Available online at www.sciencedirect.com
ScienceDirect
Blood Cells, Molecules, and Diseases 37 (2006) 77–81
BLOOD CELLS, MOLECULES, & DISEASES
www.elsevier.com/locate/ybcmd

Enhanced oxidative cross-linking of hemoglobin E with spectrin and loss of erythrocyte membrane asymmetry in hemoglobin Eβ-thalassemia

Poppy Datta [a], Sumanta Basu [a], Sudipa Basu Chakravarty [c], Amit Chakravarty [c], Debashis Banerjee [d], Sharmila Chandra [d], Abhijit Chakrabarti [a,b,*]

[a] *Biophysics Division, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Kolkata 700064, India*
[b] *Structural Genomics Section, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Kolkata 700064, India*
[c] *Thalassaemia Foundation, Kolkata-700064, India*
[d] *Clinical Haematology Service, 4 Gorky Terrace, Kolkata-700017, India*

Submitted 21 April 2006; revised 6 June 2006
Available online 31 July 2006
(Communicated by M. Lichtman, M.D., 22 June 2006)

Abstract

Oxidative stress to the erythrocytes is associated with formation of large molecular complexes of hemoglobin and the skeletal protein, spectrin. In this work, such complexes are formed with hemoglobin mixtures isolated from patients suffering from HbEβ-thalassemia with elevated levels of the HbE and purified erythroid spectrin in the presence of hydrogen peroxide. The complexes are separated on 4% SDS-PAGE and analyzed by densitometry. The results indicate enhanced formation of complexes with higher amounts of HbE, the most common hemoglobin variant prevalent in Southeast Asia. The binding affinity of spectrin with hemoglobin, in the absence of hydrogen peroxide, was found to increase with hemoglobin mixtures enriched with HbE. The presence of ATP was also found to decrease the overall yield of such complexes. Flow cytometric measurements of phosphatidylserine on the red cell surface also showed a lower degree of membrane asymmetry in HbEβ-thalassemic patients than in normal subjects. The present work shows enhanced formation of high molecular weight cross-linked complexes of hemoglobin derivatives with erythroid spectrin in HbEβ-thalassemia.

Keywords: Peroxide; Red cell; Membrane asymmetry; HbE level; Thalassemia

Introduction

The red cell is protected against oxidant stress by powerful reducing systems [1–3]. Oxidant balance is disturbed in conditions like oxidative hemolytic anemia [4] including thalassemia and other hemoglobinopathies with hemoglobin instability [5,6]. Various reports have already indicated the presence of cross-linked aggregates of hemoglobin and spectrin, the major component of the erythroid membrane skeleton [7–9]. The presence of such aggregates in erythrocytes has been implicated in pathophysiological effects such as reduced deformability and enhanced rigidity of erythrocytes and their enhanced phagocytosis by macrophages [10–13]. Hemoglobin E is the most common hemoglobin variant in Southeast Asia and contains an unstable mutation Glu26(B8)→Lys [14,15]. Homozygous HbE patients are minimally anemic, however, patients with HbE combined with β-thalassemia (HbEβ-thalassemia) have severe clinical features and is the most common form of hemoglobinopathy in India [16,17].

In vitro exposure of erythrocytes to peroxide results in signs of accelerated cell senescence, generation of hemoglobin–

Abbreviations: HbA, adult hemoglobin; HMWA, high molecular weight aggregate; HbF, fetal hemoglobin; F–spectrin, fluorescein-conjugated spectrin; RD, relative pixel density; ATP, adenosine 5′-triphosphate; Mg-ATP, ATP in presence of 10-fold molar excess of $MgCl_2$; PMSF, phenylmethylsuphonyl fluoride; NEM, *N*-ethylmaleimide; A23187, calcium ionophore; PS, phosphatidylserine; FITC-AV, fluorescein-labeled annexin V; SEM, standard error of mean; K_d, binding dissociation constant.

* Corresponding author. Structural Genomics Section, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Kolkata 700064, India. Fax: +91 33 2337 4637.
E-mail address: abhijit.chakrabarti@saha.ac.in (A. Chakrabarti).

