Erythroblast cell expansion as a marker for disease severity in $\beta^0$-thalassemia/Hb E disease

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Accepted 22 June, 2011

$\beta^0$-Thalassemia/Hb E disease is extremely common in many parts of Southeast Asia. In Thailand, there are believed to be approximately 100,000 people living with this disease which can present as a life threatening anemia syndrome dependent on regular blood transfusions. Determination of the state of presentation of the disease (mild, moderate or severe) is based upon a number of clinical and hematological parameters and currently, there is no accurate predictive methodology to gauge the final severity of the disease in a particular patient. While erythropoiesis, the process generating red blood cells, takes place in the bone marrow, sufficient circulating erythroid progenitor cells are found in the peripheral blood circulation to provide materials for analysis. In this study, the expansion of isolated erythroid progenitor cells was analyzed in culture and the level of expansion assessed in relation to disease severity. A clear statistically significant relationship between erythroid progenitor expansion and disease severity was observed, providing for the first time a potential predictive marker for disease severity for $\beta^0$-thalassemia/Hb E disease.

Key words: $\beta$-Thalassemia, biomarker, erythroblasts, hemoglobin E, severity.

INTRODUCTION

Thalassemia is a hereditary hematological disorder characterized by the defective production of globin chains as a consequence of a wide range of underlying genetic abnormalities (Weatherall and Clegg, 2001). The characteristic presentation of thalassemia is anemia (a reduction below normal of red blood cells or quantity of hemoglobin) which can range from a mild, essentially asymptomatic anemia to a severe, transfusion dependent state (Weatherall and Clegg, 2001). Hemoglobin, the primary oxygen transport molecule in the red blood cell is composed of four globin chains in the adult, two alpha chains and two beta chains. In the diploid cell, the beta globin genes are found on the two alleles of chromosome 11, while two closely linked alpha globin genes are located on chromosome 16, giving four gene copies for the alpha globin chain per cell. In normal erythroid cells, production of the globin chains is evenly matched. $\beta^0$-Thalassemia results from damage to the $\beta$-thalassemia genes which normally occurs as a result of point mutation or small deletions (Weatherall and Clegg, 2001), while $\alpha$-thalassemia results from damage or loss of $\alpha$-globin genes, which typically occurs by large deletions that may encompass one or both of the $\alpha$-globin genes (Weatherall and Clegg, 2001). Both $\alpha$- and $\beta$-thalassemia are common in many parts of Southeast Asia and in Thailand, nearly half a million people are believed to live with some form of symptomatic thalassemia (Fucharoen and Winichagoon, 1997).

The most common form of $\beta^0$-thalassemia in much of Southeast Asia is the compound hemoglobinopathy $\beta^0$-thalassemia/Hb E disease (Fucharoen and Winichagoon, 2000), caused by the co-inheritance of a null allele of $\beta$-globin and the hemoglobin E variant of $\beta$-globin, the disease is characterized by a particularly wide range of severity of presentation, ranging from an essentially normal condition with only a mild anemia to a severe, transfusion dependent state. Currently, there is no established methodology to predict the severity of presentation of this disease and classification of patients into mild, moderate and severe categories is based upon clinical presentation and analysis of hematological parameters (Sripichai et al., 2008). $\beta^0$-Thalassemia/Hb
E disease is characterized by ineffective erythropoiesis in the bone marrow in which cells die by apoptosis during expansion and differentiation at the polychromatic normoblast stage of erythropoiesis (Mathias et al., 2000) and cell death is believed to occur in the maturing erythroblast as a consequence of deposition of the unpaired α-globin chains within the cell (Mathias et al., 2000). The anaemic state generated leads to increased production of the glycoprotein hormone erythropoietin, which is a critical cytokine promoting erythropoiesis and which serves to stimulate expansion of the bone marrow mass (Centis et al., 2000; Kittikalayawong et al., 2005; Pootrakul et al., 2000) and significantly increased levels of erythropoietin have been documented in thalassemia patients (Chairsiripoomkere et al., 1999; Nisli et al., 1997; Paritpookee et al., 2002). However, even in the expanded mass, the erythroblasts still undergo apoptosis, maintaining the anaemic state and prompting further erythropoietin production by the kidney (Fried, 1975). The over expansion of the bone marrow mass can lead to significant deformity of the bones leading to bone fragility and classic facial deformity in severe cases (Mohamed and Jackson, 1998).

The mainstay therapy for severe cases of β0-thalassemia/Hb E in many countries is blood transfusion to correct the severe anaemia. Long term transfusion in itself however, can lead to subsequent problems such as severe iron overload, requiring further therapeutic strategies (Porter, 2009). Compete curative treatment of thalassemia is possible by the process of bone marrow transplantation (Smiers et al., 2010), but this expensive procedure is not widely available in many countries of the world.

