Methemoglobinemia and ascorbate deficiency in hemoglobin E β thalassemia: metabolic and clinical implications

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Plenary paper

During investigations of the phenotypic diversity of hemoglobin (Hb) E β thalassemia, a patient was encountered with persistently high levels of methemoglobin associated with a left-shift in the oxygen dissociation curve, profound ascorbate deficiency, and clinical features of scurvy; these abnormalities were corrected by treatment with vitamin C. Studies of erythropoietin production before and after treatment suggested that, as in an ascorbate-deficient murine model, the human hypoxia induction factor pathway is not totally dependent on ascorbate levels. A follow-up study of 45 patients with HbE β thalassemia showed that methemoglobin levels were significantly increased and that there was also a significant reduction in plasma ascorbate levels. Haptoglobin levels were significantly reduced, and the high frequency of the 2.2 haptoglobin genotype may place an additional pressure on ascorbate as a free-radical scavenger in this population.

Introduction

Although low ascorbate levels have been observed in patients with different hemoglobinopathies,1,2 there are very few reports of the clinical manifestations of scurvy in these conditions.3 During an analysis of the mechanisms for the broad phenotypic diversity of hemoglobin E (HbE) β thalassemia in Sri Lanka, a patient was encountered with profound ascorbate deficiency and clinical features of scurvy who also had a high level of methemoglobin. This unusual combination of findings has raised several important questions. First, to what extent does ascorbate deficiency interfere with the hypoxia-sensing mechanism in humans, particularly with respect to erythropoietin response to anemia? The key players in this pathway are the prolyl hydroxylase domain-containing enzymes that catalyze the prolyl-4-hydroxylation of the hypoxia-inducible factor in the presence of oxygen and 2-oxoglutarate as cosubstrates with iron and ascorbic acid as cofactors.4-6 Recent work in ascorbate-deficient mice suggests that other fail-safe mechanisms are involved in this reaction and that erythropoietin response is not altered in ascorbate deficiency.7,4; is this the case in humans? The second question raised by this unusual patient report is, because of the potential deleterious effects of methemoglobin on a patient’s response to anemia9 and the vascular endothelium,10 (1) how common are increased levels of methemoglobin in HbE β thalassemia and related disorders, (2) to what extent might this depend on ascorbate deficiency, and (3) what other factors may be involved?

The results of these studies suggest that, with respect to hypoxia recognition, humans are able to compensate for ascorbate deficiency in the same way as the murine model. There is a highly significant increase in methemoglobin production in HbE β thalassemia and a significant reduction in plasma ascorbate levels, although not to those observed in the patient whose findings initiated this study. There is, however, a highly significant relationship between the level of methemoglobin and splenectomy and also with the factors that modify globin-chain imbalance. A further complication in this population was the finding that the haptoglobin genotype was nearly all of the 2.2 variety, which is less effective at hemoglobin binding11,12 and may place an additional pressure on ascorbate as a free radical scavenger.

Clearly, there are multiple factors involved in the increased level of methemoglobin production in this form of thalassemia and because of its potential effects on adaptation to anemia and vascular damage, further studies of the mechanisms involved in its increased production are required in other types of thalassemia and sickle-cell anemia.

Methods

Patients

The subject in whom the findings initiated this study was a 19-year-old female patient attending the National Thalassemia Center, Kurunegala, Sri Lanka. She had presented at 2 years of age with anemia and splenomegaly, and later the diagnosis of HbE β thalassemia was established. For the next...
7 years, she received intermittent transfusion, after which she underwent splenectomy on the basis of a steady-state hemoglobin level of 5-6 g/dL and a spleen that was enlarged to 19 cm below the costal margin. For the next 10 years, despite hemoglobin values in the 7-8 g/dL range, her growth and sexual maturation were delayed, although by the age of 19 years, she had achieved midparental height and the menarche. Only when she reached the age of 17 years did the patient and her family disclose that she had had painful and enlarged gums and intermittent mucosal bleeding for several years. A detailed dietetic history at this time revealed that she ate no fruit of any form or vegetables, a diet that had persisted for several years. The results of a screening for environmental factors that might induce methemoglobinemia were negative.

