrocyte aging since Piomelli et al. [4] documented strong correlation between density and aging of erythrocytes containing [^{14}C]glycine and ^{59}Fe as markers. In the present study, varying aged erythrocytes were separated by layering on the top of discontinuous density gradients of stractan (Sigma). Four layers (8 ml each) of stractan with densities 1.043, 1.053, 1.081 and 1.111 g/ml were placed upon a dense cushion of 1.165 g/ml to prevent packing of the cells against the bottom of the centrifuge tube. On the top, 8 ml of 30% cell suspension were layered. The gradient was centrifuged at 4°C in a Sorvall RC-5B centrifuge at 11 000 rpm for 30 min using HB-4 swinging rotor. Three erythrocyte fractions were isolated with a Pasteur pipette: (I) light — cells staying between stractan densities 1.053 and 1.043, (II) pre-densest — cells passing stractan density 1.081, and (III) densest — cells passing stractan density 1.111. The lightest cell fraction rich in white blood cells and reticulocytes present above the light fraction was discarded (Fig. 1). Erythrocyte fractions were washed four times after 1 : 20 dilution with the buffer and centrifugation at 2000 rpm for 7 min in a refrigerated centrifuge to remove stractan. Mean cell volume, and mean cell hemoglobin concentration of the washed erythrocyte fractions were measured with an electronic Coulter counter.

FRACTION	MCHC (g%)	MCV (μm^3)
DISCARDED LIGHT, #1	32.1 ± 0.3	91.8 ± 1.6
DISCARDED PREDENSEST, #2	34.3 ± 0.4	88.6 ± 0.8
DENSEST, #3	34.9 ± 0.2	84.8 ± 1.1

Fig. 1. Fractionation of varying aged erythrocytes on the discontinuous stractan density gradients. Values are mean ± S.E. of five experiments. Mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were measured using an electronic counter.

Treatment with malondialdehyde

Unfractionated erythrocyte suspensions (5% hematocrit) in phosphate-buffered saline were treated with various concentrations of malondialdehyde in 25-ml Erlenmeyer flasks for 24 h in a shaking water bath at 37°C. Malondialdehyde was freshly prepared by acid-hydrolysis of malonaldehyde bis(dimethyl acetal) (Aldrich) by mixing 0.2 ml of the latter compound with 0.8 ml of 0.15 M sodium chloride and 0.03 ml of 6 M HCl and then vortexing. At the end of incubation with malondialdehyde, erythrocytes were washed twice with cold 0.15 M sodium chloride before the lipid extraction. All incubations of erythrocytes with malondialdehyde contained 10 μl of penicillin/streptomycin per ml cell suspension to vitiate any microbial growth during overnight incubations. Penicillin/streptomycin was made up of 300 mg penicillin G + 500 mg streptomycin dissolved in 10 ml distilled water.

Measurement of lipid peroxidation

Membrane peroxidative damage in the erythrocytes was determined by the following methods.

Thiobarbituric acid reactivity. Malondialdehyde, an end product of fatty acid peroxidation reactions, can react with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm. Thiobarbituric acid reactivity of erythrocyte was determined by the modified method of Stock and Dormandy [5]. For this purpose, 0.2 ml of packed cells was suspended in 0.8 ml phosphate-buffered saline made up of 8.1 g NaCl + 2.302 g Na_2HPO_4 + 0.194 g NaH_2PO_4 in 1000 ml distilled water (pH 7.4). To this, 0.5 ml of 30% trichloroacetic acid was added. Tubes were vortexed and allowed to stand in ice for at least 2 h. Tubes were centrifuged at 2000 rpm for 15 min. 1 ml each of the supernatant was transferred into another tube. To this was added 0.075 ml of 0.1 M EDTA and 0.25 ml of 1% thiobarbituric acid/0.05 M NaOH. Tubes were mixed and kept in a boiling water bath for 15 min. Absorbance was read at 532 nm after tubes were cooled to room temperature. EDTA was added to chelate any metal, such

as iron or copper, in the extract, which, otherwise, may initiate lipid peroxidation during boiling and result in falsely elevated thiobarbituric acid reactivity. An addition of EDTA to standard malondialdehyde did not have any effect on its color development with the thiobarbituric acid.