As noted earlier, the presentation of β0-thalassemia/Hb E disease is particularly wide, even after other modulating factors such as HbF production have been taken into consideration (Fucharoen et al., 2000; Fucharoen and Winichagoon, 2000). The HbE variant of β-globin results from the substitution of a lysine for glutamic acid at codon 26 of β-globin resulting in an unstable β-globin molecule (Weatherall and Clegg, 2001). Whether inherited as a heterozygous trait or as a homozygous HbE genotype, this variant normally only causes a mild anaemia, but coupled with a β0-thalassemia mutation; this compound heterozygous state causes a wide ranging and unpredictable anaemia (Rees et al., 1998).

While numerous studies have looked for genetic or other markers that allow prediction of the severity (Nuinoon et al., 2010; Sherva et al., 2010), till date no such marker has been found. In this study, we examined the expansion in culture of isolated erythroid progenitor cells from normal controls, as well as from β0-thalassemia/Hb E patients with mild and severe disease presentation and found that the degree of expansion correlated closely with the disease severity, suggesting that this methodology can provide the first predictive marker of β0-thalassemia/Hb E disease severity.

MATERIALS AND METHODS

Patients and samples

This study was undertaken after formal approval by the Committee for Human Rights related to Experimentation, Mahidol University, Thailand. After individual were informed written consent, approximately, 30 ml of patient peripheral blood and 50 ml of normal control blood were collected. Diagnosis and severity grading (mild or severe) of β-thalassaemia/Hb E was as described previously (Sripichai et al., 2008). All normal controls were screened to be normal for red blood indices and hemoglobin (Langlois et al., 2008).

Isolation and propagation of erythroblasts

To isolate CD34+, hemopoietic progenitor cells (HPCs) circulating in the peripheral blood, peripheral blood mononuclear cells were isolated from venous blood by layering over Lymphoprep™ (Axis- Shield PoC AS, Oslo, Norway) according to the manufacturer’s protocol. CD34+ cells were selected for using a direct CD34 progenitor cell isolation kit with MACS™ isolation system (Miltenyi Biotech, Auburn, CA, USA) according to manufacturer’s protocol. The purity of selected CD34+ HPCs was more than 85% as evaluated by flow cytometry using a phycoerythrin (PE)-conjugated mouse anti-human CD34 monoclonal antibody and a fluorescein isothiocyanate (FITC)-conjugated mouse anti human CD45 monoclonal as described elsewhere (Wannatung et al., 2009). These cells were then cultured according to the protocol of Choi et al. (2000) in which the cells were grown at a density of 105/ml in Iscove’s modified Dulbecco medium (IMDM; GIBCO BRL, Grand Island, NY, USA) containing 15% heat-inactivated fetal calf serum (FCS; GIBCO BRL, Grand Island, NY, USA), 15% human AB serum, 2 U/ml recombinant human (rHu) EPO (CILAG GmbH, Zug, Switzerland), 20 ng/ml rHu stem cell factor (SCF; Promokine, Heidelberg, Germany) and 10 ng/ml rHu interleukin-3 (IL-3; Promokine) at 37°C in a high-humidity, 5% CO2 incubator for the for 3 days after which the medium was replaced (without rHuIL-3) and the cells cultured for a further 4 days. Wright-Giemsa’s staining established that at least 95% of the cells showed erythroblast morphology, in agreement with our previous study (Wannatung et al., 2009).

Expansion determination

The cell number of erythroblasts from 12 normal controls and 12 β0-thalassemia/Hb E patients was determined on day 0 (after CD34+ cell selection) and 7 of culture by trypan blue staining using and using a haemocytometer. The fold expansion was calculated from the total cell number counted at day 7 relative to cell number counted at day 0.