On the basis of subsequent findings in this patient and her family, detailed studies were conducted on 45 patients attending the National Thalassemia Center who were chosen at random from more than 200 patients with \( \text{HbE} \) \( \beta \) thalassemia who were being followed at the Center. Clinical and hematologic data on this group of patients have been published previously, together with a detailed account of a classification system directed at defining the phenotypic variability of the disease.13 In short, a “mild” phenotype covers those who never required transfusion, had stopped transfusion with no ill effects after several years follow-up, or had required no further transfusion after splenectomy. Patients with “severe” phenotypes were defined as those who relied on long-term transfusion. There were 25 of the former and 20 of the latter in this study. In addition, methemoglobin values were estimated in 17 normal adult volunteers and 17 patients with different types of hemoglobinopathy at the Center.

### Procedures

Venous blood was collected into heparin and EDTA from all study participants. Duplicate measurements of methemoglobin levels and \( P_50 \) values were estimated in 17 normal adult volunteers and 17 patients with the former and 20 of the latter in this study. In addition, methemoglobin levels and \( P_50 \) were measured with the use of a Rapidpoint 405 analyzer with an integral co-oximeter (Bayer). This instrument incorporates a polychromator that allows the simultaneous measurements across the various fractions of hemoglobin in the range of 473-671 nm. To confirm that methemoglobin levels were being measured accurately, blood samples from the propositus and a group of patients with \( \text{HbE} \) \( \beta \) thalassemia and normal controls were analyzed via the manual method of Evelyn and Lewis.19 Both cytochrome \( b_5 \) reductase and cytochrome \( b_5 \) reductase deficiency were sequenced.20

A urine sample was collected from the patient and tested with Comb 10 diagnostic strips (Roche Diagnostics) for the presence of nitrates and hemoglobin. The urine sediment was examined by microscopy for the presence of red blood cells.

### Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (Release 16.0.1; SPSS Inc). Differences between median methemoglobin concentrations were assessed with the Mann-Whitney \( U \) test. We used multiple regression analysis to explore the relationship between methemoglobin and the potential predictor variables severity, transfusion status, and splenectomy. \( P < .05 \) was considered statistically significant.

### Ethical approval

Approval for the research program on \( \text{HbE} \) \( \beta \) thalassemia was obtained from the Ethical Committee of the College of Pediatricians, Colombo, Sri Lanka, and the Oxford Tropical Research Ethical Committee, Oxford, United Kingdom. This study was conducted in accordance with the Declaration of Helsinki.

### Results

The findings in the family that led to these studies are summarized in Table 1 and further biochemical data of the propositus, including findings before and after treatment, in Table 2. The propositus had a hemoglobin pattern typical of \( \text{HbE} \) \( \beta \) thalassemia; sequencing of the \( HBB \) genes revealed the \( \beta \) mutation on 1 chromosome and the severe \( \beta \) thalassemia mutation, IVS1-5 (G-C), which is very common in the Sri Lankan population,16 on the other. Further
sequencing of the \textit{HBA} and \textit{HBB} genes revealed no other abnormalities, excluding hemoglobin M mutations. The mother had findings typical of \(\beta\) thalassemia trait, although complicated by iron deficiency anemia because of menorrhagia. The father had hemoglobin E trait, whereas the patient’s 2 siblings were healthy. Multiple estimations indicated that the propositus had markedly increased levels of methemoglobin in the range of 10.7%-13.6%. The patient’s mother with \(\beta\) thalassemia trait had a slightly increased level of methemoglobin, whereas the levels in other family members were normal. The cytochrome \textit{b5} reductase levels were increased in the propositus and normal in the other family members. Both cytochrome \textit{b5} and cytochrome \textit{b2} reductase genes from the propositus were sequenced and showed no abnormality. Glyceraldehyde phosphate dehydrogenase and glutathione reductase levels were within the normal range in the propositus and normal in her parents. The patient’s \(P_50\) was significantly reduced compared with other family members, resulting in a marked left shift in the oxygen-dissociation curve compared with those of patients in the same population with \(HbE\) \(\beta\) thalassemia recently reported.\textsuperscript{21} Screening for glucose-6-phosphate dehydrogenase deficiency was negative in all family members.

To determine the reason for the extremely high methemoglobin level in the propositus, we performed further investigations, as shown in Table 2. Her plasma ascorbate level was extremely low, whereas G6PD levels were increased. Reduced glutathione levels were slightly reduced, and there was an increased level of IL-8. Treatment with vitamin C, 50 mg on alternate days, resulted in a dramatic decrease in the methemoglobin level and an increase in the ascorbate level to above the lower range of normal. There was also a shift to the right in the oxygen dissociation curve associated with a significant increase in the \(P_50\). There was no significant change in the relationship between hemoglobin and erythropoietin levels estimated on 5 samples before and after treatment.

