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Altered membrane phospholipid organization and erythrophagocytosis in Eβ-thalassemia

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Abstract

The underlying cause behind the accelerated destruction of erythrocytes in the bone marrow and in the peripheral circulation, accompanying the β-thalassemic syndromes is still not clearly understood. The present investigation demonstrates that increased phagocytosis of erythrocytes in Eβ-thalassemia is inhibited by the presence of phosphatidylserine (PS) vesicles, suggesting a PS–'PS-receptor' type of interaction in premature recognition of these erythrocytes by macrophages. Increased exposure of both aminophospholipids phosphatidylethanolamine (PE) and PS was demonstrated by fluorescamine labeling and annexin binding, respectively. The slower rate of translocation of PS across the bilayer suggested that this contributed towards the increased exposure of PS in Eβ-thalassemic erythrocytes.

Keywords: Phosphatidylserine; Erythrocyte; Phagocytosis; Aminophospholipid translocase; Thalassemia, Eβ-

1. Introduction

Thalassemias result from inherited defects in the rate of synthesis of one or more of the globin chains. This causes ineffective erythropoiesis, hemolysis and a variable degree of anemia. Thalassemia is the most frequent autosomal recessive inherited disease that is distributed worldwide. Hemoglobin E (HbE) is a globin β-chain variant resulting from the substitution of glutamic acid by lysine in codon 26. Heterozygous Eβ-thalassemia is common in South-east Asia [1]. Studies of the thalassemic syndromes have concentrated intensively on the primary defects in hemoglobin synthesis. It is generally assumed that the extra unmatched globin chains precipitate in the erythrocyte leading to membrane dysfunction. The mechanisms leading to the premature destruction of erythrocytes in the bone marrow sinusoids and the peripheral circulation, are less clearly understood, other than the possible involvement of hemichrome-induced integral protein aggregation [2]. Most of the work on the erythrocyte membrane has focused on defects in associations of cytoskeletal proteins, studied particularly in mouse models of β-thalassemia [3–7]. However, the possible involvement of loss of phospholipid asymmetry in the shortening of the life-span of thalassemic erythrocytes, has not been explored.

Different phospholipid classes are asymmetrically distributed over the two halves of the lipid bilayer in blood cells [8]. The choline phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) are concentrated in the outer leaflet, whereas the aminophospholipids phosphatidylethanolamine (PE)

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and phosphatidylserine (PS) are concentrated in the inner leaflet. It is now well established that this asymmetric distribution of phospholipids is common to many different eukaryotic cell types [9–12]. Several mechanisms of maintenance of phospholipid asymmetry have been proposed [13]. Earlier it was felt that specific associations of aminophospholipids and some cytoskeletal proteins contribute towards maintenance of phospholipid asymmetry [14]. However, recent work has shown that a perturbation in cytoskeleton proteins does not affect the phospholipid asymmetry [15–17]. The existence of an aminophospholipid translocase capable of translocating PS from the outer to the inner leaflet of the lipid bilayer, is now well documented [18–21]. Lipid asymmetry is, on the other hand, rapidly abolished upon elevation of intracellular Ca^{2+} in erythrocytes or platelets [21,22]. A major consequence of this process of lipid scrambling is the exposure of PS on the outer surface of erythrocytes or platelets.

The presence of PS in the outer leaflet of cells results in their endocytosis by phagocytes [23–26]. PS exposure has been demonstrated in red blood cells from β-thalassemia patients [27]. However, a study using model β-thalassemic erythrocytes showed a normal steady-state distribution of PS [28]. The present investigation presents evidence in favor of involvement of PS externalisation and loss of phospholipid asymmetry in increased erythrophagocytosis in heterozygous Eβ-thalassemia. The rate of PS translocation was found to be slower in these erythrocytes.

2. Materials and methods

2.1. Materials

Fatty acid-free BSA, fluorescamine, egg PC and 4,4′-diaminobenzidine were purchased from Sigma. RPMI 1640 was from Gibco, brain PS and 1-palmitoyl-2-[6-[7(nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-caproyl]-*sn*-glycerol-3-phosphoserine (NBD-PS) were products of Avanti Polar Lipids, annexin V (from human placenta) was purchased from Serbio, France. IODOBEADS iodination reagent was from Pierce, and carrier free $Na^{125}I$ was from Amersham, UK. All other reagents were of analytical grade.

