Serum Erythropoietin and Erythropoiesis in High- and Low-Fetal Hemoglobin β-Thalassemia Intermedia Patients

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Clinical data suggest that in β-thalassemia–intermedia patients, higher levels of circulating fetal hemoglobin (HbF) are associated with greater disease severity at comparable degrees of anemia. We assessed the influence of the amount of circulating HbF on serum erythropoietin (s-Epo) levels and on serum transferrin receptor, a measure of erythropoiesis, in 30 β-thalassemia–intermedia patients. Twenty-four showed more than 40% HbF (21 of whom with β-thalassemia) and 6 presented lower HbF levels (β+–thalassemia). The two groups of patients did not differ in age (15.3 ± 19 years, respectively) or degree of anemia (Hb = 8.8 g/dL in both groups). Log (s-Epo) was correlated inversely with Hb (r = -0.47; P < .01), and directly with HbF (r = .55; P < .001). Multivariate regression analysis showed that Hb and HbF were independently correlated with s-Epo levels. High-HbF patients had greater s-Epo values at the same Hb level than low-HbF patients. Considering that iron-deficiency anemia control patients represented the predicted physiologic response of s-Epo to anemia, the observed/predicted s-Epo ratio in low-HbF thalassemic patients was no different from controls, but was increased in the high-HbF group. High-HbF patients also showed an expansion of erythropoiesis as much as four to nine times the normal value at the same Hb level as low-HbF patients. We conclude that HbF exerts an independent regulatory effect on erythropoietin production and erythropoiesis that is detectable only when HbF levels exceed 40%.

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Materials and Methods

Patients

Thirty patients with thalassemia intermedia followed at the Hematology Department of the Istituto di Clinica e Biologia dell'Eta' Evolutiva of Cagliari were investigated. Informed consent was obtained from each subject or from the parents of patients under the age of 18 to draw blood for the study while they were under routine care. There were 18 males and 12 females, from 1.5 to 39 years of age (median = 15.5 years); all were of Sardinian descent and fulfilled accepted criteria for a diagnosis of thalassemia intermedia. The patients had never been transfused, but 18 (60%) had been splenectomized at least 1 year before the study. None of them showed abnormal renal function (blood urea nitrogen and serum creatinine were normal) at sample collection.

Methods

Red blood cell indices were obtained with a Coulter Counter Model S (Coulter Immunology, Hialeah, FL). Reticulocyte counts were performed by microscopic observation after staining with brilliant cresyl blue. Serum ferritin was determined using a radioimmunoassay (RIA) method (Ramco Lab, Houston, TX). Globin-chain synthesis analysis was performed according to Kan et al. HbF was assessed with the alkali denaturation method or high performance liquid chromatography (HPLC). Hb patterns were determined by electrophoresis on cellulose acetate (pH 8.4) and on citrate agar (pH 6.0) or with HPLC. DNA was extracted from peripheral blood.

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leukocytes in all patients and a-globin genotype determination and b-thalassemia defect characterization were performed according to previously described procedures.19,20

S-Epo assay. S-Epo was determined using venous blood sampled without anticoagulant, and serum was separated by centrifugation within 12 hours and stored at 20°C until thawed for assay. S-Epo was measured by means of the RIA currently used in our laboratory (EPO-Trap; Incstar, Stillwater, MN). This procedure is a competitive binding, disequilibrium RIA using human recombinant Epo for both tracer and standards. All samples were assayed in duplicate, and estimates outside the standard curve (higher than 250 mU/mL) were reassayed after dilution with a serum of known S-Epo concentration. Intra-assay and interassay variation coefficients differed at the different sample concentrations, but were always less than 10%.

Normal values, as assayed in 46 healthy volunteers (21 men and 25 women, 24 to 70 years of age: mean 32.2 ± 9 SD) whose Hb levels exceeded 12 g/dL in women and 14 g/dL in men (12.1 to 16.0 g/dL), ranged between 5.3 and 29 mU/mL (mean 18.1 ± 8.3 mU/mL). There was no difference between men and women.

To establish the expected S-Epo response to anemia, 42 patients with iron-deficiency anemia not caused by cancer who had not received red blood cell transfusions in the preceding week were chosen as the reference anemic population. Their Hb ranged from 1.4 g/dL.

S-TfR. The amount of S-TfR was estimated by an enzyme-linked immunosorbent assay (CLINIGEN, Amgen Diagnostics, Thousand Oaks, CA). The normal range, as determined in 40 healthy adult volunteers (16 men and 24 women), was 2,240 ± 510 µg/dL (range 1,470 to 3,400), without significative differences between men and women.