To explore further the significance of the findings in this patient, 45 patients with \(HbE\) \(\beta\) thalassemia were chosen at random from the 200 or more patients with this condition who were being followed in Kurenegala. Their division into strictly defined severity groups, as described in “Procedures,” main clinical and hemoglobinologic findings and some of the genetic modifiers responsible for the variation in their phenotypic severity have been reported previously.\textsuperscript{13,16} The major findings in these patients in relationship to the present study are summarized in Tables 3 and 4. As shown in Table 3, those with \(HbE\) \(\beta\) thalassemia had a significant increase in methemoglobin compared with healthy controls and univariate analysis showed that there was a highly significant increase in methemoglobin levels in those who had been splenectomized compared with those who had intact spleens. As shown in Table 3, there was also a significant relationship between methemoglobin levels and phenotypic severity, as judged by the findings in the mild and severe groups and mirrored by the transfusion requirements. In multiple regression analysis, only splenectomy was statistically significantly related to methemoglobin level (standardized \(\beta = 0.64, t = 3.68, P < .01\)).

As shown in Table 4, the mean plasma ascorbate level in this group of patients was at the bottom limit of the normal range; in 10 cases it was subnormal. Although the number of cases available with matched plasma ascorbate and methemoglobin levels was too

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**Table 2. Further biochemical and related analyses of the propositus, including, in some cases, data obtained before and after treatment with vitamin C**

<table>
<thead>
<tr>
<th>Variable (units), normal range</th>
<th>Ascorbate (nmol/mL), 28-84</th>
<th>Met Hb (%), 0.1-0.6</th>
<th>(P_{50}) (mm Hg), 24.1-27.2</th>
<th>Mean Hb (g/dL), 12-16</th>
<th>Mean Epo (IU/mL), 2-14</th>
<th>G6PD (U/gHb), 4.6-13.5</th>
<th>Pyruvate kinase (IU/gHb), 7.2-14.0</th>
<th>GSH, ((\mu)mol/gHb), 6.6-10.0</th>
<th>Haptoglobin (g/dL), 0.3-2.0</th>
<th>Hepatic iron (mL/g dw), 0.6-1.2</th>
<th>IL-8, (pg/mL), &lt; 10</th>
<th>Urinary, nitrite screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>2.1-6.7</td>
<td>10.7-13.6</td>
<td>21.3-23.6</td>
<td>6.2</td>
<td>94.3</td>
<td>21.1</td>
<td>7.7</td>
<td>6.18</td>
<td>0.26</td>
<td>2.9</td>
<td>179.6</td>
<td>Negative</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>30.2-36.6</td>
<td>0.85</td>
<td>26.9</td>
<td>7.0</td>
<td>76.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hb and Epo values are the means of 5 estimations. Epo indicates erythropoietin; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; and Met, methemoglobin.

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**Table 3. Analysis of methemoglobin levels in 45 patients with HbE \(\beta\) thalassemia with a breakdown of cases into splenectomized and nonsplenectomized, low and high transfusion rates, and mild and severe phenotypes as defined in the text**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>(n)</th>
<th>Median Met Hb, %</th>
<th>Interquartile range</th>
<th>Range</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>17</td>
<td>0.3</td>
<td>0.25-0.4</td>
<td>0.1-0.6</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (all cases)</td>
<td>45</td>
<td>2.7</td>
<td>1.9-3.65</td>
<td>0.9-6.3</td>
<td></td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (spleenectomized)</td>
<td>20</td>
<td>3.7</td>
<td>3.1-4.2</td>
<td>0.9-6.3</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (spleen intact)</td>
<td>25</td>
<td>2.3</td>
<td>1.5-2.8</td>
<td>0.9-4.8</td>
<td></td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (mild)</td>
<td>25</td>
<td>2.45</td>
<td>1.9-3.6</td>
<td>0.9-4.8</td>
<td>.004</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (severe)</td>
<td>20</td>
<td>3.1</td>
<td>1.9-3.9</td>
<td>0.9-6.3</td>
<td></td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (0-20 blood transfusions)</td>
<td>21</td>
<td>2.5</td>
<td>1.85-3.1</td>
<td>0.9-3.6</td>
<td>.001</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (&gt; 20 blood transfusions)</td>
<td>18</td>
<td>3.65</td>
<td>3.4-4.5</td>
<td>1.6-6.3</td>
<td></td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (mild, spleen intact)</td>
<td>13</td>
<td>2.2</td>
<td>1.75-2.5</td>
<td>0.9-3.6</td>
<td>.018</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (mild, spleenectomized)</td>
<td>12</td>
<td>3.6</td>
<td>2.65-4.05</td>
<td>0.9-5.0</td>
<td></td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (severe, spleen intact)</td>
<td>12</td>
<td>2.65</td>
<td>1.38-3.18</td>
<td>0.9-4.8</td>
<td>.002</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (severe, spleenectomized)</td>
<td>8</td>
<td>4.0</td>
<td>3.7-4.45</td>
<td>3.5-6.3</td>
<td></td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (mild, spleenectomized)</td>
<td>13</td>
<td>2.2</td>
<td>1.75-2.5</td>
<td>0.9-3.6</td>
<td>.32</td>
</tr>
<tr>
<td>HbE (\beta) thalassemia (severe, spleen intact)</td>
<td>12</td>
<td>2.65</td>
<td>1.38-3.18</td>
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<td></td>
</tr>
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<td>.01</td>
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<td>8</td>
<td>4.0</td>
<td>3.7-4.45</td>
<td>3.5-6.3</td>
<td></td>
</tr>
</tbody>
</table>

