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SERUM ERYTHROPOIETIN AND CIRCULATING TRANSFERRIN RECEPTOR IN THALASSEMIA INTERMEDIA PATIENTS WITH HETEROGENEOUS GENOTYPES

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ABSTRACT

Background. **Thalassemia intermedia patients usually do not require blood transfusions; however, all show variable degrees of erythropoietic marrow expansion to compensate for more or less marked anemia, and this represents the major cause of complications in untransfused individuals.**

Materials and Methods. **To assess the degree of erythropoietic expansion in thalassemia intermedia, serum erythropoietin (sEpo) and serum transferrin receptor (sTfr) were determined in thirty Italian patients characterized by their β-globin genotype.**

Results. **Six patients showed inappropriately low sEpo levels (O/P ratio < 0.85). Even excluding these cases, no clear relationship was observed between Hb levels and sEpo or sTfr. Two groups of patients were compared: the first with low HbF (< 40%) that included the majority of β^+ genotypes, and the second with high HbF (> 40%) that contained a prevalence of β° genotypes. Hb levels were similar in the two groups: 8.09±1.15 g/dL in low HbF and 8.82±1.28 g/dL in high HbF patients. Mean sEpo was 112±78.02 mU/mL (O/P ratio = 0.98±0.22) in the first and 246.62±184.30 mU/mL (O/P ratio = 1.25±0.30) in the second group, with a statistically significant difference, as expected, because of HbF oxygen hyperaffinity. No significant difference in sTfr levels was observed, indicating a comparable erythropoietic response in the two groups.**

Conclusions. **The relationships between anemia, HbF and total erythropoiesis in thalassemia are more complex than expected. Further studies of subjects with high HbF and benign conditions, such as HPFH, could be of help in clarifying this point, to the aim of safely increasing HbF in thalassemia intermedia.**

Key words: thalassemia intermedia, fetal hemoglobin, serum transferrin receptor, erythropoiesis

Thalassemia intermedia is a clinical definition applied to thalassemic patients who present a milder clinical course than those with thalassemia major.[1] Thalassemia intermedia patients usually do not require blood transfusions; however, all show variable degrees of erythropoietic marrow expansion to compensate for more or less marked anemia. Marrow expansion is the major cause of complications in untransfused patients, leading to increased intestinal iron absorption, megaloblastic crisis following folic acid deficiency, bone reabsorption and fractures, and the development of heterotopic marrow masses.[1] An analysis of the factors that influence bone marrow expansion in thalassemia may provide insights into the pathogenesis of anemia and aid in developing guidelines for the treatment of this category of patients.[2]

Thalassemia intermedia is heterogeneous both

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at the clinical and the genetic level.[1-5] Besides degree of anemia, high amounts of fetal hemoglobin (HbF) are an additional stimulus to erythropoiesis because of its high oxygen affinity. High levels of HbF may be associated with a favorable outcome in thalassemia intermedia since elevated γ chain synthesis compensates for decreased β production, thus reducing ineffective erythropoiesis. In this case the beneficial effect on erythropoiesis outweighs the decreased tissue oxygen unloading. This is best exemplified by conditions defined as *hereditary persistence of fetal hemoglobin* (HPFH), which show no anemia, notwithstanding the β gene deletion.[6-8] This *in vivo* observation is the rationale for the manipulation of hemoglobin switching as a potential treatment for thalassemia intermedia.[9]

Several reports in the literature suggest that serum erythropoietin (sEpo) values are quite variable in thalassemia intermedia, and not always closely related to the degree of anemia.[10,11] In addition, treatment with recombinant human Epo *in vivo* has produced contrasting effects.[12-14] The possibility of dosing both sEpo and serum transferrin receptor (sTfr) has provided a tool for investigating erythropoiesis in a simple, non-invasive method.[15-18] A pilot study of erythropoiesis in selected thalassemia intermedia patients from Sardinia has shown that for a given hemoglobin value, HbF has an independent regulatory effect on erythropoietin release and erythropoiesis expansion.[19] Patients with high HbF (> 40%) were found to have significantly greater sEpo and sTfr levels than low HbF (< 40%) patients at similar hemoglobin concentrations.[19]

