Imbalanced Globin Chain Synthesis in Cultured Erythroid Progenitor Cells From Thalassemic Bone Marrow and Peripheral Blood

By A. Kim Ritchey, Ronald Hoffman, Elaine Coupal, Victoria Floyd, Howard A. Pearson, and Bernard G. Forget

The plasma clot culture system was utilized to support the growth of erythroid-committed stem cells from the bone marrow of five patients with homozygous $\beta^+\text{-thalassemia}$ and the peripheral blood of one patient doubly heterozygous for $\beta^+$-thalassemia and Hb Lepore. Addition of erythropoietin to the cultures resulted in abundant colony growth (76–185 CFU-E and 3.5–11 BFU-E/6 x 10^6 bone marrow cells). Eight BFU-E/10^7 cells were assayed from the peripheral blood of the $\beta^+$-thalassemia/Hb Lepore patient. Globin chain synthetic ratios of CFU-E- and BFU-E-derived colonies were determined by CMC column chromatography after labeling of the cultures with H^-leucine for 24 hr prior to peak growth. Absent or decreased $\beta$-globin chain synthesis was observed in all cases; $\beta/\alpha$ synthetic ratios obtained in culture were quantitatively similar to those obtained in the starting bone marrow or peripheral blood cells. Study of globin chain synthesis in colonies derived from peripheral blood BFU-E of the patient with $\beta^+$-thalassemia/Hb Lepore demonstrated synthesis of a labeled peak consistent with Lepore $\beta$ chains; a similar labeled peak was barely discernable in the study of the starting peripheral blood. We conclude that thalassemic erythroid progenitor cells express in vitro the same program of abnormal globin chain synthesis as in vivo and that the plasma clot culture technique provides a valuable system for the study of the molecular defect in nucleated thalassemic erythroid cells.

THE $\beta$-THALASSEMIA syndromes are a heterogeneous group of disorders characterized by anemia and ineffective erythropoiesis. The common pathophysiologic mechanism underlying these disorders is a decreased or absent production of $\beta$-globin chains and the resultant accumulation of $\alpha$-chains and the resultant accumulation of $\alpha$-chains. The ability to demonstrate this basic biochemical defect in the progeny of hematopoietic stem cells could provide a new approach to study the molecular basis of this disease: nucleated thalassemic erythroid precursors could be analyzed and manipulated in an in vitro system in a manner not feasible with the use of total aspirated marrow.

Utilizing the plasma clot culture system, we were able to support the growth of thalassemic stem cells committed to erythroid differentiation yet not producing hemoglobin in vivo. Stem cells were obtained from the bone marrow of five patients homozygous for $\beta^+$-thalassemia and the peripheral blood of one patient doubly heterozygous for $\beta^+$-thalassemia and Hb Lepore. Colonies of hemoglobin-producing cells derived from these stem cells were then labeled with tritiated leucine while actively proliferating in culture. Analysis of the biosynthesis of globin chains from stem-cell-derived colonies revealed the imbalance of globin chain synthesis characteristic of the thalassemia syndrome.

MATERIALS AND METHODS

The five patients with homozygous $\beta^+$-thalassemia ranged in age from 6 to 21 yr (Table I). Four of the five were on a program of monthly transfusions, while one had never received a transfusion. Bone marrow cells were obtained by aspirations from the posterior iliac crest of these patients and two controls. Peripheral blood was obtained from one control and an untransfused 9-yr-old patient doubly heterozygous for $\beta^+$-thalassemia and Hb Lepore. The patients are all of Mediterranean ancestry (Greek or Italian). The four transfused patients have the typical clinical features of patients with transfusion-dependent $\beta$-thalassemia major. The fifth patient with homozygous $\beta^+$-thalassemia and the patient with Hb Lepore/ $\beta^+$-thalassemia have clinical syndromes typical of $\beta$-thalassemia intermedia with moderately severe anemia, ineffective erythropoiesis, and progressive splenomegaly that eventually necessitated splenectomy. Informed consent was obtained from the patients and controls.

The plasma clot culture technique was employed for measuring the growth of erythroid colonies from human bone marrow, as described by Tepperman. RPMI 1640 was substituted for NCTC 109 when peripheral blood was used as the source of stem cells. Bone marrow cells and peripheral blood cells in a final concentration of $6 \times 10^6/1.1$ ml and $10 \times 10^6/1.1$ ml, respectively, were cultured in quadruplicate in the presence of 2 IU of human urinary erythropoietin (NIH lot M-12-tu-LSL). Cultures were maintained in a humidified atmosphere of 4% carbon dioxide at 37°C. At 6 and 12 days of incubation for bone marrow and 14 days of incubation for peripheral blood, the clots were assessed for growth of erythroid colonies. Sample clots were removed and transferred to glass slides, fixed in glutaraldehyde, and stained with benzidine and hematoxylin. Under 100 X magnification, each clot was examined, and erythroid colonies of $\geq$ 49 benzidine-positive cells, appearing on day 6, were counted as CFU-E (colony forming unit-erythroid) derived colonies. Colonies consisting of 50–500 benzidine-positive cells or clusters of 3 or more CFU-E-derived colonies appearing on day 12 or 14 were considered to be BFU-E (burst forming unit-erythroid) derived colonies.
ERYTHROID CELL COLONIES IN \(\beta\)-THALASSEMIA