2.2. Phagocytosis assay

Macrophages were obtained from the peritoneal cavity of 7–10-week-old Balb/C male mice, 3 days after an intraperitoneal injection of 1–2 ml of 2% starch in sterile phosphate-buffered saline (PBS). Cells were washed with PBS and spun down at $500 \times g$ for 5 min. The cells were then resuspended in RPMI 1640 medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin and 10% fetal calf serum. Viability was assessed by trypan blue staining. Cells were layered on coverslips ($5 \cdot 10^5$ cells/coverslip) and incubated for 4 h in 5% CO_2 at 37°C. The coverslips were then washed with RPMI 1640 to remove any cells which had not adhered. Erythrocytes ($2 \cdot 10^6$ cells/coverslip) were layered over the macrophages and incubated for 45 min in 5% CO_2 at 37°C. Non-adherent cells were removed by gentle washing with PBS and surface-bound erythrocytes were lysed by treatment with 140 mM NH_4Cl/17 mM Tris-HCl, pH 7.6 for 5 min. Coverslips were dried, fixed with methanol, stained with 4,4′- diaminobenzidine for erythrocytes and counterstained with Giemsa for macrophages. Ingested erythrocytes were counted by microscopy. For each set, the number of macrophages that had ingested one or more erythrocytes was determined. Phagocytosis was expressed in terms of erythrocytes ingested per 100 macrophages [24].

2.3. Preparation of vesicles

Phospholipids were dried as films under a stream of N_2 and then dried under vacuum. The films were rehydrated by the addition of buffer containing 137 mM NaCl, 10 mM KCl, 7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, pH 7.4 and vortexing. Small unilamellar vesicles (SUVs) were made from these multilamellar vesicles by sonication in a bath-type sonicator and final vesicle concentrations were determined by the method of Ames and Dubin [29].

2.4. Preparation of lipid-symmetric and asymmetric erythrocytes

These were prepared according to the method of Schlegel et al. [30] by lysing erythrocytes in the

presence or absence of Ca^{2+} followed by resealing with 10 × PBS to obtain lipid-symmetric and asymmetric cells, respectively. Briefly, 100 μl of packed RBC were lysed with 1 ml of lysis buffer (7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4). Ca^{2+} (1 mM) was added during vortexing for preparation of symmetric erythrocytes and kept on ice for 2 min. 100 μl of resealing buffer (10 × PBS, 1 mM $MgCl_2$) was added to restore the isotonicity, followed by incubation at 37°C for 30 min. Cells were then pelleted down at 3000 rpm and counted, followed by resuspension in RPMI 1640 and addition to the confluent layer of macrophages.

2.5. Incubation with vesicles

PC or PS vesicles were added to each macrophage-containing coverslip along with the erythrocytes. Erythrophagocytosis was assayed as described above.

2.6. Determination of phospholipid distribution in red blood cells

The steady-state transmembrane distribution of PE was determined by labeling of aminophospholipids with fluorescamine [31]. Briefly, RBCs were washed three times with PBS and resuspended in labeling buffer (100 mM KCl, 50 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Na_2CO_3, 20 mM Tricine, pH 8). Subsequently, fluorescamine (2 μmol) was added to $1.3 \cdot 10^9$ RBC in 3.3 ml of buffer from a 114 mM stock solution in DMSO/acetone (1:2.5). Labeling was carried out at room temperature for 30 s, and the reaction was stopped by adding 7.5 ml of the labeling buffer containing 6 mM glycylglycine (instead of Na_2CO_3). RBCs was pelleted by low speed centrifugation and lysed by adding 0.5 ml of 20 mM glycylglycine, pH 8. Lipids were extracted by the method of Rose and Oklander [32]. Lipids were separated by thin-layer chromatography and phospholipid phosphorus was measured.

2.7. Annexin binding assay

This was performed as described by Tait and Gibson [33]. Annexin (from human placenta) was iodinated using the Pierce IODOBEADS iodination reagent and $Na^{125}I$ [34]. Binding assays were carried out in a buffer consisting of 10 mM Hepes, pH 7.4, 135 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM glucose, 1 mg/ml BSA (fatty acid-free), 1 U/ml heparin, and 2.5 mM Ca^{2+}. Erythrocytes at a concentration of $5 \cdot 10^7$ cells/ml, were incubated with ^{125}I-annexin at a concentration of 100 nM for 15 min at 37°C, and radioactivity associated with the cells was counted after centrifugation. Nonspecific binding was measured in the presence of 5 mM EDTA.