Here we assessed the levels of sEpo and sTfr in a series of thalassemia intermedia patients heterogeneous in terms of both HbF percentage and molecular defect. The ultimate goal of this type of investigation is to evaluate the relationships between genotype, HbF levels and the degree of erythropoietic expansion in order to assess whether high HbF is useful or detrimental to erythropoiesis. This is a point of major interest in view of new modalities of treating β-thalassemia patients with pharmacological stimulation of HbF.[9]

Materials and Methods

Patients

Thirty adult patients who fulfilled the criteria for thalassemia intermedia were studied (Table 1). There were fourteen males and sixteen females, aged 22-55 (mean age 33±8 years). Sixteen had never received blood transfusions; the others had been occasionally transfused because of surgery, pregnancy or intercurrent illnessess. Patients who had received blood transfusions were studied at least two months after the last transfusion. Nineteen patients had been splenectomized and 4 of the unsplenectomized ones presented significantly enlarged spleens (++ or +++ in Table 1). Iron deficiency was excluded in all cases; the majority of patients had clinical or laboratory evidence of iron overload. None of the patients had abnormal renal function tests at the time of the study.

Hematological studies

Hemoglobin values and red blood cell indices were obtained with a Technicon H3. HbF was dosed by the alkali-denaturation technique.[20]

For sEpo and sTfr assays, serum was separated by centrifugation and stored at –20°C until the assay was performed. sEpo levels were determined by using a commercially available kit (Milenia Erythropoietin DPC, Los Angeles, CA) with an enzyme immunometric assay. Normal values for non anemic subjects were 5-40 mU/mL.

The following regression line was employed to calculate the expected sEpo values for the degree of anemia: log sEpo = –0.557(Hb)±9.2.[21] On this basis the expected log sEpo value was calculated for each case and the observed/predicted (O/P) ratio log sEpo was obtained.[17,21] Regression lines representing the limits of variability Hb/log sEpo were drawn from the literature.[21]

Serum levels of sTfR were measured using a commercially available enzyme immunoassay (R&D system, Mc Kinley Place, MN, USA) that employs a polyclonal antibody in a sandwich EIA format. Mean normal value for sTfr in non anemic subjects was 1.54±0.53 μg/mL.

For sEpo and sTfR determinations, all samples were analyzed in duplicate and values outside

Table 1. Clinical data and molecular defects of the patients studied.

Case	Age yr	Sex	Hb g/dL	HbF %	sEpo mU/L	O/P ratio	sTfR µg/mL	Blood Trans	Spleen	Mutations	Phenotype
1.	24	F	7.8	3.0	99	0.94	21.2	±	*	cd39/N.D.	β+
2.	24	F	9.4	24.0	74	1.08	24.4	±	+	cd39/N.D.	β+
3.	55	F	7.3	6.0	38	0.71	21.0	–	+	-101/cd39°	β+
4.	32	M	9.0	7.9	93	1.08	31.0	–	++	-101/IVSI-1	β+
5.	35	F	8.5	19.0	128	1.09	22.0	–	*	-101/cd39	β+
6.	33	M	8.1	5.1	95	0.97	40.0	–	*	IVS1-6/IVS1-6	β+
7.	40	M	7.4	8.0	36	0.71	13.8	–	*	IVS1-6/IVS1-6	β+
8.	32	M	5.7	12.0	58	0.67	35.0	±	*	IVS1-6/IVS1-6	β+
9.	45	M	6.6	15.0	300	1.03	21.0	–	+++	IVS1-6/IVS1-6	β+
10.	29	M	9.2	26.0	101	1.13	24.1	–	*	IVS1-6/IVS1-6	β+
11.	32	M	7.5	16.0	38	0.72	22.9	–	*	IVS1-6/cd39	β+
12.	42	F	7.8	21.0	207	1.09	20.6	±	*	IVS1-6/IVS2-1	β+
13.	33	F	7.3	25.0	55	0.78	33.0	±	*	IVS1-6/IVS110	β+
14.	25	F	8.4	26.0	103	1.02	20.8	±	+	$\delta\beta^{Sic}$/IVS110	β+
15.	22	F	10.0	32.0	109	1.29	18.5	–	+	-87/cd39	β+
16.	26	F	9.5	33.0	258	1.42	16.5	–	+	-87/cd39	β+
17.	24	M	8.7	49.0	43	0.85	25.0	–	*	IVS1-6/cd39	β+
18.	35	F	8.8	45.0	576	1.48	27.5	±	++	IVS1-6/cd39	β+
19.	34	F	8.3	58.0	374	1.29	29.4	±	*	IVS1-6/IVS110	β+
20.	26	M	8.8	86.7	86	1.04	23.0	±	*	$\delta\beta^{Sic}$/IVS110	β+
21.	29	F	7.6	>96.0	99	0.92	29.6	±	*	cd39/cd39	β°
22.	46	F	9.9	>96.0	117	1.29	28.7	±	*	cd39/cd39	β°
23.	35	M	6.5	>96.0	199	0.95	40.0	–	*	cd39/cd39 °	β°
24.	31	M	11.4	>96.0	150	1.76	16.4	–	*	cd39/cd39	β°
25.	36	F	8.7	>96.0	61	0.94	12.6	±	*	cd39/IVS1-2	β°
26.	25	F	9.5	>96.0	193	1.35	32.0	+	*	cd39/IVS2-1°	β°
27.	23	M	10.0	>96.0	352	1.61	27.3	–	+	cd39/IVS2-1	β°
28.	26	M	6.8	>98.0	200	0.98	27.0	±	*	cd39/Lepore	β°
29.	33	F	9.3	100	402	1.49	34.7	–	+	$\delta\beta^{Sic}/\delta\beta^{Sic}$	δβ°
30.	49	M	9.2	100	600	1.57	27.4	–	++	$\delta\beta^{Sic}/\delta\beta^{Sic}$	δβ°

