Antioxidant defense status of red blood cells of patients with 
β-thalassemia and Eβ-thalassemia

Dibyendu Chakraborty, Maitree Bhattacharyya*

Department of Biochemistry, University College of Science, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700 019, India

Received 14 August 2000; received in revised form 27 November 2000; accepted 5 December 2000

Abstract

Anemia in β-thalassemia is caused by a combination of ineffective erythropoiesis and premature hemolysis of RBC in the peripheral circulation. Excess of the α-globin chain present in β-thalassemic RBC is mainly responsible for oxidative damage of erythrocyte membrane protein. The activities of glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione peroxidase, and glutathione-S-transferase, and the catalytic activity of catalase and superoxide dismutase, and the concentrations of non-enzymic antioxidants such as reduced glutathione were measured to estimate the status of the antioxidant defense system in the erythrocytes for protection against oxidative stress. The extent of lipid peroxidation was also estimated in thalassemic erythrocytes. Significantly lower activities of reduced glutathione indicate the cell to be in a pro-oxidant state and decreased activity of catalase favors hydrogen peroxide-mediated lipid peroxidation in β-thalassemic and Eβ-thalassemic RBC. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Oxidative stress; Antioxidants; β-Thalassemia; Eβ-Thalassemia

1. Introduction

Beta-thalassemias are genetic disorders which encompass a wide variety of clinical phenotypes ranging in severity from clinically silent heterozygous β-thalassemia to severe transfusion-dependent thalassemia major [1]. Shortened red cell life span, rapid iron turnover, and tissue deposition of excess iron are the major factors responsible for the functional and physiological abnormalities found in the various forms of thalassemia. Beta-thalassemic RBCs have an altered morphology and exhibit a decreased deformability as measured by ektacytometry. This altered deformability is, in part, due to the rigidity of their membranes and the state of hydration of these cells [2].

Beta-thalassemia hemoglobin E is the most common form of hemoglobinopathy in southeast Asia and India, and is mostly due to the interaction of ββ-thalassemia with HbE. HbEβ-Thalassemia shows a remarkable degree of variability of clinical expression, very similar to homozygous β-thalassemia [3,4]. Severe oxidative damage is observed in erythrocytes due to the presence of an excess of α-globin chain [5]. Antioxidants are potentially protective agents which help to guard against oxidative hemolysis and its clinical manifestation in Eβ-thalassemia. This paper reports an attempt to evaluate the
enzymatic antioxidant defense system in the erythrocytes of different β-thalassemia and Eβ-thalassemia patients. The glutathione-mediated antioxidant defense mechanism was also an interesting feature of this work.

2. Materials and methods

2.1. Chemicals

Glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), glucose-6-phosphate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Tris(hydroxymethyl)aminomethane (TRIS), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) and ethylenediaminetetraacetic acid (EDTA) disodium salt were purchased from Sigma (St. Louis, MO, USA). 2-Mercaptoethanol, 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from E. Merck. The oral iron chelator deferiprone (L1) was obtained as a gift from Cipla (Mumbai Central, India). All other chemicals were of analytical grade and purchased locally.

2.2. Clinical samples

The β-thalassemia and Eβ-thalassemia patients were previously diagnosed and were under the supervision of medical professionals during this period. The patients were transfusion dependent. Blood from thalassemic patients was collected just before transfusion and none had transfused for at least 3 months. The group of carrier or trait subjects were the brothers, sisters or cousins of those diagnosed as a carrier or with a trait. All were symptom-free and lived a normal life with a normal hemoglobin concentration requiring no blood transfusions. Blood from healthy individuals was used as a normal control for this study. All individuals were aged between 5 and 15 years.

2.3. Isolation of plasma and hemolysate

Plasma was separated by centrifugation of the blood sample at 3000×g for 15 min. After separation of the buffy coat, the packed red blood cells (RBC) were washed twice with two volumes of isotonic saline solution at pH 7.0. The washed RBCs were then hemolysed by suspending them in double distilled water and centrifuged at 15 000×g for 40 min. The supernatant representing the hemolysate was collected and centrifuged two times at 15 000×g for 40 min and used directly for determinations.