Lipid fluorescence. Lipid fluorescence was measured in the lipid extracts of red blood cells. For this purpose, 0.4 ml of packed red cells was suspended into 3 ml of isopropanol and 2 ml of chloroform, as described by Rose and Oklander [6]. Tubes were vortexed and allowed to stand at room temperature for about 30 min, at which time these tubes were again vortexed and then centrifuged at 2000 rpm for 15 min in a refrigerated centrifuge. The lipid extract, i.e., clear supernatant, from each tube was transferred into another tube. Fluorescence in the lipid extracts was measured using Perkin-Elmer Spectro Fluorometer Model 650-10 at excitation wavelength of 400 nm and emission wavelength of 455 nm. These wavelengths are characteristic of lipid complex formed by the cross-linking of aldehyde groups of malondialdehyde with the amino groups of PS and PE [7,8]. Previous studies have shown a linear correlation between an increase in the lipid extract fluorescence at these excitation and emission wavelengths and the amount of exogenous malondialdehyde treatment in vitro to red blood cells [7,9]. A relative increase in the lipid fluorescence is an indirect index of peroxidation of membrane lipids and malondialdehyde accumulation. Lipid fluorescence values presented in results are the only relative Perkin-Elmer units obtained by setting the spectrofluorometer at band pass of 10 nm each of excitation and emission. Other fluorescent products with different excitation and emission wavelength may also be formed during the peroxidation of membranes [10]. However, wavelengths utilized in this assay are maximas of the predominant peak and represents the cross-linking of malondialdehyde with aminophospholipids [7,9,11].

Phospholipid malondialdehyde adduct. Peroxidative membrane damage also was assessed by determining the amount of heterologous phospholipid-MDA adduct separated by thin-layer chromatography of red cell lipid extracts on silica gel H plates (Silica 60, 0.25 mm thickness, Brinkman Instruments) using the solvent system chloroform/methanol/acetic acid/water (50 : 25 : 8 : 4, v/v). The quantitation of phospholipid-malondialdehyde adduct on TLC gives the amount of malondialdehyde cross-linked between PE and PS, whereas fluorescence in the lipid extracts presumably measures total malondialdehyde cross-linking between PE and PE, PS and PS, and PE and PS, i.e., both homologous and heterologous malondialdehyde adducts [9]. Visualization of various lipid spots on the TLC plate was carried out as described earlier [9]. Phospholipid-phosphorus determination was done by the method of Fiske and SubbaRow [12].

Statistical analyses were carried out using non-paired Student's '*t*'-test.

Results

Fig. 1 illustrates separation of varying aged (density) erythrocytes fractions on discontinuous stractan density gradients. Fractions I, II and III comprised 26, 5 and 3% of the total unfractionated erythrocytes and had mean cell hemoglobin concentration of 32.1 ± 0.3, 34.3 ± 0.4 and 34.9 ± 0.2 g%, and mean cell volume of 91.8 $\pm$ 1.6, 88.6 ± 0.8 and 84.8 ± 1.1 μm^3, respectively.

Fig. 2 illustrates thin-layer chromatography of lipids of erythrocytes of varying density. As can be seen, densest and predensest cells contain a new phospholipid 'adduct', which moved between PE and PS. This new lipid was phosphorus-positive and ninhydrin-negative. A similar lipid spot was formed when erythrocytes were treated in vitro with authentic malondialdehyde. This phospholipid has been characterized as an adduct of PE, PS and malondialdehyde [9]. Quantitation of various phospholipid classes by measuring phospholipid-phosphorus showed that this adduct constitutes 0.2 ± 0.1, 1.1 ± 0.1 and 1.5 ± 0.1% of the total phospholipids, in light, predensest and densest erythrocyte fractions, respectively (Table I). The amount of adduct formed was 0.11 ± 0.09, 0.61 ± 0.05, 1.19 ± 0.15, 1.99 ± 0.21, 3.19 ± 0.18 and 4.16 ± 0.26% of total phospholipids, when unfractionated erythrocytes were incubated in vitro for 24 h in a buffer containing 0, 1, 2, 3, 4 and 5 μmol malondialdehyde per ml, respectively. This suggests that during aging, erythrocytes undergo