1079-9796/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcmd.2006.06.001

spectrin complexes, decreased cell deformability, and, an altered cell surface leading to the enhancement of phagocytosis [18–20]. Investigators also have reported globin chain complexes with spectrin caused by an oxidative mechanism, causing damage to the erythrocyte during aging [7,21]. In the cases of thalassemia and sickle-cell anemia, the loss of phospholipid asymmetry has been implicated in the recognition of erythrocytes by macrophages. Phosphatidylserine (PS) exposure has been proposed to play a role both in the removal of erythrocytes and the formation of the hypercoaguable state that occur to in severe β-thalassemia intermedia [22–25]. In severe α-thalassemia, the membranes are hyperstable, whereas in β-thalassemia, the membranes are unstable [10,11]. Various observations support the idea that of partly oxidized globin chains cross-link with adjacent cytoskeleton proteins [26]. The motivation for the present work was to measure (1) spectrin binding affinities of hemoglobin mixtures enriched with HbE, (2) yields of cross-linked complexes of the respective hemoglobin samples and spectrin in the presence of hydrogen peroxide and (3) the extent of membrane asymmetry maintained in the erythrocytes of the HbE carriers and the HbEβ-thalassemic patients. Results indicate greater loss of membrane asymmetry, higher spectrin binding affinity and enhanced yield of cross-linked complexes than normal in presence of hydrogen peroxide with elevated levels of HbE. These observations could explain the severity of the anemia in HbEβ-thalassemia.

Methods

Percoll, A23187 and ATP and other chemicals were purchased from Sigma. Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes. FITC conjugated annexin V (FITC-AV) was obtained from BD Biosciences Pharmingen (San Diego, CA).

Collection and isolation of hemoglobin from human blood samples

Human blood samples taken for diagnosis from patients with Eβ-thalassemia were characterized by Bio-Rad Variant HPLC system. The level of each hemoglobin variant, HbA, HbE and HbF, in the blood samples of β-thalassemia carriers, HbE carriers and Eβ-thalassemic patients was estimated from the HPLC system [27]. The blood samples were taken from patients at the time of diagnosis and who have not had transfusions. Human erythrocytes, after removal of the buffy coat and plasma, were extensively washed with phosphate-buffered saline (5 mM phosphate, 0.15 M NaCl, pH 7.4). Hemoglobin was isolated from packed erythrocytes by osmotic lysis using three volumes of 1 mM Tris, pH 8.0, at 4°C for 1 h. The hemoglobin mixture was purified by gel filtration on Sephadex G-100 column (30 × 1 cm) in a buffer containing 5 mM Tris, 50 mM KCl, pH 8.0. The hemoglobin samples were stored in oxy-form at −70°C for not less than 7 days and characterized by the measurements of absorption at 415 nm and 541 nm, respectively. The protein concentration was determined spectrophotometrically using a molar extinction coefficient of 125,000 M^{-1} cm^{-1} at 415 nm and 13,500 M^{-1} cm^{-1} at 541 nm respectively [28].

For flow cytometric measurements, the blood samples were collected in Na_2EDTA and reached us within 3–4 h of its collection. The blood sample was diluted by addition of equal volume of phosphate buffer containing 2.5 mM KCl, 1.5 mM KH_2PO_4, 135 mM NaCl, 9 mM NaH_2PO_4, 1.0% dextrose, 0.01% NaN_3, pH 7.4 and was kept on 75% iso-osmotic percoll, 0.9% NaCl, 0.01% NaN_3. Erythrocytes were collected from the bottom of the percoll gradient, washed and kept in the same buffer.

Isolation and purification of spectrin

White ghosts from ovine and human blood were prepared by hypotonic lysis in 5 mM phosphate, 1 mM Na_2EDTA containing 20 g/ml of PMSF at pH 8.0 (lysis buffer) and dimeric spectrin was isolated at 37C following the protocol of Gratzer [29]. Details of purification and characterization of the ovine spectrin and its molecular identity with the human spectrin have been published in an earlier study [30]. Covalent labeling of spectrin was done with fluorescein isothiocyanate (FITC), in a buffer ~pH 9.0 by following a procedure previously described [31]. The labeling ratio of fluorescein to spectrin was determined to be 2 to 3 fluorescein molecules per spectrin dimer.