2.4. Isolation of erythrocyte ghost membranes

The erythrocyte membrane was isolated according to the procedure Kuross et al. [6] with some modifications. After removal of the buffy coat, 0.5 ml of washed RBC was added to 45 ml cold lysing buffer (5 mmol/l sodium phosphate, 1 mmol/l EDTA, 1 mmol/l PMSF, pH 8.0). Extensive washes with the same buffer at 0°C were performed by 4 min centrifugation at 13 000×g in a refrigerated centrifuge. Between each wash the ghost pellet was passed five times through a 22-ga needle to assist lysis of any residual red pellet material. Three washes are usually sufficient to obtain hemoglobin-free membranes, but sometimes additional washes are needed until no hemoglobin can be detected in the supernatant.

2.5. Assay of enzymic antioxidants

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) was measured by the method of Lohr and Waller [7]. Glutathione reductase (GR, EC 1.6.4.2) was assayed by the method of Delides et al. [8]. Glutathione peroxidase (GPx, EC 1.11.1.9) was assayed by the method of Paglia and Valentine [9]. Superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were assayed following the method of Paoletti et al. [10] Beers et al. [11], respectively. Glutathione-S-transferase (GST, EC 2.5.1.18) was measured according to the method of Levender et al. using 1-chloro-2,4-dinitrobenzene as the substrate [12].

2.6. Determination of non-enzymic antioxidants

Reduced glutathione (GSH) was measured in RBC by the method of Beutler et al. [13] using Ellman’s reagent.
2.7. Estimation of MDA

Lipid peroxidation was assayed by measuring the formation of malondialdehyde (MDA), which was estimated following the method of Takayama et al. [14] using thiobarbituric acid (TBA) and trichloroacetic acid (TCA). Erythrocyte membranes in 5 mmol/l phosphate buffer (pH 7.4) were incubated with 5 mmol/l H₂O₂ for 30 min at 37°C. The reaction was stopped by adding trichloroacetic acid (TCA) and TBA. After boiling for 20 min, the samples were cooled, centrifuged, and the absorbance of the supernatant was determined at 532 nm. To quantify lipid peroxidation, we calculated the concentrations of malondialdehyde (MDA), a major semicarbazone (BHA) and trichloroacetic acid (TCA), using an extinction coefficient of 1.56×10³ (mol/l⁻¹ cm⁻¹) and expressed the results in nanomoles of MDA per milligram membrane protein.

2.8. Estimation of protein

Protein was estimated according to the method of Lowry et al. using bovine serum albumin (BSA) as standard [15].

2.9. Measurement of membrane iron

The free iron on ghost membranes was determined by its reactivity with ferrozine (which does not detect heme iron) in the presence of the denaturant sodium dodecyl sulfate and the reducing agents ascorbate and sodium metabisulphite [16]. The term ‘free iron’ is used here according to the previously validated convention [17] and refers to non-heme non-ferritin iron that reacts within 2 min in the ferrozine assay.

2.10. Statistical analysis

The values are expressed as mean±S.D. and statistically significant differences between values were obtained using the two-tail Student’s t-test.

3. Results

Hematological parameters determined from thalassemic patients, carrier subjects and controls are presented as mean±S.D. in Table 1. Table 2 shows the enzymatic antioxidant status and reduced glutathione concentration in the red blood cells of patients with β-thalassemia, Eβ-thalassemia, with β-thalassemia trait, HbE carrier and control subjects. The activities of G6PD, GR, GPx and SOD were higher (P < 0.001) in β-thalassemic RBC [degrees of freedom (df) = 93, t = 11.74, 26.98, 23.66, 9.36, respectively] and Eβ-thalassemic RBC [df = 96, t = 15.11, 4.59, 22.25, 11.50, respectively] compared to controls. The reduced glutathione concentration was significantly decreased (P < 0.001) in β-thalassemic (df = 93, t = 24.89) and Eβ-thalassemic RBC (df = 96, t = 23.39) compared to control subjects. The reduced glutathione (GSH) level was increased in β-thalassemia trait (P < 0.01, df = 86, t = 3.208) and, in HbE carriers, SOD activities were increased (P < 0.01, df = 88, t = 3.15) compared to control subjects.