Data Analysis

Statistical analysis was performed with MICROSTA software (Ecosoft, Inc) running on an IBM PC (IBM, Indianapolis, IN). The Student’s t-test was used for comparisons between means, Pearson’s test for calculating correlation coefficients, linear regression analysis for assessing correlation between variables, and analysis of covariance for comparing regression slopes. A P value of less than .05 was considered significant.

RESULTS

Patient Characteristics

Table 1 summarizes the results of a- and b-globin gene mapping in the patients. In synthesis, 21 patients had a b-thalassemia and 9 had a b-thalassemia, with different a-globin genotypes. HbF concentration ranged from 3.7% to 99% of the total Hb level. This marked variation in the percentage of HbF was in accord with the thalassemic genotype. All 21 cases with b-thalassemia had a percentage of HbF greater than 96%. Three b-thalassemia cases presented a value ranging from 46% to 60%, and the remaining six b-thalassemia patients showed concentrations under 10%. For consistency of reporting and discussion, we divided the patients into two groups: those with HbF greater than 40% (high-HbF) and those with less than 40% (low-HbF). The characteristics of these patients, including Hb, mean corpuscular volume (MVC), reticulocyte count, and serum ferritin, are presented in Table 2. The two populations of patients had comparable degrees of anemia. High-HbF patients had greater MVC values than low-HbF patients; however, the uneven distribution of splenectomized cases (70.8% vs 16.6%) contributed to this difference. As a matter of fact, splenectomized patients had higher MVC (79 ± 64 fl) than nonsplenectomized ones. Hematologic data before splenectomy were available for 14 out of the 17 high-HbF patients who had been splenectomized. Mean presplenectomy Hb was 8.3 (range 7.1 to 10 g/dL), showing that the two patient populations actually represent constitutionally similar cases as far as severity of anemia is concerned.

s-Epo Levels as a Function of Anemia and HbF

Thalassemic patients showed a higher mean s-Epo value than normal individuals (126.2 ± 99.7 mU/mL, range 19 to 435 mU/mL), and log (s-Epo) levels increased as Hb decreased (r = - .47; P = .008). Among the other variables considered in this study, s-Epo was also found to be correlated with the percentage of HbF (r = .55; P = .001), and high-HbF patients had a s-Epo value significantly greater than low-HbF patients (144 ± 102 mU/mL, range 40.2 to 435, 54.9 ± 37.1 mU/mL, range 19 to 102; P = .02). Multiple regression analysis, considering s-Epo as the dependent variable, showed that both the Hb and HbF levels were significantly and independently correlated variables.

Table 1. b-Thalassemia Mutation and a-Globin Genotype in b-Thalassemia–Intermedia Patients

<table>
<thead>
<tr>
<th>b-Thalassemia Mutation</th>
<th>a-Globin Genotype</th>
<th>No. of Cases</th>
<th>a/b Ratio</th>
<th>HbF%</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-thalassemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b\textsuperscript{39}/b\textsuperscript{39}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>3</td>
<td>1.9 ± 0.4</td>
<td>&gt;96</td>
</tr>
<tr>
<td>b\textsuperscript{39}/b\textsuperscript{39}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>9</td>
<td>1.8 ± 0.5</td>
<td>&gt;96</td>
</tr>
<tr>
<td>b\textsuperscript{39}/a\textsuperscript{a}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>1</td>
<td>1.2</td>
<td>&gt;96</td>
</tr>
<tr>
<td>b\textsuperscript{39}/a\textsuperscript{a}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>1</td>
<td>ND</td>
<td>&gt;96</td>
</tr>
<tr>
<td>b\textsuperscript{39}/a\textsuperscript{a}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>4</td>
<td>2.4 ± 0.5</td>
<td>&gt;96</td>
</tr>
<tr>
<td>b\textsuperscript{39}/a\textsuperscript{a}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>1</td>
<td>2.6</td>
<td>&gt;96</td>
</tr>
<tr>
<td>b\textsuperscript{39}/b\textsuperscript{39}</td>
<td>a\textsuperscript{a}/a\textsuperscript{a}</td>
<td>2</td>
<td>3.0</td>
<td>&gt;96</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

Table 2. Clinical and Hematologic Data (Mean and Range) of 30 Thalassemia-Intermedia Patients According to HbF Levels