2.8. Determination of PS translocation rates in red blood cells

The translocation of PS from the outer to the inner leaflet was measured using fluorescent NBD-labeled PS [35]. Briefly, PS was added to a final concentration of 1 μM to 20 μl packed RBC and incubated on ice for 5 min in 5 mM Hepes, pH 7.5, 145 mM NaCl, 5 mM KCl, 1 mM Mg^{2+}, 10 mM glucose (Buffer A). Cells were spun down, washed and reconstituted in 2 ml of Buffer A. 100-μl aliquots were removed at different time intervals into 1 ml of buffer A containing 1% fatty acid-free BSA, in order to remove NBD-PS from the outer monolayer. Samples were pelleted and fluorescence associated with the supernatant was measured in a spectrofluorimeter (excitation wavelength: 472 nm, emission wavelength: 534 nm).

3. Results

3.1. Uptake of erythrocytes by macrophages

Eβ-thalassemic erythrocytes were phagocytosed to a greater extent than normal erythrocytes (Table 1). The role of PS externalisation in increased phagocytosis was investigated. Assuming that externalised PS interacts with a PS receptor on macrophages, it was argued that PS vesicles would competitively inhibit binding and uptake of the thalassemic erythrocytes by macrophages. When macrophages were coincubated with thalassemic erythrocytes and PS vesicles, the extent of phagocytosis was reduced almost to the extent seen in the case of normal erythrocytes (Table 1). The inhibition of phagocytosis showed a

Table 1
Uptake of erythrocytes by macrophages

Group	Number	Number of erythrocytes/100 macrophages
Normal	6	7.0 ± 0.9
Symmetric	6	17.0 ± 2.0
Symmetric + PS (4 nmol)	6	12.7 ± 0.4
Symmetric + PS (8 nmol)	6	9.5 ± 0.4
Symmetric + PS (16 nmol)	6	6.9 ± 0.7
Symmetric + PC (8 nmol)	6	14.1 ± 0.2
Symmetric + PC (16 nmol)	6	13.9 ± 1.2
Eβ	6	20.3 ± 0.7
Eβ + PS (4 nmol)	6	14.0 ± 1.0
Eβ + PS (8 nmol)	6	10.3 ± 0.1
Eβ + PS (16 nmol)	6	6.7 ± 1.8
Eβ + PC (8 nmol)	6	17.2 ± 0.1
Eβ + PC (16 nmol)	6	16.5 ± 0.6

Results represent the means ± S.D.
'Eβ' represents Eβ-thalassemic erythrocytes.

dose effect, i.e., increasing amounts of PS vesicles caused increased inhibition. No such effect was observed with PC vesicles. The observation was similar to that seen in the case of lipid-symmetric erythrocytes where co-incubation with PS vesicles reduced phagocytosis almost to background levels observed in the case of lipid-asymmetric erythrocytes. These findings strengthened the view that at least in vitro, enhanced uptake of thalassemic erythrocytes by macrophages involves a PS–'PS-receptor' interaction.

3.2. Steady-state distribution of phosphatidylethanolamine (PE)

The stationary distribution of PE in the outer and inner leaflet of the bilayer was studied by fluorescamine labeling. There was a significant increase in PE in the outer leaflet of the bilayer in Eβ-thalassemic erythrocytes compared to normal erythrocytes (Table 2). The distribution of PS could not be measured by the fluorescamine labeling technique.

Table 2
Steady-state distribution of PE in normal and Eβ-thalassemic erythrocytes

Group	Number	% PE in the outer monolayer
Normal	6	18.6 ± 1.5
Eβ-thalassemic	6	23.4 ± 1.9

Results represent the means ± S.D. $0.001 < P < 0.01$.

Table 3
Annexin binding to normal and Eβ-thalassemic erythrocytes

Group	Number	^{125}I-Annexin binding
Normal	9	1230 ± 270
Eβ-thalassemia	12	14424 ± 7620

Results represent the means ± S.D.