Table 1
Hematological data and diagnostic indicators (mean±S.D.) of thalassemic patients, carriers and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 50)</th>
<th>β-Thalassemia trait (n = 38)</th>
<th>β-Thalassemia (n = 45)</th>
<th>HbE carrier (n = 40)</th>
<th>Eβ-Thalassemia (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Hb (g/dl)</td>
<td>12.8±0.3</td>
<td>10.6±0.4</td>
<td>7.3±0.6</td>
<td>11.2±0.3</td>
<td>8.1±0.2</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>1.2±0.1</td>
<td>1.4±0.2</td>
<td>40.4±18.7</td>
<td>1.44±0.65</td>
<td>24.14±5.59</td>
</tr>
<tr>
<td>HbA₂ (%)</td>
<td>2.42±0.25</td>
<td>4.6±0.3</td>
<td>2.3±0.2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HbE + HbA₂ (%)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>23.56±2.49</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>HbA (%)</td>
<td>96.4±0.3</td>
<td>94.1±0.5</td>
<td>57.3±18.9</td>
<td>75.0±3.1</td>
<td>73.5±5.9</td>
</tr>
<tr>
<td>Mean corpuscular volume (fl)</td>
<td>82.2±1.3</td>
<td>60.3±1.5</td>
<td>73.4±1.4</td>
<td>74.2±1.4</td>
<td>69.7±1.1</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>26.7±0.2</td>
<td>21.8±1.4</td>
<td>23.02±0.02</td>
<td>23.5±0.1</td>
<td>20.6±0.23</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39.4±1.3</td>
<td>33.4±1.2</td>
<td>26.3±1.4</td>
<td>34.4±1.4</td>
<td>27.4±1.1</td>
</tr>
</tbody>
</table>

n.d., not detectable; n, number of subjects studied.
Table 2
Glutathione status and the specific activity of the different enzymes of the glutathione-mediated antioxidant defense system in RBC of β-thalassemia, Eβ-thalassemia, β-thalassemia trait, HbE carrier and control groups

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Controls (n = 50)</th>
<th>β-Thalassemia trait (n = 38)</th>
<th>β-Thalassemia (n = 45)</th>
<th>HbE carrier (n = 40)</th>
<th>Eβ-Thalassemia (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD (nmol of NADPH produced/mg protein/min)</td>
<td>1.59±0.26</td>
<td>1.52±0.12</td>
<td>2.31±0.32*</td>
<td>1.50±0.12</td>
<td>2.69±0.40*</td>
</tr>
<tr>
<td>GR (nmol of NADPH oxidised/mg protein/min)</td>
<td>1.72±0.17</td>
<td>1.71±0.13</td>
<td>2.86±0.23*</td>
<td>1.38±0.17*</td>
<td>1.88±0.17*</td>
</tr>
<tr>
<td>GST (nmol product formed/mg protein/min)</td>
<td>1.97±0.25</td>
<td>1.97±0.19</td>
<td>1.96±0.15</td>
<td>1.95±0.14</td>
<td>1.94±0.11</td>
</tr>
<tr>
<td>GPx (nmol NADPH oxidised/mg protein/min)</td>
<td>7.58±0.45</td>
<td>7.69±0.54</td>
<td>10.35±0.67*</td>
<td>7.21±0.58**</td>
<td>10.65±0.85*</td>
</tr>
<tr>
<td>SOD (units/mg protein/min)</td>
<td>1.48±0.15</td>
<td>1.49±0.16</td>
<td>1.79±0.17*</td>
<td>1.59±0.18**</td>
<td>1.85±0.16</td>
</tr>
<tr>
<td>CAT (mol H₂O₂ consumed/mg protein/min)</td>
<td>0.119±0.013</td>
<td>0.1137±0.012</td>
<td>0.1168±0.007</td>
<td>0.1201±0.012</td>
<td>0.113±0.012**</td>
</tr>
<tr>
<td>GSH (μmol/mg protein)</td>
<td>4.42±0.26</td>
<td>4.64±0.41**</td>
<td>2.95±0.31*</td>
<td>4.54±0.42</td>
<td>3.25±0.22*</td>
</tr>
</tbody>
</table>

Significantly different from control subjects: *P < 0.001, **P < 0.01.