Table 1. Clinical Characteristics of Thalassemic Patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Hyper-transfusion</th>
<th>Source of Stem Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Homozygous (\beta)-thal</td>
<td>Yes</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Homozygous (\beta)-thal</td>
<td>Yes</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>Homozygous (\beta)-thal</td>
<td>Yes</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>Homozygous (\beta)-thal</td>
<td>Yes</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>Homozygous (\beta)-thal</td>
<td>No</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>(\beta^2)-thal/Hb Lepore</td>
<td>No</td>
<td>Peripheral blood</td>
</tr>
</tbody>
</table>

For determination of globin chain synthesis in the peripheral blood and bone marrow, aliquots of peripheral blood, whole bone marrow, or the red cell pellet recovered following Ficoll-Hypaque separation of the marrow were initially washed three times in Krebs-Ringer bicarbonate buffer solution. Samples were then incubated for 2 hr at 37°C with shaking in the presence of a mixture of amino acids excluding leucine and 100 \(\mu\)Ci of \(^3\)H-leucine (120 Ci/m mole, NEN, Corp., Boston, Mass.). Following incubation, the sample was washed three times in cold normal saline and lysed by addition of 4 vol of 0.005 \(M\) MgCl\(_2\) prior to globin chain extraction. For determination of globin chain synthetic ratios in CFU-E and BFU-E-derived colonies, 10 \(\mu\)Ci of tritiated leucine were added to the plasma clots near peak growth of the erythroid colonies. Following 24 hr of incubation, the clots were collected and washed three times in phosphate-buffered saline. Hemoglobin was extracted by trisice thawing and homogenizing the clots in the presence of 0.005 \(M\) MgCl\(_2\) and \(1/16\) triton solution. Carrier hemoglobin was added, and the hemoglobin fraction was then purified by Sephadex G100 gel filtration; the hemoglobin peak and trailing fractions containing free globin chain were pooled.

Globin chains were extracted from the pooled fractions by acid-acetone precipitation and then separated by carboxymethyl cellulose column chromatography in the presence of 8 \(M\) urea, as previously described.

RESULTS

Erythroid Colony Formation

Greater than normal numbers of bone marrow CFU-E-derived colonies were found in all patients except one, who formed a normal number of colonies (Table 2). This last hypertransfused patient had a normal bone marrow myeloid to erythroid ratio of 2:1, while the others had pronounced erythroid hyperplasia.

Numbers of bone marrow BFU-E-derived colonies were less than normal in all instances except one; while peripheral blood BFU-E were similar to controls. At the time of bone marrow BFU-E enumeration, however, numerous small colonies—not in clusters—were present, but not enumerated as BFU-E-derived colonies.

Globin Chain Synthetic Ratios

Figure 1 is a representative composite of radiochromatograms from one patient with \(\beta\)-thalassemia. The top graph represents the newly synthesized radioactive globin following 2-hr incubation of the bone marrow with \(^3\)H-leucine. Distinct \(\gamma\), \(\beta\), and \(\alpha\) peaks are seen, and imbalance of chain synthesis is evident with a \(\beta/\alpha\) ratio of 0.113. The middle graph represents radioactive globin produced in culture by BFU-E-derived colonies. Imbalanced globin is present with a \(\beta/\alpha\) ratio of 0.173. The bottom graph demonstrates that globin chain imbalance is evident in the more primitive BFU-E-derived colonies with a \(\beta/\alpha\) ratio of 0.232.

Table 3 shows the globin chain synthetic ratios from the thalassemic patients studied plus two control subjects. Bone marrow ("0 time") \(\beta/\alpha\) synthetic ratios were less than 0.15 in all patients except the untransfused patient with thalassemia intermedia. \(\beta/\alpha\) ratios from CFU-E-derived colonies were quantitatively similar in all cases. The slightly increased \(\beta/\alpha\) ratio noted in the BFU-E of patient one was not paralleled in two other study patients.