3.3. Annexin binding

Annexin binding was routinely performed after storage of the samples for 24 h at 4°C, since it was not possible to perform assays with patient samples on the day of drawing blood. The thalassemic population showed an average of almost twelve times more annexin binding when compared with normal donors (Table 3). This supported the view that exposed PS was significantly higher in thalassemic erythrocytes compared to normal erythrocytes.

3.4. Inward translocation of fluorescent NBD-PS

The translocation of PS from the outer to the inner monolayer was assessed after loading of cells with fluorescent NBD-PS. The rate of translocation was found to be slower in the case of Eβ-thalassemic erythrocytes compared to normal ones (Table 4). The initial velocity of translocation was slightly higher in normal (2.9%/min) compared to patient (1.8%/min) erythrocytes. The average times taken for translocation of 50% of the NBD-PS from the outer to the

Table 4
Translocation rates of NBD-PS from the outer to the inner monolayer of erythrocytes

Group	Number	Initial velocity (% translocation per min)	Time taken for 50% translocation
Normal	6	2.9 ± 0.3	16.1 ± 1.3
Eβ-thalassemia	6	1.8 ± 0.3	28.8 ± 4.4

Results represent the means ± S.D. $0.001 < P < 0.01$.

inner leaflet were 16.1 and 28.8 min for normal and Eβ-thalassemic erythrocytes, respectively.

4. Discussion

The mechanisms leading to the premature destruction of thalassemic erythrocytes, and consequent anemia, remain poorly understood. In the present investigation, attempts were made to analyze the possible role of PS exposure on the outer surface of thalassemic erythrocytes in the recognition of these cells by macrophages and their clearance from the circulation. Transmembrane lipid organization had previously shown to be altered in mouse β-thalassemic cells [36], although no obvious differences in stationary distribution of PS had been detected. The present study focused on heterozygous Eβ-thalassemia, a condition abundantly found in South-east Asia and associated with severe anemia. Eβ-thalassemic erythrocytes, not unexpectedly, showed increased erythrophagocytosis compared to normal erythrocytes. The extent of phagocytosis could be reduced down to the levels observed in normal erythrocytes when incubations with macrophages were performed in the presence of PS (but not PC) vesicles, suggesting a role of PS exposure in recognition of these erythrocytes by macrophages. The stationary distribution of PE in thalassemic erythrocytes also differed from that of normal cells. Annexin binding was performed as a measure of the extent of PS exposure. The average annexin binding was twelve times higher in thalassemic erythrocytes compared to normal cells, lending support to the view that PS was significantly more exposed to the outer surface in thalassemic erythrocytes. This was in agreement with previous investigations on red blood cells from β-thalassemic patients [27]. However, model β-thalassemic erythrocytes prepared by the introduction of excess α-globin chains did not show any changes in the steady-state distribution of PS [28]. The net transmembrane movement and equilibrium distribution of PS and PE are the result of active transport mediated by the aminophospholipid translocase, and the passive diffusion of these phospholipids. The role of the translocase was assessed by measuring the inward movement of fluorescent NBD-PS incorporated into the outer leaflet of normal and thalassemic erythrocytes. Dramatic differences were not observed. However, the initial rates of translocation were slower, and the times required for inward translocation of 50% of the NBD-PS were consistently longer in the thalassemic erythrocytes, unlike the observations reported in mouse β-thalassemic cells [36] or in model β-thalassemic erythrocytes [28]. The slower kinetics of translocation could contribute towards the net alteration in transmembrane distribution of aminophospholipids in these cells. The lack of adequate amounts of samples prevented measurements to be performed on density-separated cells. However, the results obtained in the present study warrant further analysis of the distribution and movement of PS in human β-thalassemic cells by flow cytometry.