TABLE I

PHOPSHOLIPID COMPOSITION OF VARYING AGED (DENSITY) ERYTHROCYTE FRACTIONS

Values are mean ± S.E. of five blood samples Differences between values marked [a] and [b], [a] and [c], and [d] and [c] are statistically significant ($P < 0.05$).

Fractions	Sphingomyelin	PC	PS + PI (% of total)	Phopsholipid malondialdehyde adduct	PE
Light (1)	24.1 ± 0.4	27.8 ± 0.8 [a]	16.1 ± 0.1 [a]	0.2 ± 0.1 [a]	31.8 ± 0.6
Predensest (2)	22.3 ± 0.2	30.5 ± 0.5 [b]	13.7 ± 0.5 [b]	1.1 ± 0.1 [b,d]	33.3 ± 0.7
Densest (3)	23.0 ± 0.3	31.2 ± 0.3 [c]	12.9 ± 0.5 [c]	1.5 ± 0.1 [c]	32.2 ± 0.2

significant peroxidative membrane lipid damage and accumulation of malondialdehyde, an end product of lipid peroxidation. Table I also shows a significantly decreased PS and increasd PC in aged (densest) erythrocytes when compared to young (light) erythrocytes.

Table II gives fluorescence in the lipid extracts of varying aged erythrocyte fractions. This shows progressive accumulation of lipid fluorescent products and, thus, malondialdehyde in the

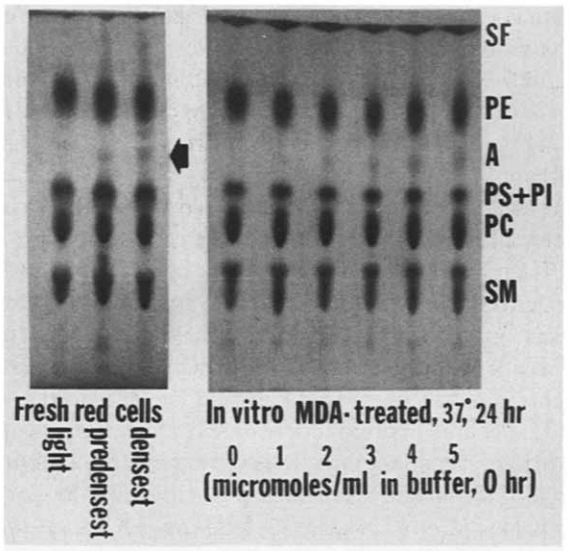

Fig. 2. Thin-layer chromatography of lipids of varying aged erythrocytes (left) and in vitro malondialdehyde-treated erythrocytes (right). Details of fractionation of varying aged erythrocytes, malondialdehyde (MDA) treatment in vitro and chromatography are given in Materials and Methods. Phospholipids on the thin-layer chromatogram were visualized after its exposure to iodine vapors. Note the presence of a new phospholipid 'A' (shown by arrow) in the aged erythrocytes. SF, solvent front; A, phospholipid-malondialdehyde adduct; SM, sphingomyelin.

TABLE II

LIPID EXTRACT FLUORESCENCE IN THE VARYING AGED (DENSITY) ERYTHROCYTE FRACTIONS

Values are mean ± S.E. of five experiments. Differences among the values marked [a], [b] and [c] in the same column were statistically significant ($P < 0.05$). 0.4 ml packed red cells was vertexed with 3 ml isopropanol and 2 ml chloroform. Fluorescence was read in the supernatant after centrifugation as described in Materials and Methods. Relative lipid fluorescence values were multiplied with 2.5 to calculate fluorescence values in per ml packed cells.