Variable levels of \(\gamma\)-chain synthesis were observed in cultures of both CFU-E and BFU-E-derived colonies of our thalassemic patients. In some cases the \(\gamma/\alpha\) synthetic ratio was similar to that observed in the starting material, while in other cases it was substantially higher. The initial double peak of radioactivity preceding the \(\gamma\)-chain peak in the studies of CFU-E and BFU-E-derived colonies presumably contains nonglobin proteins synthesized by nonerythroid cells present in the plasma clot cultures, since it is obtained as the major product in cultures grown in the absence of erythropoietin. If, instead of partial purification of hemoglobin as was done in our study, more extensive purification is carried out prior to CMC chromatography, then this pre-\(\gamma\) peak is greatly reduced or can be totally eliminated, as has been demonstrated by Papayannopoulos et al.

Figure 2 shows the in vitro globin chain synthesis from the patient heterozygous for \(\beta\)-thalassemia and Hb Lepore. The lines with open circles represent newly synthesized radioactive globin chains, while the lines with closed circles represent known globin chains.

Table 2. Erythroid Colonies Formed per 6 x 10\(^6\) Bone Marrow Cells (PTs 1–5) or 10\(^6\) Peripheral Blood Cells (PT 6) in the Presence of 2U of Erythropoietin

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>CFU-E</th>
<th>BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>81.0 ± 8.0</td>
<td>12.0 ± 1.5</td>
</tr>
<tr>
<td>PT 1</td>
<td>146.5 ± 5.6</td>
<td>11.5 ± 0.6</td>
</tr>
<tr>
<td>PT 2</td>
<td>185.25 ± 26.3</td>
<td>5.25 ± 0.6</td>
</tr>
<tr>
<td>PT 3</td>
<td>37.0 ± 9.6</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>PT 4</td>
<td>76.0 ± 3.9</td>
<td>5.25 ± 1.9</td>
</tr>
<tr>
<td>PT 5</td>
<td>112.25 ± 4.5</td>
<td>3.5 ± 1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peripheral blood</th>
<th>CFU-E</th>
<th>BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>N.A.</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td>PT 6</td>
<td>N.A.</td>
<td>7.7 ± 1.2</td>
</tr>
</tbody>
</table>

Result represents the mean ± SEM of quadruplicate determinations. (NA, not applicable.)
Table 3. Globin Chain Synthetic Ratios in Controls and Patients With Thalassemia

<table>
<thead>
<tr>
<th>Patients</th>
<th>0 Time</th>
<th>CFU-E</th>
<th>BFU-E</th>
<th>0 Time</th>
<th>CFU-E</th>
<th>BFU-E</th>
<th>0 Time</th>
<th>CFU-E</th>
<th>BFU-E</th>
</tr>
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<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>1.148</td>
<td>0.873</td>
<td>1.194</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>0.885</td>
<td>0.714</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

ND, not done; * no gamma chain synthesis documented.

Fig. 1. Composite of radiochromatograms demonstrating globin chain synthesis from a patient with β⁺-thalassemia.
Globin chain synthesis in \( \beta^-\)-thalassemia

**HETEROZYGOUS \( \beta^-\)-THAL / Hb LEPORE**

**PERIPHERAL BLOOD**

\[ \frac{\delta \beta}{\alpha} = 0.005 \]

**BFU-E (PB.)**

\[ \frac{\delta \beta}{\alpha} = 0.15 \]

**Fig. 2.** Radiochromatograms from a patient with \( \beta^-\)-thalassemia/Hb Lepore demonstrating active synthesis of Lepore \( \delta \beta \) chains in erythroid colonies derived from peripheral blood BFU-E.

added as marker. The top graph of globin chain synthesis from peripheral blood reveals a prominent \( \gamma \), no \( \beta \), a barely discernable peak consistent with Lepore \( \delta \beta \) chains, and an \( \alpha \) chain peak; the \( \delta \beta/\alpha \) synthetic ratio was 0.005. Similar results were obtained in four different studies of labeled peripheral blood cells from this patient, and the \( \delta \beta/\alpha \) synthetic ratio in these studies always ranged between 0 and 0.01. Globin synthesis from peripheral blood BFU-E-derived colonies also shows prominent \( \gamma \) and absent \( \beta \) chain synthesis. However, unlike the pattern noted from peripheral blood, a clearly defined peak of newly synthesized Lepore \( \delta \beta \) chains can now be seen. The \( \delta \beta/\alpha \) chain ratio from the BFU-E-derived colonies was 0.15. The \( \delta \beta/\alpha \) synthetic ratio of labeled bone marrow cells, obtained from the patient at a different time from the culture study, was also 0.15. Globin synthetic studies on peripheral blood and peripheral blood BFU-E of a normal control revealed \( \beta/\alpha \) ratios of 0.738 and 0.789, respectively. There was no evidence of globin synthesis in the \( \delta \beta \) globin region.