** indicates splenectomy; °α-thalassemia carriers.*

the standard curve (higher than 200 mU/mL for sEpo and higher than 5 µg/mL for sTfr) were reanalyzed after appropriate dilution (1:6).

There were no differences in sTfR or sEpo between males and females.

Molecular studies

DNA was obtained from the peripheral blood buffy coat. Mutations in the β gene were identified after PCR amplification of the β gene sequences, either by ASO as described,[22] or by denaturing gradient gel electrophoresis and direct sequencing.[23] δβ-thalassemia Sicilian type was assessed either by Southern blot or by PCR as previously described.[24,25] Family analysis was carried out when possible, particularly in patients heterozygous for a single β-thalassemia mutation. α gene number was determined by Southern blotting or PCR as described.[26]

Statistical studies

Mean and standard deviation were calculated for each variable. The Student's t-test was used for comparisons between means. The relationships between sEpo (or sTfr) and total Hb and between sEpo and sTfr were determined by linear regression analysis after log transformation of sEpo or sTfr values. A p value of less than 0.05 was considered significant.

Results

The main hematological features of the patients as well as their molecular defects are listed in Table 1. Hb values were extremely variable, ranging from 5.7 to 11.4 g/dL (mean 8.43±1.25); the majority of patients (25/30) showed Hb levels ranging from 7.3 to 10 g/dL.

The molecular defects were highly heteroge-

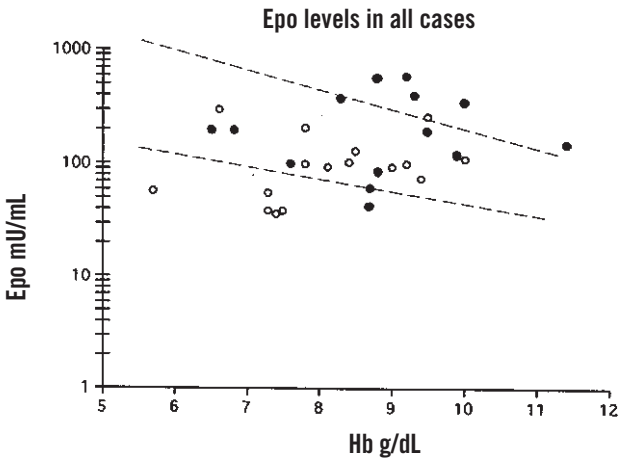

Figure 1. Relationship between serum erythropoietin (s-Epo) and total hemoglobin (Hb) in all the cases studied. Empty circles indicate low Hb F cases. Full circles indicate high Hb F subjects. Dotted lines indicate the limits of variability of the regression log sEpo/Hb taken from the literature (ref. #21).