Fractions	Relative lipid fluorescence		
	per ml packed cells	per 10^{10} cells	per mg phospholipid
Light	22.50 ± 0.75 [a]	14.75 ± 0.49 [a]	25.71 ± 0.86 [a]
Predensest	29.25 ± 0.50 [b]	16.71 ± 0.29 [b]	30.79 ± 0.53 [b]
Densest	33.40 ± 0.75 [c]	20.94 ± 0.47 [c]	34.35 ± 0.77 [c]

TABLE III

THIOBARBITURIC ACID REACTIVITY (MALONDIALDEHYDE) OF VARYING AGED (DENSITY) ERYTHROCYTES FRACTIONS

Values are mean ± S.E. of five experiments. Differences in the same column are statistically non-significant ($P > 0.05$). Malondialdehyde values in the fractionated, untreated red blood cells were obtained by its thiobarbituric acid reactivity and by using molar extinction coefficient of malondialdehyde-thiobarbituric acid complex at 532 nm = $1.56 \cdot 10^5$/cm per molar solution.

Fractions	Thiobarbituric acid reactivity (malondialdehyde) (nmol)		
	per ml packed cells	per 10^{10} cells	per mg phospholipid
Light	3.22 ± 0.57	3.68 ± 0.65	2.10 ± 0.37
Predensest	3.23 ± 0.40	3.40 ± 0.43	1.85 ± 0.23
Densest	3.24 ± 0.47	3.34 ± 0.22	2.04 ± 0.13

erythrocyte membrane with aging. This also suggests significant peroxidative membrane lipid damage during erythrocyte aging. To rule out the effect of differences in the mean cell volume and total phospholipid per cell, values have been expressed as per ml packed cells, per cell, as well as per mg phospholipid. Thiobarbituric acid reactivity of varying aged erythrocytes was similar (Table III).

Discussion

Various investigators agree that with increasing erythrocyte age there are decreases in the total lipid, cholesterol and phopsholipid of the cell [1,13]. Among the phospholipid classes, a relative increase of the PC fraction has been reported with erythrocyte aging [14]. During the erythrocyte aging process, an increase in the linoleate and a decrease in arachidonic acid and other long-chain fatty acids also has been reported [13,14].

The present study has documented formation of a new phospholipid adduct and increased fluorescence in the lipid extracts of human erythrocytes aged in vivo. Both formation of phospholipid-malondialdehyde adduct and fluorescence represent cross-linking of membrane components with malondialdehyde, an end product of lipid peroxidation [7–9,11], and, thus, occurrence of membrane lipid peroxidation and malondialdehyde accumulation in aging erythrocytes. On the other hand, in vitro malondialdehyde treatment to erythrocytes also has been shown to result in reduced cell deformability and survival [15,16]. This suggests that membrane lipid peroxidation and accumulation of malondialdehyde observed during the in vivo aging of human erythrocytes can affect cell deformability and survival. Whether or not any of malondialdehyde has been cross-linked with the membrane proteins in aged human erythrocytes is not known. Increased fluorescence in the lipid extracts of aged erythrocytes of rats [7] and indirect observations on the membrane proteins also have suggested age-related memrbane lipid peroxidation in rat and bovine erythrocytes [7,17]. Membrane lipid peroxidative damage in in vivo aged erythrocytes seems to be a result of an age-associated decrease in the activities of enzymes such as glucose-6-phosphate dehydrogenase and glutathione peroxidase [3,18,19], responsible for the detoxification of oxygen radicals generated by the homoglobin [20,21].

Free malonyldialdehyde is water-soluble and could dissipate from erythrocyte into plasma. Thus, malonyldialdehyde detected in the membrane probably is not all that was formed during the aging of the erythrocyte.