Oxidative cross-linking and binding studies of hemoglobin and spectrin

Hemoglobin mixtures, in different amounts, were incubated with 1 μM spectrin in presence of 1 mM H_2O_2, at 37°C for 15 min in phosphate-buffered saline containing 1 mM sodium azide [32]. After the incubation, the reaction mixtures were treated with 0.1 mM DTT, and 1% SDS and then subjected to electrophoresis on 4% acrylamide gel after staining with Coomassie blue. The incubation was carried out in presence of different concentrations of ATP with 10-fold molar excess of $MgCl_2$ ranging from 0.5 to 1.0 mM ATP. Analysis of band intensities was done with the bands *b* and *c* in presence of 0.1 mM DTT showing the best reproducibility. Since *band c* is localized along side α–spectrin, *band b*, which is separated in the gel, remains the best choice for the densitometry analysis. Human erythroid spectrin was also used to check the cross-linking experiments, showed indistinguishable, identical results from that of the ovine erythroid spectrin.

The densitometry analyses of the relative pixel density (RD) of respective bands were done using Quantity One software of BioRad. The error bars associated with the density values are the mean density with standard error of mean (SEM). RD represents the relative pixel density, after subtracting the observed pixel intensity values from the respective backgrounds. The ΔRD (%) has been defined as the relative pixel density of the isolated hemoglobin mixtures, normalized by using hemoglobin from normal individuals. Four to six gels with hemoglobin mixtures isolated from three HbEβ-thalassemic patients containing HbE ranging from 50 to 60% and

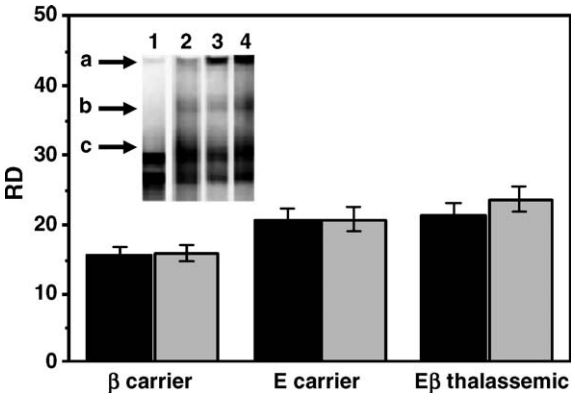

Fig. 1. Extent of formation of the spectrin–hemoglobin cross-linked complexes. Variation of relative pixel densities of *band b* (black bar) and *band c* (grey bar) of the complexes generated from the oxidative cross-linking of hemoglobin mixtures (22.5 μM) from β-thalassemia carrier, E carrier and HbEβ-thalassemic individuals with spectrin (1 μM) in presence of 1 mM hydrogen peroxide and 0.1 mM DTT. The inset shows a representative 4%SDS-PAGE of the oxidative cross-linked products of spectrin without hemoglobin (lane 1) and with hemoglobin from β-thalassemia carrier (lane 2), hemoglobin from HbE carrier (lane 3) and hemoglobin from HbEβ-thalassemic patients (lane 4).

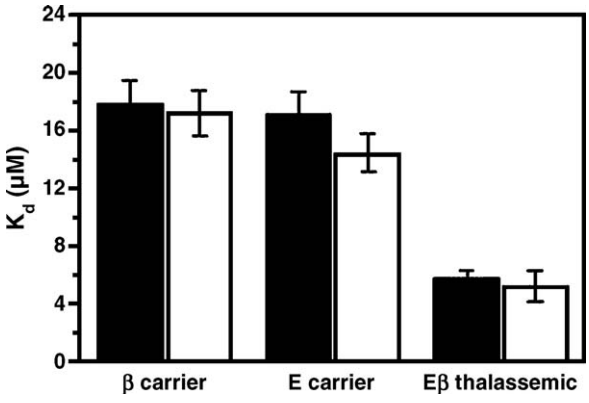

Fig. 3. Levels of HbE on the spectrin–hemoglobin binding dissociation constants and the effect of ATP. K_d (μM) values of spectrin with various hemoglobin samples of the β-thalassemia carrier, HbE carrier and HbEβ-thalassemic patients, in presence (empty bar) and absence (filled bar) of ATP (0.6 mM).

fetal hemoglobin ~5% with the remainder of HbA, density determined by Variant™.

Interaction of F–spectrin with hemoglobin was studied by fluorescence measurements. The extent of fluorescence quenching as a function of increasing concentrations of different hemoglobin was analyzed by the following method. The apparent dissociation constant of hemoglobin binding to spectrin (K_d) was determined using non-linear curve fitting analysis described previously [31].

