ORIGINAL ARTICLE

Oxidation Status of β-Thalassemia Minor and Hb H Disease, and Its Association with Glycerol Lysis Time (GLT_{50})

Chris Adhiyanto1,2, Yukio Hattori1, Yasuhiro Yamashiro1, Ferania Mella1, Takenori Nitta1, Mizuki Iihoshi1, Satuki Araki1, Maryam Matar3, and Fumiya Takagi4

1Department of Health Science, Yamaguchi University Graduate School of Medicine, Ube, Japan,
2Faculty of Medicine and Health Science, Universitas Islam Negeri Syarif Hidayatullah, Jakarta, Indonesia,
3United Arab Emirates Genetic Disease Association, Dubai, United Arab Emirates,
4Fukuyama Rihshou KK, Fukuyama Medical Center, Fukuyama, Japan

Abstract

β-Thalassemia (β-thal), especially β-thalassemia major (β-TM), is reported to be related to reactive oxygen species (ROS) and enhanced oxidation status. It is reflected by increased malondialdehyde (MDA), by membrane lipid peroxidation and decreased by the newly developed total antioxidant capacity (TAC). However, there is less evidence for β-thal minor and Hb H (β4) disease on its association with oxidation status. On the other hand, hemolysis by glycerol lysis time (GLT_{50}) is invariably prolonged in thalassemia. The reason for the prolongation of GLT_{50} is not well understood. The aim of this study was to investigate the oxidation state in β-thal minor and Hb H disease and to find out the association of the oxidation with the prolongation of GLT_{50}. Blood samples from 39 subjects (33 with β-thal minor, six with Hb H disease) were collected from individuals living in Japan. The clinical screening tests and molecular identification of the thalassemias were performed. Malondialdehyde and TAC were measured using spectrophotometric analyses. In β-thal minor and Hb H disease, the plasma MDA level was significantly elevated and the TAC reduced. A highly reversed correlation between MDA and TAC was noted. Their GLT_{50} levels were evidently prolonged, and the GLT_{50} has significant correlations with MDA and TAC. β-Thalassemia minor and mild Hb H disease are evidently in a milieu of reduced redox state, and GLT_{50} prolongation in β-thal minor and Hb H disease has a close correlation with the oxidation state, possibly by oxidative impairment of the membrane protein of the red cell.

Introduction

β-Thalassemia (β-thal) is a hereditary hemoglobinopathy, and common in tropical and subtropical areas. In Japan it is less common and mostly β-thal minor, a clinically milder form. Most thalassemias are caused by defective production of either α- or β-globin chains that form Hb tetramers (α2β2). Another globin produced from intact genes is normally produced in half the amount of a normal person. The net reduction of hemoglobin (Hb) tetramer brings about microcytic red cells (microcytosis) that are universally seen in all types of thalassemias. When the imbalance is extreme, such as in β-thal major (β-TM), it is clinically of a more severe type, the normally produced α-globin denatures in the erythroblasts and eventually lets the red blood cells (RBCs) undergo hemolysis. These hemolytic involvements are not seen in β-thal minor, presumably because the excess globin normally produced in any amount would completely undergo hydrolysis by the proteolytic enzymes in the erythroblast.

On the other hand, the RBCs cells in patients with β-TM are exposed to a high oxidation state in the blood. The oxidation of the denatured globin in erythroblasts or RBCs causes release of free iron. In turn, free iron, by non enzymatic reaction, reacts with oxygen and releases reactive oxygen species (ROS) that initiate the process of oxidation of lipids and proteins the of the red cell membrane, and render the membrane rigid. Membrane rigidity may cause the RBCs to be susceptible to lysis in the circulatory system (1). However, there is still very little known about the association of the oxidation states for β-thal minor, which actually has no hemolytic involvement. Oxidation of RBCs is often measured by the malondialdehyde (MDA) method. Malondialdehyde is a natural organic compound and one marker for oxidative stress. In RBCs, ROS degrade polyunsaturated lipid membrane to form MDA (2,3) whose levels are elevated in β-TM.
(4, 5), while it is uncertain in β-thal minor. Total antioxidant capacity (TAC) is a new method used to measure the total antioxidant status in biological samples, and is the representative of all antioxidant states in a sample (6). On the other hand, the rigidity of the RBC membranes could be checked by glycerol lysis time (GLT 50), where the time (in seconds) for half lysis of RBCs on exposure to the solution composed of high-concentration of glycerol and low-concentration of salt is measured spectrophotometrically (7,8). The GLT 50 in β-thal minor is invariably longer than normal (9). The principle of GLT 50 is that the glycerol in hypotonic-buffered saline competes with water in influx and delays the osmotic burst of RBCs. Although the reason is uncertain, the thalassemic red cells take more time before lysis, possibly due to the effect of membrane oxidation. Here, the plasma MDA and TAC, as indicators of the oxidation level and GLT 50, were measured for β-thal minor and Hb H (β4) disease, and the redox state were studied. In addition, the association of GLT 50 with the oxidation state was also investigated. The correlation of Hb level as an indicator of phenotypic severity to the oxidation state was also studied.