Table 3 presents the ghost membrane-bound free iron in β-thalasemic and Eβ-thalassemic RBC. Incubation in the presence of the oral iron chelator deferoxamine (L1) reduced the concentration of membrane-bound free iron. Fig. 1 illustrates lipid peroxidation expressed as MDA concentration under hydrogen peroxide stress. Prior incubation of β-thalasemic and Eβ-thalassemic red blood cells with deferoxamine reduced MDA production of the cell under H₂O₂ stress.

4. Discussion

Reduced glutathione (GSH) is an important scavenger of free radicals and a potent endogenous antioxidant which helps to protect cells from oxidative injury. Besides its role in the maintenance of the redox potential within the cell, it is also a key component of the enzymatic antioxidant system. The reduced glutathione (GSH) concentration was observed to be significantly lower in β-thalassemic and Eβ-thalassemic RBC compared with carrier and control subjects, suggesting that the erythrocyte is in a pro-oxidant condition which may be a partial cause of the increased hemolysis and shortened RBC survival observed in β-thalassemic and Eβ-thalassemic RBC.

The activity of glutathione reductase and glucose-6-phosphate dehydrogenase was increased when measured in β-thalassemic and Eβ-thalassemic erythrocytes compared to carrier and control sub-

Table 3
Membrane-bound free iron in thalassemic and normal RBC

<table>
<thead>
<tr>
<th></th>
<th>Incubation without deferiprone (−L1)</th>
<th>Incubation with deferiprone (+L1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (N = 10)</td>
<td>0.32±0.08</td>
<td>0.27±0.02**</td>
</tr>
<tr>
<td>β-Thalassemia (N = 12)</td>
<td>18.72±6.34</td>
<td>9.46±2.15*</td>
</tr>
<tr>
<td>Eβ-Thalassemia (N = 15)</td>
<td>12.18±4.41</td>
<td>8.70±1.43**</td>
</tr>
</tbody>
</table>

* Membrane-bound free iron is expressed in nmol/mg protein.
* Membrane-bound free iron, incubation without deferiprone (−L1) at 37°C for 2 h.
* Membrane-bound free iron, incubation with 600 μM deferiprone (+L1) at 37°C for 2 h.
* Membrane-bound free iron, incubation with deferiprone (−L1) at 37°C for 2 h.
* Membrane-bound free iron, incubation with deferiprone (−L1) at 37°C for 2 h.
* Indicates significantly (P < 0.001) from β-thalassemia without deferiprone (−L1); **indicates significantly (P < 0.01) from Eβ-thalassemia without deferiprone (−L1).
Fig. 1. Deferiprone (L1) inhibits the peroxide response of β-thalassemic and Eβ-thalassemic membranes to H₂O₂ stress. MDA content in H₂O₂-stimulated RBC membranes obtained from thalassemic and normal RBCs previously incubated without deferiprone (−L1) and with deferiprone (+L1, 600 μM) for 2 h at 37°C.

jects. It can be inferred that, in β-thalassemic and Eβ-thalassemic RBC, increased activities of enzymes that contribute to regeneration of GSH, either directly by glutathione reductase or indirectly by glucose-6-phosphate dehydrogenase, are not sufficient to regenerate GSH to meet the cell’s requirement. Glucose-6-phosphate dehydrogenase generates NADPH, which is important in maintaining the integrity of certain proteins in mature erythrocytes. The observed increased activity of glucose-6-phosphate dehydrogenase in β-thalassemic and Eβ-thalassemic patients may be rationalized by the increased need of the erythrocytes for NADPH or pentose for maintaining cell integrity.

This work investigated the antioxidant status of thalassemic erythrocytes by direct measurement of cytoprotective enzymes. During normal cellular metabolism, cellular defenses consist of the cytoprotective enzymes superoxide dismutase, catalase and glutathione peroxidase. A response to oxidative stress perturbs the normal cellular metabolism, including an increase in intracellular free calcium, concentration damage to membrane ion transporter proteins and peroxidation of lipids.