Flow cytometry of red cells

The extent of red cell membrane asymmetry was determined within 4–6 h of collection of the blood sample by flow cytometry in FACS Calibur (Becton Dickinson) with freshly isolated red cells after labeling with FITC-AV. About 2×10^5 cells were suspended in 200 μl of the buffer of 10 mM HEPES, pH 7.4, containing 2.5 mM $CaCl_2$ and 0.14 M NaCl, labeled with 10 μl of FITC-AV solution by incubating for 15 min in the dark, and then brought to 1 ml for final measurements. Intact erythrocytes were analyzed for fluorescence intensity using the same standard settings on the calibrated flow cytometer. For each sample, 30,000 events were acquired and analyzed by CellQuestPro™ software (BD Biosciences). A clustered cell population was selected and analyzed for FL1 histogram expressed in logarithmic scale. The contribution of autofluorescence was corrected by selecting the marker, M1 for each sample taking 0.2% of the cells against respective isotype controls. The percent event found in the positive region of the selection marker is considered to be the percent binding of FITC-AV to the red cells [26]. The red cells were also treated with NEM, the calcium ionophore, A23187 and calcium chloride, used as the positive control for the measurement of maximum binding of annexin V [33,34].

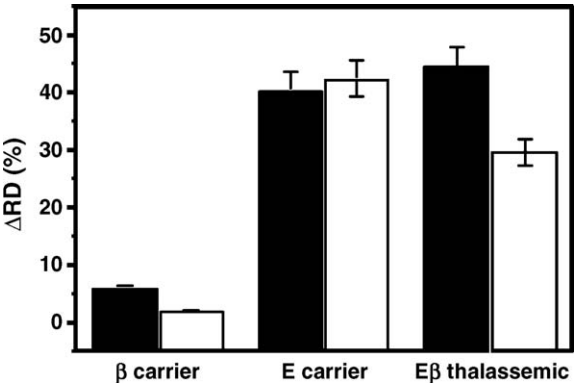

Fig. 2. Effects of ATP on the yield of the spectrin–hemoglobin cross-linked complexes. Variation of normalized relative pixel densities, ΔRD (%), of *band b*, in presence (empty bar) and absence (filled bar) of Mg-ATP (0.6 mM). The complexes were generated from 1 μM spectrin and 22.5 μM hemoglobin in presence of 0.1 mM DTT and 1 mM hydrogen peroxide.

Results

SDS-PAGE analysis of cross-linked hemoglobin–spectrin complexes in presence of H_2O_2: effect of ATP

When hemoglobin and spectrin were allowed to react in presence of 1mM H_2O_2 and 0.1mM DTT, two additional bands other than α- and β-spectrin appeared, one, just above the band of α-spectrin at ~300 kDa (designated as *band c*), and another at ~410 kDa (designated as *band b*). Also, an additional high

Table 1
Annexin binding to erythrocytes from thalassemic individuals

Blood samples	% Annexin V binding (*n*)
Normal red cells	1.53±0.06 (116)
β-Carrier (HbA2 >3.5%)	1.76±0.32 (14)
E-carrier (HbE 25–30%)	1.63±0.25 (3)
Eβ-thalasssemics (HbE 40–70%)	5.67±0.67 (25)

n denotes the number of different blood samples used for analysis.

molecular weight aggregate (HMWA, designated as *band a*) appeared on the top of the gel that did not enter into the gel (inset of Fig. 1, lanes 3 and 4), when hemoglobin samples contained ~30% HbE, from the HbE carriers and ~60% HbE from the HbEβ-thalassemic patients. The relative densities (RD) of *band b* and *band c*, in the presence of DTT, in cases of the β-thalassemia carriers, HbE carriers, and the HbEβ-thalassemics are shown in Fig. 1. Further analysis of band *b* alone, shown in Fig. 2, indicated the amount of cross-linked complexes in cases of the HbE carriers and HbEβ-thalassemic patients compared to the β-thalassemia carriers. Fig. 2 also shows the ΔRD% values of *band b*, indicating a large decrease of cross-linked complex, in the presence of 0.6 mM ATP and 6 mM $MgCl_2$, for the Eβ-thalassemic samples compared to both the β-thalassemia and hemoglobin E carriers.