Hb, hemoglobin; and Met, methemoglobin.
small to determine whether there was a significant relationship between the 2 at this level of plasma ascorbate, the mean level of methemoglobin in those in whom these measurements were available and who had all been splenectomized was 4.24%, and the mean level of plasma ascorbate was 23.1 nmol/mL, that is in the subnormal range.

Overall, the level of haptoglobin was subnormal in the patients with HbE β thalassemia, although no case of absent haptoglobin was encountered. The majority of the patients had the 2.2 haptoglobin genotype. There was a wide range of hepatic iron concentrations that were not significantly related to the methemoglobin level. There was also a highly significant elevation of IL-8 levels in this condition and are the levels related to the methemoglobin levels in this low-normal range. Before treatment the propositus also had a low P50 and a marked left shift in her oxygen dissociation curve, a finding that has been previously observed in association with increased methemoglobin levels.9 The ferric (Fe³⁺) hemes are unable to reversibly bind oxygen, and they increase the oxygen affinity of the associated ferrous hemes (Fe²⁺) in the hemoglobin tetramer, causing a left shift in the oxygen dissociation curve.25

There was a significant increase in the P50 with a right shift in the oxygen dissociation curve after treatment with ascorbate. In short, this reflects a more effective adaptation to anemia, as recently described in patients with HbE β thalassemia.21

The finding that multiple estimations of the erythropoietin response to a particular hemoglobin level did not change in the propositus before and after treatment with ascorbate is of particular interest with regard to response to hypoxia. These findings are similar to those reported recently in an ascorbate-deficient mouse model.7,8 An increased erythropoietin response to anemia depends on the oxygen-sensing properties of the prolylhydroxylase domain-containing enzymes that catalyze the prolyl-4-hydroxylation of the hypoxia-inducible factor and require oxygen and 2-oxoglutarate as cosubstrates with iron and ascorbic acid as cofactors.4,6 The erythropoietin response to anemia in the murine model was normal despite profound ascorbate deficiency.8 A major compensatory mechanism appeared to be the action of reduced glutathione, the levels of which remained normal or increased in the propositus in the present study.8 These observations suggest that the relationship between ascorbate and hypoxia response in humans and mice are similar.

What are the broader issues resulting from these findings? In particular, because the results of the studies in this unusual patient provide clear evidence that ascorbate deficiency can induce methemoglobinemia in HbE β thalassemia, how common are increased methemoglobin levels in this condition and are the levels related to hypoxia response in humans and mice are similar.