**DISCUSSION**

Imbalanced globin chain synthesis in the \( \beta^-\)-thalassemias has been demonstrated in peripheral blood reticulocytes and bone marrow erythroblasts. Utilizing short-term suspension cultures, Kim and coworkers have furthered our understanding of globin synthesis in these disorders by showing imbalanced synthesis in early erythroblasts and during their maturation in culture. We have been able to extend these observations by culturing bone marrow and peripheral blood erythroid-committed stem cells from patients with \( \beta^-\)-thalassemia in a plasma clot system. Our studies indicate that the same program of abnormal globin chain synthesis expressed in thalassemic bone marrow cells and reticulocytes can be demonstrated in colonies derived from both early and late erythroid progenitor cells.

It has previously been demonstrated that, in homozygous \( \beta^-\)-thalassemia, \( \alpha/\beta \)-globin chain imbalance can be greater in reticulocytes than in bone marrow cells, although such a phenomenon is not universally observed. Furthermore, partially purified proerythroblasts and orthochromatic erythroblasts isolated from bone marrow of patients with homozygous \( \beta^-\)-thalassemia have been shown to display less globin chain imbalance than whole bone marrow preparations. Increased \( \alpha \)-chain proteolysis in nucleated erythroid cells is the most likely explanation for the commonly
observed phenomenon of apparently balanced globin chain synthesis in bone marrow of individuals with heterozygous β-thalassemia. A similar phenomenon may account for the previously reported differences in globin chain synthetic ratios between marrow and peripheral blood cells in homozygous β-thalassemia. In our study, the relatively minor variability of β/α ratios found between starting bone marrow cells and erythroid cell colonies may reflect the fact that colonies derived from both early and late committed erythroid stem cells are a composite of nucleated erythroid cells at differing stages of development, with variable degrees of proteolytic activity for excess free α-chains that accumulate in β-thalassemic erythroid cells.

In normal adults, synthesis of γ-chains has been shown by others to be higher in colonies derived from both peripheral blood BFU-E and from bone marrow CFU-Es or BFU-Es than it is in erythroid cells obtained from the same individuals in vivo, although some controversy exists concerning the universality of this phenomenon. Colonies derived from peripheral blood BFU-E of patients with thalassemia have also been shown by different workers to synthesize increased amounts of γ-chain compared to the patient’s erythroid cells in vivo. Since β-thalassemia major is a process characterized by erythropoietic stress and increased F cell production and/or selective F cell survival in vivo, one would expect to find higher levels of γ-chain synthesis in cultures of thalassemic cells than of normal cells. In our studies, we did in fact find higher levels of γ-chain synthesis in cultures of thalassemic cells than of normal control cells (Table 3). In some cases, the γ/α synthetic ratios obtained from cultured thalassemic cells were similar to those of the starting bone marrow cells, but in other cases there was no significant difference. It has been previously observed that γ/α synthetic ratios are usually much lower in total bone marrow than in peripheral blood reticulocytes of patients with homozygous β-thalassemia, because of selective survival of cells that synthesize increased numbers of γ-chains and therefore have fewer excess free α-chains. Therefore, the increased levels of γ/α chain synthesis in some cultures of thalassemic erythroid cells compared to starting total bone marrow cells could be related to selective survival of thalassemic F cells in those cultures. Other factors that could also contribute to fluctuations in measurements of γ/α synthetic ratios include: (1) variable degrees of proteolysis of excess α-chains in nucleated thalassemic erythroid cells at different stages of maturation in culture and (2) variable degrees of contamination of the γ peak region of the CMC chromatograms by nonglobin proteins from the pre-γ peak.

Hemoglobin Lepore is an abnormal hemoglobin resulting from a nonhomologous crossing over of mispaired δ and β loci. The gene product (δβ chain) is synthesized at a low level. Studies of globin synthesis in patients with Hb Lepore reveal that its synthesis occurs primarily within nucleated bone marrow cells; reticulocytes display little or no Lepore δβ globin chain synthesis. The finding of active synthesis of δβ globin chains in colonies derived from peripheral blood BFU-E of the patient with β-thalassemia/Hb Lepore implies that the pattern of globin chain synthesis observed in our cultures is similar to that of nucleated erythroid precursor cells rather than that of more mature reticulocytes. Similarly, in cultures of thalassemic marrow stem cells, a small peak of radioactivity was frequently seen following the β-chain peak, in the position expected for δ chains (Fig. 1 and additional data not shown). This post-β peak accounted for 2%–5% of the α-chain radioactivity, but insufficient material could be obtained for structural studies to confirm that the material in fact constituted authentic δ chains. These observations indicate that potential utility of cultures of peripheral blood (and bone marrow) derived stem cells for the study of molecular processes in nucleated thalassemic erythroid precursor cells.

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