<table>
<thead>
<tr>
<th></th>
<th>Low HbF (N = 6)</th>
<th>High HbF (N = 24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>2/4</td>
<td>8/16</td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>19 (8-37)</td>
<td>15.3 (1.5-39)</td>
<td>NS</td>
</tr>
<tr>
<td>Splenectomy (Y/N)</td>
<td>1/5</td>
<td>17/7</td>
<td>.01</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>8.8 (7.5-10.3)</td>
<td>8.8 (7.3-10.7)</td>
<td>NS</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>61.7 (55-73.6)</td>
<td>76.1 (57.3-91)</td>
<td>.0004</td>
</tr>
<tr>
<td>Retics (x10\textsuperscript{3}/L)</td>
<td>48.4 (32-68)</td>
<td>113 (48-400)</td>
<td>NS</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>6.33 (3.7-10)</td>
<td>92.2 (40-98)</td>
<td></td>
</tr>
<tr>
<td>s-Ferritin (µg/dL)</td>
<td>288 (49-783)</td>
<td>405 (15-1,300)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.
ERYTHROPOIESIS IN \( \beta \)-THALASSEMIA INTERMEDIA

Fig 1. The relationship between serum erythropoietin (s-Epo) and hemoglobin concentration (Hb) in specimens with HbF concentrations less than 40% or greater than 40%.

Together, they justified 75% of the variance in s-Epo; 44% was caused by HbF alone. The Hb/s-Epo relationship was stronger when high-HbF \((r = -.48; P = .01)\) and low-HbF patients \((r = -.78; P = .05)\) were considered separately. The better result was caused by two different regression lines. In fact, as shown in Fig 1, the parameters of the curve describing the regression between s-Epo and Hb were \(\log (\text{s-Epo}) = 8.3 - 0.51 \text{ (Hb)}\) in high-HbF patients and \(\log (\text{s-Epo}) = 8.6 - 0.43 \text{ (Hb)}\) in the low-HbF group. Both the slope and the intercept values of these two lines were significantly different \((P < .01\) and \(P < .001\), respectively). The difference in s-Epo concentration for high- versus low-HbF patients was as great as 30 and 200 mU/mL (82% and 200%) for expected Hb values of 5 and 10 g/dL, respectively.

In the reference anemic population, s-Epo ranged between 39 and 942 mU/mL. The anemic control subjects yielded the following regression \((r = -.76; P = .000)\) between Epo and Hb: \(\log (\text{s-Epo}) = 8.2 - 0.47 \text{ (Hb)}\). Based on this formula, a predicted log (s-Epo) value was derived for each thalassemic patient sample, and the ratio of observed/predicted (O/P) log (s-Epo) was calculated. This ratio had a mean value of 1.12, range 0.78 to 1.47. There was a significant difference among the O/P ratios in low- and high-HbF patients \((0.93 v 1.17, P < .000)\), and these values were correlated with the HbF level \((r = .66; P = .000)\). Whereas the O/P ratio of low-HbF patients did not differ from that of the anemic control population, high-HbF patients showed greater values, documenting that s-Epo levels in this population are higher than physiologically expected.

No effect of splenectomy on the O/P ratio was found. Indeed, the splenectomized patients presented values that were not significantly different from those of the nonsplenectomized ones.

S-TfR

Thalassemic patients had a s-TfR level ranging from 9,310 to 28,085 µg/dL, ie, from 4.1 to 12.5 times the normal mean value. In the entire group of patients, s-TfR values were correlated inversely with Hb levels \((r = -.46; P = .03)\) and directly with s-Epo values \((r = .56; P = .005)\), showing a physiologic regulation of erythropoiesis. An independent regulatory effect on the extent of erythropoiesis (s-TfR) was also documented by the levels of circulating HbF. As a matter of fact, high-HbF patients had a curve describing the Hb/s-TfR regression slope \([\log (\text{s-TfR}) = 11.2 - 0.14 \text{ (Hb)}; r = -.52; P = .01]\) that was greater than that for low-HbF patients \([\log (\text{s-TfR}) = 10.9 - 0.15 \text{ (Hb)}; r = -.31; P = .05]\) (Fig 2). The expected difference in s-TfR concentration for high-versus low-HbF patients was as great as 8,996 and 18,286 µg/dL, ie, from four to nine times normal, for Hb values of 10 and 5 g/dL, respectively.

DISCUSSION

The objective of this work was to provide a quantitative analysis of the factors governing erythropoiesis in \( \beta \)-thalassemia intermedia. In particular, we assessed how circulating
HbF influences the production of Epo and, consequently, the extent of erythropoiesis.