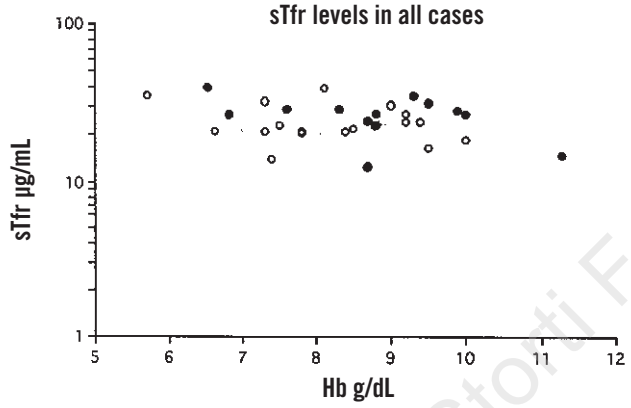

Figure 2. Relationship between serum transferrin receptor (sTfr) and total hemoglobin (Hb) in all the cases studies. Empty circles indicate low Hb F cases. Full circles indicate high Hb F subjects.

neous. Twenty patients (1-20) had β^+ thalassemia. The majority were homozygotes (5) or compound heterozygotes (6) for IVS 1 nt 6 T→C; five were compound heterozygotes for at least one promoter mutation (either -101 C→T or –87 G→C). In two subjects a single β defect was characterized. Eight patients (21-28 in Table 1) had β° thalassemia: 4 were codon 39 homozygotes; the others were codon 39 compound heterozygotes with IVS 2 nt 1 (2), IVS 1 nt 2 (1) or Hb Lepore-Boston (1). Two patients (29-30) were homozygotes for Sicilian δβ-thalassemia.

$-\alpha^{3.7}$ thalassemia was identified in three patients (indicated with ° in Table 1).

HbF concentrations ranged from 5.1% to 100 %. β^+/β^+ homozytgotes or $\beta^+/\beta°$ compound heterogotes showed values from 5.1 to 86.7%, $\beta°/\beta°$ >96% HbF, and Sicilian δβ-homozygotes had 100% HbF.

S-Epo values in all series were spr
174.8±151.81), a remarkable increase over normal controls. However, 6 patients had sEpo levels inappropriately low for their degree of anemia (O/P ratios < 0.85) (Table 1) and below the limits of variability reported for Epo/Hb regression lines in the literature (Figure 1). These values were obtained repeatedly. We were unable to find an inverse correlation between log Epo and Hb ($r = 0.258$; $p = 0.168$), even excluding the above mentioned cases (Figure 1). sTfr levels ranged from 12.6 to 40 µg/mL (mean values 25.5±7.02 µg/mL), several times greater than normal controls but without a correlation with Hb values ($r = –0.224$; $p = 0.233$) (Figure 2).

In order to analyze the results further, patients were classified into two groups according to HbF percentage and, partially, according to the genetic defect. Group 1 (HbF < 40 %) included 16 patients and group II (HbF > 40%) 14 patients. The distinction may appear arbitrary

but it was useful for comparing the data with a previous publication.[19] Mean, SD and ranges of age, Hb, HbF, sEpo and sTfr, sex and genotype distribution in the two groups are shown in Table 2. Hb levels were comparable in the two groups (8.09±1.15 g/dL vs 8.82±1.28 g/dL). Genotypes differed significantly; all the patients in the first group had β^+ thalassemia, whereas there was a prevalence of β° mutations in the second group. S-Epo values in the low HbF group were 112±78.02 mU/mL (O/P ratio 0.98±0.22) and 246.62±184.30 mU/mL (O/P ratio 1.25±0.30) in the high HbF group, and the difference was statistically significant (p = 0.01).

There was no significant difference between sTfr levels in the low HbF (24.11±7.07 μg/mL) and the high HbF (27.19±6.84 μg/mL) groups. sTfr levels were not related to sEpo values. No relationship was observed between the degree of anemia and log sTfr, even when only the sTfr values from subjects with adequate Epo production were considered (not shown).

Table 2. Main characteristics of the study patients grouped according to HbF concentration.