### Materials and methods

Blood samples from 39 subjects suspected of carrying thalassemia and 12 normal controls were collected from individuals living in Japan. The blood sample, 5 mL, was collected from each individual with EDTA as an anticoagulant. Informed consent was obtained prior to the blood sampling. A complete blood count (CBC) was performed using conventional methods at Fukuyama Rinshou KK, Fukuyama Medical Center, Fukuyama, Japan. Hb F, Hb A2, isopropanol test, GLT 50 and isoelectric focusing (IEF) were performed as the clinical screening tests for thalassemias. Molecular identification of the thalassemias was carried out by DNA sequencing for β-thal and gap-polymerase chain reaction (gap-PCR) for α-thalassemias. The DNA analysis conducted for the β-globin gene was amplified by PCR using the following primers: forward (5'-AGT AGC AAT TTA TGG TAC TGA TGG TAT GG-3'; reverse, 5'-TTT CCC AAG GTT TGA ACT AGC TCT T-3'). The PCR product was isolated by agarose gel electrophoresis, excised and purified by QIAquick gel extraction kit (Qiagen, Tokyo, Japan; www.qiagen.com). After dideoxy reaction using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan), it was subjected to DNA sequencing (ABI PRISM™ 3100 Avant Genetic; Applied Biosystems, Tokyo, Japan; customerservice@lifetech.com). Meanwhile, the primers for gap-PCR for α-thal and α triplication were: 5'-GAT GCA CCC ACT GGC ATC CTT G-3' and 5'-CCC ATG CTG C-3' and 5'-GAT GCA CCC ACT GGC ACT CCT GC-3' and 5'-AAC ACC TCC ATT GTT GGC ACA TCC C-3' (for anti-α3.7).

Malondialdehyde in plasma was measured using Cayman’s TBAR Assay Kit (Cat. #10009055; Cayman Chemical Company-Funakoshi Co. Ltd., Osaka, Japan; cayman@caymanchem.com). Total antioxidant capacity was measured using Abcam (Cat. #ab65329; Abcam Company, Cambridge, MA, USA; webmaster@abcam.com). They were in equilibrium with the RBCs. Statistical analysis was performed using StatFlex V6 English version (Artech Co., Ltd, Osaka, Japan; statflex@statflex.net).

### Results

Genetic evaluation of 39 blood samples detected 33 β-thal minor and six Hb H disease patients. To investigate an oxidation process associated with β-thal minor, the level of MDA and TAC in plasma of the affected individuals was examined (Table 1).

(i) The MDA and TAC in β-thal minor were evidently higher and lower, respectively than in the normal controls. Thus, it was suggested that the ROS also affected the β-thal minor, which seemed to hold true for Hb H disease too. However, no statistical difference was observed between β-thal minor and Hb H disease. (ii) The GLT 50 of β-thal minor and Hb H disease also demonstrated evident prolongation (p < 0.001). However, no significant difference was found between β-thal minor and Hb H disease.

Table 2 shows all of the samples together and the correlation between the parameters. (iii) The MDA and TAC displayed a remarkably significant reverse correlation (r: −0.786, p < 0.001*). Thus, when the oxidation state is enhanced, the TAC is decreased. (iv) The GLT 50 has considerable correlation with TAC and MDA (p < 0.01*), especially with the former. Thus, GLT 50 seems to be associated with MDA and TAC, or oxidation (redox) status. (v) The Hb level has no significant correlation with GLT 50, MDA and TAC.

### Table 1. Glycerol Lysis Time, Malondialdehyde and Total Antioxidant Capacity Values for β-Thalassemia Minor, Hb H Disease and the Normal Controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>β-Thal Minor (n = 33)</th>
<th>Hb H Disease (n = 6)</th>
<th>Normal Controls (n = 12)</th>
<th>p Value β-Thal Minor vs. Hb H Disease vs. Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>10.90 ± 2.10</td>
<td>9.40 ± 1.42</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>GLT 50</td>
<td>146.30 ± 83.90</td>
<td>178.00 ± 80.00</td>
<td>31.30 ± 1.40</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MDA serum (μM)</td>
<td>9.62 ± 2.80</td>
<td>8.60 ± 2.50</td>
<td>4.50 ± 0.90</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TAC serum (nM TE)</td>
<td>5.62 ± 0.72</td>
<td>5.66 ± 0.46</td>
<td>7.20 ± 0.96</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

N.D.: not determined; GLT 50: glycerol lysis time (in seconds); MDA: malondialdehyde; TAC: total antioxidant capacity; TE: trolox equivalent; p < 0.001* showed significance.