The catalytic activities of superoxide dismutase and glutathione peroxidase in β-thalassemic and Eβ-thalassemic RBC were also increased significantly ($P < 0.001$) compared to control and carriers. It is interesting that no such enhanced catalytic activity was observed for catalase in thalassemic erythrocytes. Glutathione peroxidase activity increased significantly in β-thalassemia and Eβ-thalassemia patients compared to carrier and control subjects. The increased activity of SOD in patients with β-thalassemia and Eβ-thalassemia may be involved in scavenging the superoxide radical ($O_2^-$), thereby producing more hydrogen peroxide in the erythrocytes. Catalase counteracts hydrogen peroxide and protects the cell from peroxidative damage. However, no such significant increase of catalase activity was observed, which suggests that the complete removal of hydrogen peroxide, produced by increased superoxide dismutase activity in thalassemic RBCs, was not possible. The activity of glutathione-$S$-transferase was not significantly variable in thalassemic RBC.

The carrier subjects with the β-thalassemia trait showed increased concentrations ($P < 0.01$) of re-
duced glutathione (GSH), but HbE carriers showed lower glutathione reductase ($P < 0.001$) and GPx ($P < 0.01$) activity, but higher SOD ($P < 0.01$) activity compared to control subjects.

Oxidative stress is a consequence of the disease process in β-thalassemia [18], which is evident as an increase in the plasma lipid peroxidation product MDA [19]. Lipid peroxidation of erythrocytes is manifested by MDA production (Fig. 1), but freshly drawn RBCs from β-thalassemic and Eβ-thalassemic patients do not exhibit any significant increase in the concentration of MDA. This may be due to the prior removal of oxidatively injured cells from the circulation. The carbonyl groups of MDA are highly reactive and are capable of cross-linking with protein and phospholipid amino groups [20]. Since cross-linking of membrane components may stiffen the red cell membrane, injured MDA-rich cells may be removed by splenic entrapment.

The ghost membrane of β-thalassemic and Eβ-thalassemic RBC contains large amounts of free iron. Our studies showed that (Fig. 1) pre-exposure of both β-thalassemic and Eβ-thalassemic RBC to an iron-chelating drug, deferiprone (L1), decreased the extent of the $H_2O_2$-induced generation of MDA by the membrane. Since MDA is a typical product of free iron-mediated oxidative damage [21], these findings support the contention that, by chelating free iron, L1 removes a significant fraction of redox-active iron from the membrane (Table 3), and thereby attenuates its susceptibility to oxidant-induced damage. The enhanced activity of superoxide dismutase in β-thalassemic and Eβ-thalassemic RBC saves the cell by scavenging superoxide radical ($O_2^-$), but as the catalase activity is not enhanced, the higher $H_2O_2$ concentration is not totally removed. It is likely to react with membrane-bound free iron, enabling free iron-mediated oxidative damage of thalassemic RBC membranes. Thus, the removal of allegedly injurious iron from thalassemic membranes can be of benefit for RBC survival. The oral iron chelator deferiprone (L1) binds to hemoglobin and alters its conformation [22]. This therapeutically important drug not only reduces transfusional systemic iron overload [23,24], but also removes iron from its potentially toxic location on the membranes of hemoglobinopathic RBCs [25]. Deferiprone (L1) is only partially effective in removing abnormal RBC membrane iron in the model murine β-thalassemia and provides the first evidence in an in vitro model that abnormal free iron deposits contribute to RBC membrane pathobiology and that chelation of such iron may improve RBC function [26].

Beta-thalassemia and other hemoglobinopathies are widespread on the Indian subcontinent [27,28] and appear to be predominant in certain communities, where the reported incidence is about 6–10% [29]. Thus, understanding the clinical manifestations of β-thalassemia major, β-thalassemia trait HbE carrier and Eβ-thalassemia from the viewpoint of the enzymatic antioxidant defense system in diseased erythrocytes is of great significance for the future management of these patients.

Acknowledgements

We are indebted to Dr. R.N. Ghosh, Department of Hematology, Calcutta Hospital, and Dr. S.B. Dutta and Dr. K. Das, Life Care Blood Transfusion Centre, for providing the thalassemic blood samples. This work was supported financially by the University Grants Commission, New Delhi.

References


Bartlett AN, Hoffbrand AV, Kontoghiorghes GJ. Long term trial with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1). II. Clinical observations. Br J Haematol 1990;76:301–5.