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References

[1] Winichagoon, P., Fucharoen, S., Wilairat, P., Chihara, K., Fukumaki, Y. and Wasi, P. (1992) Biochim. Biophys. Acta 1139, 280–286.
[2] Yuan, J., Kannan, R., Shinar, E., Rachmilewitz, E.A. and Low, P.S. (1992) Blood 79, 3007–3013.
[3] Shinar, E., Rachmilewitz, E.A. and Lux, S.E. (1989) J. Clin. Invest. 83, 404–410.
[4] Advani, R., Sorensen, S., Shinar, E., Lande, W., Rachmilewitz, E. and Schrier, S.L. (1992) Blood 79, 1058–1063.
[5] Advani, R., Rubin, E., Mohandas, N. and Schrier, S.L. (1992) Blood 79, 1064–1067.
[6] Yuan, J., Rubin, E., Alijurf, M., Ma, L. and Schrier, S.L. (1994) Blood 84, 632–637.
[7] Yuan, J., Bunyaratvez, A., Fucharoen, S., Fung, C., Shinar, E. and Schrier, S.L. (1995) Blood 86, 3945–3950.
[8] Devaux, P.F. (1994) Chem. Phys. Lipids 73, 107–120.
[9] Schroit, A.J. (1994) Curr. Top. Membr. 40, 47.
[10] Chahine, J.M.E.H., Cribier, S. and Devaux, P.F. (1993) Proc. Natl. Acad. Sci. USA 90, 447–451.

[11] Muller, K., Pomonski, T., Muller, P., Zachowski, A. and Hermann, A. (1994) Biochemistry 33, 9968–9974.
[12] Calvez, J.Y., Zachowski, A., Hermann, A., Morrot, G. and Devaux, P.F. (1988) Biochemistry 27, 5666–5670.
[13] Devaux, P.F. (1993) Curr. Opin. Struct. Biol. 3, 489–494.
[14] Kuypers, F.A., Lubin, B.H., Yee, M., Agre, P., Devaux, P.F. and Geldwerth, D. (1993) Blood 81, 1051–1055.
[15] Beleznay, Z., Zachowski, A., Devaux, P.F., Navazo, P. and Ott, P. (1993) Biochemistry 32, 3146–3152.
[16] Gudi, S.R.P., Kumar, A., Bhakuni, V., Gokhale, S.M. and Gupta, C.M. (1990) Biochim. Biophys. Acta 1023, 63–72.
[17] Seigneuret, M. and Devaux, P.F. (1994) Proc. Natl. Acad. Sci. USA 81, 3751–3755.
[18] Zachowski, A. (1993) Biochem. J. 294, 1–14.
[19] Zachowski, A., Favre, E., Cribier, S., Herve, P. and Devaux, P.F. (1986) Biochemistry 25, 2585–2590.
[20] Connor, J. and Schroit, A.J. (1988) Biochemistry 27, 848–851.
[21] Williamson, P., Kulick, A., Zachowski, A., Schlegel, R.A. and Devaux, P.F. (1992) Biochemistry 31, 6355–6360.
[22] Smeets, E.F., Comfurius, P., Bever, E.M. and Zwaal, R.F.A. (1994) Biochim. Biophys. Acta 1195, 281–286.
[23] Allen, T., Williamson, P. and Schlegel, R.A. (1988) Proc. Natl. Acad. Sci. USA 85, 8067–8071.
[24] Pradhan, D., Williamson, P. and Schlegel, R.A. (1994) Mol. Membr. Biol. 11, 187–188.
[25] Schroit, A.J., Madsen, J.W. and Tanaka (1985) J. Biol. Chem. 260, 5131–5138.
[26] Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L. and Henson, P.M. (1992) J. Immunol. 148, 2207–2216.
[27] Yashar, V.B., Barenholz, Y., Hy-Am, E. and Rachmilewitz, E.A. (1993) Am. J. Hematol. 44, 63–65.
[28] Kuypers, F.A., Schott, M.A. and Scott, M.D. (1996) Am. J. Hematol. 51, 45–54.
[29] Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775.
[30] Schlegel, R.A., Reed, J.A., McEvoy, L., Algarin, L. and Williamson, P. (1987) Methods Enzymol. 149, 281–293.
[31] Geldwerth, D., Kuypers, F.A., Butikofer, P., Allary, M., Lubin, B.H. and Devaux, P.F. (1993) J. Clin. Invest. 92, 308–314.
[32] Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431.
[33] Tait, J.F. and Gibson, D. (1994) J. Lab. Clin. Med. 123, 741–748.
[34] Pierce Instruction Manual (1991) for IODOBEADS Iodination Reagent.
[35] Collean, M., Herve, P., Fellmann, P. and Devaux, P.F. (1991) Chem. Phys. Lipids 57, 29–37.
[36] Muller, P., Zachowski, A., Beuzard, Y. and Devaux, P.F. (1993) Biochim. Biophys. Acta 1151, 7–12.