Fluorescence analysis of the interaction of hemoglobin with spectrin and effects of ATP

Interactions of increasing concentrations of HbE-rich samples from the HbE carriers and the HbEβ-thalassemic patients with spectrin have been estimated by fluorescence technique, in presence and absence of Mg-ATP [31]. The histogram representation of the binding dissociation constants shows in Fig. 3, that the K_d (in μM) decreased considerably from 17.9±1.5 μM for the β-thalassemia carriers (with >95% HbA with 3% HbA2) to 5.2±0.5 μM in case of the HbEβ-thalassemics (with ~50% HbE and HbF<10%). ATP showed a small but a significant effect in increasing the binding affinities to spectrin.

Flow cytometric analysis of red cell membrane asymmetry

Table 1 summarizes the data on FITC-labeled annexin V binding to the red cells. The FITC-AV-treated red cells showed increased fluorescence and the HbEβ-thalassemic samples showed the highest FITC-AV binding compared to normal, or either the β-thalassemia or HbE carriers. Normal red cells after treatment with NEM together with the ionophore A23187 and Ca^{+2}, served as the positive control indicated ~60% binding of FITC-AV (not shown). The percent annexin V binding is expressed as the mean±SEM.

Discussion

The yield of the cross-linked complexes under oxidative stress condition, mimicked by exposure of hemoglobin samples to hydrogen peroxide, is highly dependent on the levels of HbE present in the blood samples of patients suffering from HbEβ-thalassemia. The yield of such complexes increased substantially in the hemolysate of both the HbE carriers (HbE 25–32%) and the HbEβ-thalassemic patients (HbE 40–50%) compared to β-thalassemia carriers and normal individuals. HMWA formation in both the HbE carrier and HbEβ-thalassemia patients samples, indicated by *band a*, was favored even in presence of the disulfide reducing agent DTT (inset of Fig. 1).

Intracellular concentration of ATP ranges from 1.2 to 1.8 mM in normal red cells [35]. In this work, we have used ATP concentration typically in the low millimolar range at around 0.5–1.0 mM. ATP decreased the yield of formation of the cross-linked products of spectrin and hemoglobin, under oxidative conditions only in case of samples from HbEβ-thalassemic patients with higher levels of HbE (50–60%) as compared to HbE carriers, shown in Fig. 2. However, the effect of Mg-ATP on the binding affinities were only marginal, the K_d decreasing from 17.2±1.5 μM to 14.4±1.2 μM in case of the HbE carriers (Fig. 3). We have chosen a hemoglobin concentration above 10 μM in order to assure the presence of significant amount of hemoglobin tetramers preventing the formation of hemoglobin dimers on the basis of earlier thermodynamic studies indicating the equilibrium constant of the conversion of hemoglobin from dimer to tetramer to be in the range of $1.0–1.2\times10^6$ M^{-1} between 20 and 27°C [36].

There are reports correlating the oxidative damage of erythrocytes and the alteration of phospholipid asymmetry. Hydrogen peroxide and hemoglobin oxidation products have both direct and indirect effects in extracting phospholipids from the membrane of the human erythrocytes [37–39]. The extent of PS exposure could act as the index of instability of the cytoskeleton protein network resulting from the deposition of oxidative cross-linked products inside the cell [40–42]. In the present work, we have observed a strong correlation between extent of PS exposure with the level of HbE in the thalassemic red cells. The extent of PS exposure increased from normal individuals to β-thalassemia carriers and HbE carriers with HbE levels <30% to the Eβ-thalassemic patients with HbE levels ranging from 50 to 60% with HbF levels <5% (Table 1).