This study shows that a significant relationship between Hb and s-Epo exists in β-thalassemia intermedia patients, but that considerable variation between s-Epo levels is present at a given Hb value. The HbF level proved to be important for the scattering of s-Epo values: at comparable Hb concentrations, patients with high HbF percentages presented more s-Epo than those with lesser quantities of HbF. To compare the Epo response in thalassemic patients with the physiologic response of Epo production to anemia, the Hb/s-Epo relationship in thalassemic patients was compared with that found in patients with iron-deficiency anemia. Whereas thalassemic patients with HbF values under 40% displayed a Hb/s-Epo dependence that did not differ significantly from that of the control anemic population, the high-HbF group showed greater s-Epo levels than the control population at the same Hb value. High-HbF patients had s-Epo concentrations from 80% to 200% above those of low-HbF patients with the same Hb level.

The evidence that thalassemic patients present an adequate or overexpressed s-Epo concentration is at variance with some previously published results14,15 that showed low s-Epo levels for the degree of anemia in both thalassemia-major and thalassemia-intermedia patients. This discrepancy is not easy to explain. One hypothesis could be that other investigators used patients with aplastic anemia as controls, and these patients show higher s-Epo levels than those with iron-deficiency anemia at comparable Hb concentrations.16

The ultimate consequence of an altered regulation of Epo production in thalassemic patients with high HbF is a proportional increase in erythropoiesis. In this study, we assayed the extent of erythropoiesis by measuring s-TfR concentration. Circulating TfR is derived primarily from erythroid precursors in the bone marrow, and its level, in the absence of iron deficiency, provides a reliable measure of total erythropoiesis.15 In the overall population of thalassemic patients, we observed an s-TfR increase from 4 to 12 times normal values. The increase in s-TfR generally paralleled the severity of anemia, but different Hb/s-TfR response curves were obtained in patients with high and low HbF values. The difference seems to be not only statistically significant, but biologically meaningful as well. High-HbF patients had s-TfR levels four to nine times above normal with respect to low-HbF patients with the same Hb value. The possibility that differences in the efficiency of erythropoiesis between the two groups of patients could influence the difference in s-TfR levels has to be ruled out. As a matter of fact, evidence exists that s-TfR levels in patients with hemolytic anemia and in those with ineffective erythropoiesis do not differ.17

The detrimental effects of high levels of HbF in β-thalassemia-intermedia patients would seem to contradict the fact that high γ-chain production in β-gene cluster defects is known to produce a thalassemia with a milder phenotype, and that genetic manipulation with drugs that increase HbF levels has proved to be beneficial for the Hb concentrations of patients with the disease.18 However, our results must not be interpreted in terms of the effects of HbF synthesis on the severity of anemia, but in terms of how Epo production and erythropoiesis are regulated at a given Hb level. Our data are very similar to those obtained in premature infants.25 In infants with HbF values less than 30%, the Hb concentration decreased 2 to 3 g/dL lower than that of infants with HbF levels greater than 60% before comparable Epo responses were noted. A hypothesis that could explain both these data and our results is that high HbF levels act on Epo production and erythropoiesis by shifting the hemoglobin-oxygen dissociation curve to the left, thus producing lower tissue oxygen availability at equivalent levels of circulating Hb.

There are relatively few data available that offer any insight into the real clinical significance of changes related to HbF concentration in thalassemic patients.34 Subjects with high levels of HbF have been reported to show an earlier presentation of the disease, a quicker progression to splenomegaly, and a higher incidence of extramedullary erythropoiesis. This is confirmed by the present data, which show 7% of splenectomized patients in the high-HbF group versus 14% in the low-HbF one. Nevertheless, large studies concerning this point are still needed, because this knowledge would be useful for both classificatory and prognostic purposes.

Our results do shed light on the effects of changes in the erythropoietic profiles of β-globin disorders resulting when Hb is increased with therapies like butyrate, hydroxyurea, or erythropoietin.7-14 These clinical interventions increase both circulating Hb and the fraction of HbF, and the results should theoretically be evaluated in terms of overall oxygen delivery. From our data, only HbF concentrations above 40% are responsible for greater s-Epo activity and erythropoietin expansion than expected for the degree of anemia. In the reported results up to now in the literature, however, in no case has γ-chain manipulation resulted in an increase of HbF of this magnitude. Thus, the amelioration of anemia overwhelms the shifting of oxygen dissociation, and this is documented by the clinical benefits reported.

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