### Table 2. Correlation of Membrane Rigidity and Oxidation Status.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GLT 50</th>
<th>MDA</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLT 50</td>
<td>r: 0.381; p &lt; 0.01*</td>
<td>r: −0.572; p &lt; 0.01*</td>
<td>r: −0.786; p &lt; 0.001*</td>
</tr>
<tr>
<td>MDA</td>
<td>r: −0.372; p &lt; 0.01*</td>
<td>r: −0.786; p &lt; 0.001*</td>
<td>—</td>
</tr>
</tbody>
</table>

GLT 50: glycerol lysis time (in seconds); MDA: malondialdehyde; TAC: total antioxidant capacity; r: regression; p < 0.001* showed significance.
Discussion

Several studies have reported that increased oxidative stress in β-TM is related to an excess of denatured α- or β-globin chains, iron overload and decreased Hb level (10). The oxidation that occurs in the erythroblasts is expected to cause changes in membrane structure of the erythroblasts and induces premature apoptosis (4,11), leading to intramedullary hemolysis, or ineffective erythropoiesis. Although β-thal minor may share the same pathophysiology, the amount of the denatured α- or β-globin chain would be much less than in β-TM, and the clinical picture often becomes asymptomatic. However, in our experiments, it was found that even the oxidation status of β-thal minor was under enhanced, which was reflected by higher MDA and lower TAC levels (Table 1). Our Hb H disease cases were all compound heterozygotes for −α^3.7 and the Southeast Asian α^0-thal (−SEAs) type that tends to manifest a milder clinical severity (12,13). The RBCs of β-thal minor and mild Hb H disease are thus not only in a milieu of decreased redox state. In addition, even slight oxidative stress may lead to precipitation of Hb that is seen in the in vitro addition of brilliant cresyl blue (BCB), mild oxidant in Hb H disease. In our study, the dominant β-thal that is characterized by hemolytic involvement demonstrated extraordinary elevated MDA levels. Excess burden of elimination of denatured, highly unstable β-globin, in addition to unpaired α-globin, may give rise to increased ROS generation.

β-Thalassemia minor demonstrates poikilocytosis, mainly of target cells and decreased osmotic fragility. Therefore, the membrane of thalassemic RBCs is suggested to be altered. The GLT50 is known to have close association with the osmotic fragility test where the thalassemic red cells are resistant to osmotic lysis (14). Thus, it is suspected that the membranes of the thalassemic RBCs are osmotically ‘rigid.’ The GLT50 is invariably prolonged in β-thal minor or heterozygous β-thal (8,9), and has been used for the screening of thalassemia. We examined the correlation of GLT50 to oxidation markers, or MDA and TAC in β-thal minor and Hb H disease. It was highly correlated with both MDA and TAC (Table 2). Therefore, GLT50 is very likely to be associated with oxidation. Kahan and Rachmilewitz (14) described only 50.0% of thiol groups in thalassemia compared to normal membranes and osmotic fragility was normalized by administration of vitamin E. The authors concluded that resistance to osmotic fragility was caused by oxidative stress leading to cross-linked and rigid membranes.

It was reported that both glycerol and water flow across the red cell membrane via Aquaporin 3 (AQP3) that lies on the red cell membrane as an oligomeric integral water channel protein (WCP) (15). The excess of free α-globin in β-thal is very susceptible to oxidation and the generation of globin radicals is well established. The latter react more easily with cytoskeletal and membrane proteins rather than penetrate into the lipid bilayer. McMillan et al. (16) reported that the Hb radical is less lipophilic and difficult to penetrate the lipid bilayer. However, interaction between globin radicals and membrane proteins such as AQP3 may cause impaired function from the membrane surface. It might be assumed that AQP3 in thalassemic RBCs denatured by oxidation of its thiol groups, resulting in delayed entrance of glycerol and delayed of hemolysis. The presence of up to six thiols in APQ (16) make, besides intramolecular adducts, intramolecular reaction possible between thiols forming cystine from two cysteins with essential impact on protein conformation and function (16–18).

Similar to Kahan and Rachmilewitz (14), Freisleben et al. (19) reported changes in thiol status in RBC membranes indicating oxidation processes and rigidified environment in membrane proteins. Concomitantly, changes in lipid domains of the RBC membranes of thalassemia patients were detected (20). In β-thal minor patients, isolated RBC membranes exhibited increased order parameters, i.e., increased rigidity (20) correlated with oxidative stress (21). Thus, it may be assumed that the membrane of thalassemic RBCs undergo not only the oxidation by membrane lipid peroxidation suggested by increased MDA level, but also impairment of membrane protein that gives rise to delayed water influx through the membrane, delayed hemolysis, and prolonged GLT50.

However, other WCPs such as Aquaporin 1 (AQP1) that is specific to water channels may still be intact. Hence, GLT50 might be more sensitive to thalassemia than to osmotic fragility tests (22). Although the cause of the prolonged GLT50 in thalassemia is not certain, it may be related to AQP3 denaturation. Further studies on permeability of glycerol as well as water in thalassemic RBCs are necessary.

The correlation of Hb level as a marker of phenotypic severity with MDA and TAC was not significant. The reason is unknown, but there may be various factors that influence the levels of MDA as discussed in (23) and TAC which was reassessed in (24).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

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