References

[1] M.D. Scott, J.W. Eaton, F.A. Kuypers, et al., Enhancement of erythrocyte superoxide dismutase activity: effects on cellular oxidant defense, Blood 74 (1989) 2542–2549.
[2] M.D. Scott, B.H. Lubin, L. Zuo, F.A. Kuypers, Erythrocyte defense against hydrogen peroxide: preeminent importance of catalase, J. Lab. Clin. Med. 118 (1991) 7–16.
[3] D.T.-Y. Chiu, B.H. Lubin, S.B. Shohet, Peroxidative reactionsin red cell biology, in: W. Pryor (Ed.), Free Radicals in Biology, Vol. 5, Academic Press Inc., San Diego, 1982, pp. 115–160.
[4] E.C. Gordon-Smith, J.M. White, Oxidative haemolysis and Heinz body haemolytic anaemia, Br. J. Haematol. 26 (1974) 513–517.
[5] R.W. Carrell, C.C. Winterbourn, E.A. Rachmilewitz, Activated oxygen and haemolysis, Br. J. Haematol. 30 (1975) 259–264.
[6] I. Kahane, A. Shifter, E.A. Rachmilewitz, Cross-linking of red blood cell membrane proteins induced by oxidative stress in beta thalassemia, FEBS Lett. 85 (1978) 267–270.
[7] N. Sauberman, N.L. Fortier, W. Joshi, J. Piotrowski, L.M. Snyder, Spectrin–haemoglobin crosslinkages associated with in vitro oxidant hypersensitivity in pathologic and artificially dehydrated red cells, Br. J. Haematol. 54 (1983) 15–28.
[8] L. Leb, P. Beatson, N.L. Fortier, L.M. Snyder, The phagocytosis of circulating red blood cells (RBC) by fresh human monocytes, Blood 62 (1983) 5a.
[9] L.M. Snyder, S.C. Liu, F. Garver, et al., Direct demonstration of hemoglobin associated with purified spectrin from senescent human red cells, Blood 62 (1983) 40.