The absence of an increase in the thiobarbituric acid reactivity of aged erythrocytes is consistent with a previous report [14]. This absence could be due to the fact that during the course of erythrocyte aging, malondialdehyde formation parallels its cross-linking with the lipids or other membrane components and, thus, is not available in the free form to react with the thiobarbituric acid. The increase in PC in aged erythrocytes also has been observed previously [14], but the reason for this increase is not known. Decreased PS levels in aged erythrocytes could be partly due to the fact that PS is rich in unsaturated fatty acids, which are most vulnerable to peroxidative breakdown, and to the fact that PS does not participate in the exchange process with plasma phospholipids for its renewal [22].

Thus, cross-linking of membrane components due to lipid peroxidation can impede cell deformability and passage through the narrow splenic sinusoids, which can contribute to the senescence of aging human erythrocytes. Malondialdehyde containing aged human erythrocytes also may expose new antigen on their surface [23,24], presumably due to destablization of the membrane bilayer [25,26], and become susceptible to phagocytosis by the reticuloendothelial system.

Acknowledgements

This study was supported in part by NIH grant 1R01 HL 30247, a grant-in-aid from Hoffmann-La Roche, and the American Diabetes Association, and an Edward P. Stiles Award from the Louisiana State University Medical Center at Shreveport. The author is grateful to Kathy Jones for typing and to Barbara MacRoberts for editing the manuscript.

References

1 Westerman, M.P., Pierce, L.E. and Jensen, W.N. (1963) J. Lab. Clin. Med. 62, 394–400.

2 Van Deenen, L.L.M. and DeGier, J. (1974) in The Red Blood Cell (Surgenor, D.M., ed.), pp. 147–211, Academic Press, New York.
3 Clark, M.R. and Shohet, S.B. (1985) Clin. Haematol. 14, 223–257.
4 Piomelli, S., Lurinsky, G. and Masserman, L.R. (1967) J. Lab. Clin. Med. 69, 659–674.
5 Stocks, J. and Dormandy, T.L. (1971) Br. J. Haematol. 20, 95–111.
6 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 528–531.
7 Jain, S.K. and Hochstein, P. (1980) Biochem. Biophys. Res. Commun. 92, 247–254.
8 Bidlack, W.R. and Tappel, A.L. (1973) Lipids 8, 203–207.
9 Jain, S.K. and Shohet, S.B. (1984) Blood 63, 362–367.
10 Esterbauer, H., Koller, E., Slee, R.G. and Koster, J.F. (1986) Bochem. J. 239, 405–409.
11 Beppu, M., Murakami, K. and Kikugawa, K. (1987) Biochim. Biophys. Acta 897, 169–179.
12 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
13 Gastel, C.V., Berg, V.D., Gier, J.D. and Van Deenen, L.L.M. (1965) Br. J. Haematol. 11, 193–199.
14 Phillips, G.B., Dodge, J.T. and Howe, C. (1969) Lipids 4, 544–549.
15 Pfafferott, C., Meiselman, H.J. and Hochstein, P. (1982) Blood 59, 12–15.
16 Jain, S.K., Mohandas, N., Clark, M.R. and Shohet, S.B. (1983) Br. J. Haematol. 53, 247–255.
17 Bartosz, G., Soszynski, M. and Wasilwski, A. (1982) Mechan. Aging Dev. 19, 45–52.
18 Bartosz, G., Tannert, C. and Fried, R. (1978) Experientia 38, 575.
19 Glass, G.A. and Gershon, D. (1984) Biochem. J. 218, 531–537.
20 Scarpa, M., Viglino, P., Contri, D. and Rigo, A. (1984) J. Biol. Chem. 259, 10657–10659.
21 Winterbourne, C.C. (1983) Biomed. Biochim. Acta 42, 5134–5138.
22 Jain, S.K. and Hochstein, P. (1978) Blood 51, 769–770.
23 Hebbel, R.P. and Miller, W.J. (1984) Blood 64, 733–741.
24 Kay, M.M.B., Bosman, G.C., Shapiro, S.S., Bendich, A. and Bassel, P.S. (1986) Proc. Natl. Acad. Sci. USA 83, 2463–2467.
25 Jain, S.K. (1984) J. Biol. Chem. 259, 3391–3394.
26 Jain, S.K. (1985) J. Clin. Invest. 76, 281–286.