Discussion

There seems little doubt that the high level of hemoglobin in the patient whose findings initiated these studies resulted from profound ascorbate deficiency. It has been estimated that ascorbate is responsible for approximately 16% of methemoglobin reduction in red cells,22 with the remainder relying on several enzymes, notably cytochrome b₅, reductase, glyceraldehyde-3-phosphate dehydrogenase, and glutathione reductase.23 The levels of these enzymes were all increased or normal in the propositus and her family members, and structural studies of the cytochrome b₅ reductase gene of the propositus were normal. Sequencing of the HBA and HBB genes excluded HbM. Furthermore, the administration of cautious doses of ascorbate, because of the possible deleterious effects of rapid iron mobilization,24 rapidly reversed the methemoglobin levels into the low-normal range. Before treatment the propositus also had a low P50 and a marked left shift in her oxygen dissociation curve, a finding that has been previously observed in association with increased methemoglobin levels.9 The ferric (Fe³⁺) hemes are unable to reversibly bind oxygen, and they increase the oxygen affinity of the associated ferrous hemes (Fe²⁺) in the hemoglobin tetramer, causing a left shift in the oxygen dissociation curve.25

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Table 5. Miscellaneous methemoglobin levels in conditions related to HbE β thalassemia obtained from patients attending the same clinic

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
<th>Methemoglobin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, median; range</td>
<td>17</td>
<td>0.3; 0.1-0.6</td>
</tr>
<tr>
<td>β thalassemia intermedia</td>
<td>4</td>
<td>1.4, 2.9, 4.7, 4.8</td>
</tr>
<tr>
<td>Hb E/β thalassemia</td>
<td>1</td>
<td>2.4</td>
</tr>
<tr>
<td>ß/ß/ß thalassemia</td>
<td>3</td>
<td>2.0, 2.8, 2.8</td>
</tr>
<tr>
<td>Hb SS disease</td>
<td>2</td>
<td>0.3; 1.2</td>
</tr>
<tr>
<td>Hb S/ß thalassemia</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Hb SD disease</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Hb SE disease</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>ß thalassemia trait</td>
<td>2</td>
<td>0.4, 0.5</td>
</tr>
<tr>
<td>Hb E trait</td>
<td>2</td>
<td>0.3; 0.3</td>
</tr>
</tbody>
</table>

Hb indicates hemoglobin.
degree of globin-chain imbalance, it seems likely that splenic function and the degree of excess α-chain synthesis play a major role in determining the level of methemoglobin, at least in HbE β thalassemia.

What is the source of the increased methemoglobin? As in other forms of β thalassemia, excess α-chains are produced in HbE thalassemia with the production of red cell inclusions; despite the mild instability of HbE β chains are not found in these precipitates. One of the major degradation products of excess α-chains are hemichromes, which bind to the red cell membrane and promote sequestering of band 3. As they form, they go through reversible and irreversible phases during which methemoglobin is produced as an intermediate. It is, therefore, possible, that abnormal red cells exposed to this mechanism are recognized and sequestered in the spleen and hence the level of methemoglobin is increased after splenectomy. Because, like other forms of thalassemia, there is a significant hemolytic component in HbE β thalassemia, it follows that the circulation will be continuously exposed to increased levels of methemoglobin.

Another potential source of methemoglobin, in this case in plasma, is the further oxidation of hemoglobin released during hemolysis, the fail-safe mechanism in this case again is binding by haptoglobin. In the present study the haptoglobin levels were reduced in the patients with HbE β thalassemia, although only to a minor degree. However, molecular analysis showed that in almost every case the haptoglobins were of the 2.2 variety, which has been shown to be less effective than the 1.1 variety with respect to hemoglobin binding and which occurs commonly in some Asian countries. Recent studies suggest that because of its reduced binding properties, it may put greater pressure on the use of ascorbate as a free radical scavenger and, indeed, may be associated with increased frequency of the clinical manifestations of ascorbate deficiency.

Methemoglobin is a significant activator of endothelial cells by stimulation of E-selectin, IL-6, and IL-8 production. It is of interest therefore that the IL-8 levels in this series of patients with HbE β thalassemia were considerably increased. Because of increasing evidence for vascular complications in other forms of thalassemia intermedia and in sickle cell disease, and because of the results of the small pilot study shown in Table 5, further investigation of the potential pathologic role of methemoglobin is indicated, particularly in conditions with reduced splenic function or in which splenectomy is commonly practiced.

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Authorship

Contribution: A.A. and C.F. performed the laboratory studies; S.A. conducted the statistical analysis; A. Premawardhena, D.B., A. Perera, T.S.P., and N.O. collected and analyzed the clinical data on the patients, and A.A. and D.J.W. designed the study and wrote the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Methemoglobinemia and ascorbate deficiency in hemoglobin E β thalassemia: metabolic and clinical implications

Angela Allen, Christopher Fisher, Anuja Premawardhena, Dayananda Bandara, Ashok Perera, Stephen Allen, Timothy St Pierre, Nancy Olivieri and David Weatherall