[10] S.L. Schirer, E. Rachmilewitz, N. Mohandas, Cellular and membrane properties of alpha and beta thalassemic erythrocytes are different: implication for differences in clinical manifestations, Blood 74 (1989) 2194–2202.
[11] S.L. Schrier, Thalassemia: pathophysiology of red cell changes, Annu. Rev. Med. 45 (1994) 211–218.
[12] A. Knyszynski, D. Danon, I. Kahane, E.A. Rachmilewitz, Phagocytosis of nucleated and mature beta thalassaemic red blood cells by mouse macrophages in vitro, Br. J. Haematol. 43 (1979) 251–255.
[13] E. Wiener, S.N. Wickramasinghe, The in vitro interaction between red cells from patients with homozygous β-thalassemia and normal human macrophages, Exp. Hematol. 13 (1985) 401a.
[14] U. Sen, J. Dasgupta, D. Choudhury, et al., Crystal structures of HbA2 and HbE and modeling of hemoglobin δ_4: interpretation of the thermal stability and the antisickling effect of HbA2 and identification of the ferrocyanide binding site in Hb, Biochemistry 43 (2004) 12477–12488.
[15] H. Frischer, J. Bowman, Hemoglobin E, an oxidatively unstable mutation, J. Lab. Clin. Med. 85 (1975) 531–539.
[16] D. Weatherall, J. Clegg, Thalassemia: a global public health problem, Nat. Med. 3 (1996) 847–849.
[17] D.C. Rees, J.B. Clegg, D.J. Weatherall, Is hemoglobin instability important in the interaction between hemoglobin E and thalassemia? Blood 92 (1998) 2141–2146.
[18] C.R. Kiefer, J.F. Trainor, J.B. McKenney, C.R. Valeri, L.M. Snyder, Hemoglobin–spectrin complexes: interference with spectrin tetramer assembly as a mechanism for compartmentalization of band 1 and band 2 complexes, Blood 86 (1995) 366–371.
[19] L.M. Snyder, N.L. Fortier, J. Trainor, et al., Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin–hemoglobin cross-linking, J. Clin. Invest. 76 (1985) 1971–1977.
[20] L. Leb, L.M. Snyder, N.L. Fortier, M. Andersen, Antiglobulin serum mediated phagocytosis of normal senescent and oxidized RBC: role of anti-IgM immunoglobulins in phagocytic recognition, Br. J. Haematol. 66 (1987) 565–570.
[21] L.M. Snyder, L. Leb, J. Piotrowski, et al., Irreversible spectrin–haemoglobin crosslinking in vivo: a marker for red cell senescence, Br. J. Haematol. 53 (1983) 379–384.
[22] Y.V. Borenstain-Ben, Y. Barenholz, E. Hy-Am, et al., Phosphatidylserine in the outer leaflet of red blood cells from beta-thalassemia patients may explain the chronic hypercoagulable state and thrombotic episodes, Am. J. Hematol. 44 (1993) 63–65.
[23] B. Lubin, D. Chiu, J. Bastacky, et al., Abnormalities in membrane phospholipid organization in sickled erythrocytes, J. Clin. Invest. 67 (1981) 1643–1649.
[24] F.A. Kuypers, J. Yuan, R.A. Lewis, et al., Membrane phospholipid asymmetry in human thalassemia, Blood 91 (1998) 3044–3051.
[25] B.L. Wood, D.F. Gibson, J.F. Tait, Increased erythrocyte phosphatidylserine exposure in sickle-cell disease: flow-cytometric measurement and clinical associations, Blood 88 (1996) 1873–1880.
[26] R. Advani, S. Sorenson, E. Shinar, et al., Characterization and comparison of the red blood cell membrane damage in severe human alpha- and beta-thalassemia, Blood 79 (1992) 1058–1063.
[27] H.M. Waters, J.E. Howarth, K. Hyde, et al., An evaluation of the Bio-Rad Variant Haemoglobin Testing System for the detection of haemoglobinopathies, Clin. Lab. Haematol. 20 (1998) 31–40.
[28] E.E. Di Iorio, Preparation of derivatives of ferrous and ferric hemoglobin, in: E. Antonini, L. Rossi-Bernardi, E. Chiancone (Eds.), Methods in Enzymology, vol. 76, Academic Press, New York, 1981, pp. 57–72.
[29] W.B. Gratzer, Preparation of spectrin, in: D.W. Frederiksen, L.W. Cunnigham (Eds.), Methods in Enzymology, vol. 85, Academic Press, New York, 1982, pp. 475–480.
[30] M. Bhattacharyya, S. Ray, S. Bhattacharya, A. Chakrabarti, Chaperone activity and Prodan binding at the self-associating domain of erythroid spectrin, J. Biol. Chem. 279 (2004) 55080–55088.
[31] P. Datta, S. BasuChakrabarty, A. Chakrabarty, A. Chakrabarti, Interaction of erythroid spectrin with hemoglobin variants: implications in beta-thalassemia, Blood Cells, Mol. Dis. 30 (2003) 248–253.
[32] W. Joshi, L. Leb, J. Piotrowski, et al., Increased sensitivity of isolated alpha subunits of normal human hemoglobin to oxidative damage and crosslinkage with spectrin, J. Clin. Lab. Med. 102 (1983) 46–52.
[33] F.A. Kuypers, R.A. Lewis, M. Hua, et al., Detection of altered membrane phospholipid asymmetry in subpopulation of human red blood cells using fluorescently labeled annexin V, Blood 87 (1996) 1179–1187.
[34] R.F.A. Zwall, A.J. Schroit, Pathophysiologic implication of membrane phospholipid asymmetry in blood cells, Blood 89 (1997) 1121–1132.
[35] T.A. Rapoport, R. Heinrich, S.M. Rapoport, The regulatory principles of glycolysis in erythrocytes in vivo and in vitro. A minimal comprehensive model describing steady states, quasi-steady states and time-dependent processes, Biochem. J. 154 (1976) 449–469.
[36] S.H. Ip, G.K. Ackers, Thermodynamic studies on subunit assembly in human hemoglobin. Temperature dependence of the dimer-tetramer association constants for oxygenated and unliganded hemoglobins, J. Biol. Chem. 252 (1977) 82–87.
[37] L.S. Brunauer, M.S. Moxness, W.H. Huestis, Hydrogen peroxide oxidation induces the transfer of phospholipids from the membrane into the cytosol of human erythrocytes, Biochemistry 33 (1994) 4527–4532.
[38] M.S. Moxness, L.S. Brunauer, W.H. Huestis, Hemoglobin oxidation products extract phospholipids from the membrane of human erythrocytes, Biochemistry 35 (1996) 7181–7187.
[39] K. de Jong, D. Geldwerth, F.A. Kuypers, Oxidative damage does not alter membrane phospholipid asymmetry in human erythrocytes, Biochemistry 36 (1997) 6768–6776.
[40] S. Manno, Y. Takakuwa, N. Mohandas, Identification of a functional role for lipid asymmetry in biological membranes: phosphatidylserine-skeletal protein interactions modulate membrane stability, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 1943–1948.
[41] J. Yuan, A. Bunyaratvej, S. Fucharoen, et al., The instability of the membrane skeleton in the thalassemic red blood cells, Blood 86 (1995) 3945–3950.
[42] J. Amer, A. Goldfarb, E. Fibach, Flow cytometric analysis of the oxidative status of normal and thalassemic red blood cells, Cytometry A 60 (2004) 73–80.