Wright-Giemsa’s staining

A total of 6x10⁴ erythroblasts were cytocentrifuged into a glass slide by using a cytopsin. The cytopsin erythroblasts were fixed into the slide with absolute methanol for 15 min. After the slides were completely dried, the fixed slide was stained with Wright-Giemsa stain for 15 min. The morphological features of 200 cells per slide of the stained erythroblast were examined under light microscope. Analysis showed that on day 7 of culture, at least 95% of the cells
Table 1. Clinical and genetic characterization of 12 β-thalassemia/Hb E patients used in the study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Hb (g/dl)</th>
<th>Hct (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>Severity</th>
<th>Genotype</th>
<th>ββ°° mutations</th>
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<tr>
<td>1</td>
<td>F</td>
<td>6.8</td>
<td>23.5</td>
<td>59.7</td>
<td>17</td>
<td>29</td>
<td>Mild</td>
<td>Codon 17; AT</td>
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<tr>
<td>2</td>
<td>F</td>
<td>9.1</td>
<td>27.4</td>
<td>51.9</td>
<td>17.2</td>
<td>33.1</td>
<td>Mild</td>
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<tr>
<td>3</td>
<td>F</td>
<td>6.6</td>
<td>25.9</td>
<td>59.6</td>
<td>17.5</td>
<td>29.3</td>
<td>Mild</td>
<td>Codon 17; AT</td>
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<tr>
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<td>6.5</td>
<td>17.6</td>
<td>72.5</td>
<td>23.5</td>
<td>32.5</td>
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<td>Codon 41/42; -TTCT</td>
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</tr>
<tr>
<td>5</td>
<td>F</td>
<td>8.4</td>
<td>22.9</td>
<td>54.9</td>
<td>17.1</td>
<td>31.2</td>
<td>Mild</td>
<td>Codon 41/42; -TTCT</td>
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<tr>
<td>6</td>
<td>F</td>
<td>8.4</td>
<td>24</td>
<td>72</td>
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<td>31.8</td>
<td>Mild</td>
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<tr>
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<td>M</td>
<td>7.5</td>
<td>23.3</td>
<td>60</td>
<td>19.3</td>
<td>32.1</td>
<td>Severe</td>
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<tr>
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<td>F</td>
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<td>19.2</td>
<td>62.1</td>
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<tr>
<td>12</td>
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<td>29.3</td>
<td>Severe</td>
<td>Codon 17; AT</td>
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showed erythroblast morphology, in agreement with our previous study (Wannatung et al., 2009).

Statistical analysis

Results are presented as the mean value together with the standard error of the mean (SEM) calculated by the GraphPad Prism5 software (GraphPad Software, La Jolla, CA). Statistical analysis was performed by the Student’s t-test with p < 0.05 considered to be significant.

RESULTS

Samples of peripheral blood were collected from 12 normal controls and 12 β0-thalassemia/Hb E patients (6 with a mild disease presentation and 6 with a severe disease presentation). Patient characteristics, together with the specific genotype of each patient are shown in Table 1. The β0-thalassemia/Hb E patients were screened to be free of any concurrent compound β0-thalassemia hemoglobinopathy and normal controls were free of both alpha and beta hemoglobinopathies. Haemopoietic progenitor cells, which are positive for the CD34 cell surface marker (CD34+) were isolated from samples of peripheral venous blood by first layering over lymphoprep media to isolate peripheral blood mononuclear cells and then, CD34+ cells were selected from this cell population by mixing the cells with antibodies labeled with a magnetic isolation tag linked to an anti-CD34+ antibody and passing through a magnetic field. Cells that do not express CD34 (CD34- cells) passed directly through the column, while CD34 expressing cells (CD34+) were retained in the column. After washing the column and removing the magnetic field, the CD34+ cells were eluted. This methodology generates at least 85% pure CD34+ cell population as shown previously (Wannatung et al., 2009) and this was confirmed by Wright-Giemsa’s staining (Figure 1).

The CD34+ erythroid progenitor cells were then cultured for 7 days in the presence of cytokines that drive the progenitor cells along an erythroid development pathway and on day 7 differentiation of the cells to the proerythroblast stage of erythropoiesis was again confirmed by Wright-Giemsa’s staining. Consistent with our previous report (Wannatung et al., 2009), more than 95% of cells showed erythroblast morphology (Figure 1).

Cell numbers were counted on days 0 and 7 of culture and the expansion of the cells calculated as a fold increase over the day 0 number. In agreement with our previous data (Wannatung et al., 2009), normal control erythroid cells expanded some 15 fold (14.6 ± 2.4) in culture from day 0 to day 7, while β0-thalassemia/Hb E erythroblasts expanded some 40 fold (41.8 ± 6.8) in culture, a result that was statistically significant (p < 0.05).

Analysis of the cell expansion in terms of patient severity is shown in Figure 2. As can be seen, a statistically significant difference was observed between the expansion of normal control erythroblasts and erythroblasts isolated from mild presentation β0-thalassemia/Hb E patients (p < 0.05), between normal control erythroblasts and erythroblasts from severe presentation β0-thalassemia/Hb E patients (p < 0.01) and critically, there was a significant difference in levels of expansion between erythroblasts from mild and severe presentation β0-thalassemia/Hb E patients (p < 0.05).

DISCUSSION

Hematopoietic stem cells are primarily resident in the bone marrow, but a small number of such cells circulate in the peripheral blood allowing their isolation and differentiation in culture. The cell surface antigen CD34 or hemopoietic progenitor cell antigen 1 (HPCA1), is expressed selectively on early hematopoietic stem cells and this expression can be used to isolate these cells using specific monoclonal antibodies directed against this antigen (Giarratana et al., 2005; Liu et al., 2007; Migliaccio et al., 2002; Sato et al., 1993). Hematopoietic
stem cells are induced to differentiate into an erythroid lineage and proliferate following exposure to specific cytokines including erythropoietin, stem cell factor (SCF) and interleukin 3 (IL-3). Under the appropriate cytokine treatment, isolated hematopoietic stem cells can be induced to differentiate into a dedicated erythroid lineage that subsequently follow the well characterized process of erythroblast maturation leading to the formation of mature red blood cells. Mature red blood cells or erythrocytes are enucleated cells, and ejection of the nucleus from the precursor cells is one of the final stages of erythrocyte development. Expansion of erythroid progenitor cells during culture has been well documented and we have previously established that β0-thalassemia/Hb E erythroid progenitor cells expand to a significantly greater extent than normal control progenitor cells (Wannatung et al., 2009). To date however, the expansion of these cells has not been examined in relation to disease severity in β0-thalassemia/Hb E patients and as such, this is the first report of an association between the degree of expansion of these cells in culture and disease severity. A clear statistical difference was seen between the amounts of

Figure 1. Wright-Giemsa’s staining of cells isolated from peripheral blood of normal controls (top) and β0-thalassemia/Hb E patients (bottom) on day of isolation after CD34+ cell selection (day 0) and after culture for 7 days (day 7).
Figure 2. Analysis of cell expansion of erythroblasts from normal controls, and mild and severe cases of \( \beta \)-thalassemia/Hb E disease. Statistically significant differences are indicated.

expansion of erythroblasts from normal control patients when compared with erythroblasts from both mild and severe \( \beta^0 \)-thalassemia/Hb E patients. Critically however, there was also a statistically significant difference between the expansion seen with erythroblasts isolated from mild presentation \( \beta^0 \)-thalassemia/Hb E patients and those isolated from severe presentation \( \beta^0 \)-thalassemia/Hb E patients suggesting that this method can be used to predictively determine disease severity in yet unclassified \( \beta^0 \)-thalassemia/Hb E patients. At this point, the volume of blood taken (30 mls from patients) is only within the bounds of experimental practice and application of this technique in a clinical setting would require smaller volumes of blood to be taken. Additionally, the cost of isolation and culture of the cells to assess expansion at this point is prohibitive, suggesting that further experimentation needs to be undertaken, possibly to find an easily detectable surrogate bio-marker for the expansion potential of these cells.

In patients, the expansion of the bone marrow mass can cause physical facial deformity as well as an increased tendency for bone fractures (Mohamed and Jackson, 1998). Currently, this is explained by the anaemic state feeding back to the bone through the action of the cytokine erythropoietin and causing increased expansion of the bone marrow mass, the expansion thereby, is physically causing problems with the normal formation and functioning of the bones. Our results however, imply that this model may not completely explain the full pathophysiological causes of erythroid expansion. The hematopoietic stem cells isolated in this study are cultured under identical conditions and in particular, the conditions are identical with respect to the levels of erythropoietin added to the isolated cells. If external erythropoietin was the sole driving force mediating the expansion of these cells (either alone or in conjunction with other added cytokines), it would be expected that under identical growth conditions and with identical levels of stimulatory cytokines, proerythroblasts from both normal and \( \beta^0 \)-thalassemia/Hb E patients would show similar levels of expansion and more over, as noted earlier, a marked difference was noted between the mild and severe cases, suggesting that at least a part of the increased expansion capacity of \( \beta^0 \)-thalassemia/Hb E erythroblasts is mediated by an intrinsic factor or factors associated with the phenotypic expression of the disease. Till date, there remains no explanation as to why patients with apparently similar or identical genetic defects of the \( \beta^0 \)-thalassemia gene, when coupled with Hb E, showed such a wide presentation of severity, and this issue remains the object of considerable investigation. However, as shown here, the apparently intrinsic difference in the extent of expansion of progenitor cells to constant levels of erythropoietin may provide some of the answers.
ACKNOWLEDGEMENTS

This work was supported by a Research Chair Grant from the National Science and Technology Development Agency (NSTDA) and Mahidol University. A.L. is supported by the Office of the Higher Education Commission.

REFERENCES