	Group I Hb F < 40 % (N = 16)	*Group II Hb F > 40 % (N = 14)*	*p*
Sex M/F	7/9	6/7	N.S.
Age (yrs)	33±8.8 (22-55)	33±8 (23-49)	N.S.
Hb g/dL	8.09±1.15 (5.7-10)	8.82±1.28 (6.5-11.4)	N.S.
HbF %	17.62±9.48 (5.1-33)	86.34±19.75 (45-100)	
sEpo (mU/mL)	112±78.02 (36-300)	246.62±184.30 (43-600)	0.01
sEpo (O/P ratio)	0.98±0.22 (0.67-1.42)	1.25±0.30 (0.86-1.75)	0.01
sTfr (μg/mL)	24.11±7.07 (13.8-40)	27.19±6.84 (12.6-40)	N.S.
Genotype β^+/β°	16/0	4/10	

Discussion

The relationships between erythropoietic expansion and HbF production in thalassemic patients are complex and have only been partially explored. Undoubtedly the amount of HbF influences erythropoietic activity through increased erythropoietin release because of reduced oxygen unloading in the tissues. This is clearly demonstrated by previous reports[19] and was shown here by the significantly higher sEpo (and O/P ratio) levels in high as compared to low HbF patients (Table 2), and by the remarkably increased sEpo levels in δβ homozygotes with 100% HbF. However, at variance with previous results, we were unable to demonstrate an inverse correlation between Hb and sEpo. In a significant proportion of cases Epo production was low or at least inadequate for the degree of anemia, as indicated by low sEpo O/P ratios. This occurred mainly in low HbF patients with extreme degrees of anemia (Table 1). Inadequate Epo levels in thalassemia have been reported by other investigators[10,11] and are not easily explained. We observed no correlation with spleen presence or size, iron overload or age. Renal function tests were normal, and anemia of chronic disorders was not documented in these patients. It does not appear that Epo is consumed by a high degree of erythroid hyperplasia, given the low-intermediate range of sTfr levels in these subjects (Table 1). Whether inflammatory cytokines, found to be increased in some thalassemic patients,[27] play some role in Epo reduction in these cases remains to be explored.

Circulating sTfr is derived mainly from immature erythroblasts and its level, in the absence of iron deficiency, is considered the best estimate of total erythropoiesis.[15,17] Although sTfr levels were increased several times over normal controls, no clear relationship was observed beween Hb and log sTfr in our series analyzed as a whole (Figure 2). Considering only the cases with adequate Epo production, sTfr levels increased as Hb decreased, but they were still unrelated to the degree of anemia (data not shown). sTfr levels were slightly increased in high as compared to low HbF patients, but the difference was not statistically significant, suggesting a similar erythropoietic response in the two groups.

Our results are apparently difficult to recon-

cile with the clear Hb/sEpo and Hb/sTfr relationships observed in a group of Sardinian patients with thalassemia intermedia.[19] A detailed analysis of the two studies reveals several differences. First, patients in our series were significantly older than in the Sardinian study (mean age 33 vs 15.5 years). Second and most important, high and low HbF groups in the Sardinian study were at the two extremes of the spectrum of thalassemia intermedia conditions; in the high HbF group, β° homozygotes with >96% HbF represented the majority of cases, whereas in the low HbF group only 6 cases were studied with mild mutations and HbF <12%. On the contrary, HbF levels in our series were much more spread, reflecting heterogeneous genotypes, particularly in the β^+ series. As a consequence, whereas sEpo and sTfr levels in the high HbF groups in the two studies are comparable, low HbF patients in our study had greater HbF, sEpo and sTfr values, thus reducing the differences with the high HbF group.

There is clear evidence that sTfr reflects total erythropoiesis, but it is still unclear whether differences in the ratio of ineffective to effective erythropoiesis can affect its level.[19] We cannot exclude that variable degrees of ineffective erythropoiesis influenced sTfr concentrations in our patients. An important determinant of ineffective erythropoiesis is the capability of the erythroid cell to produce HbF. It is well known that the amount of HbF measured in the blood is determined by several factors, both inherited and acquired, as witnessed by HbF level variation in patients with the same genotype (Table 1). Genetic modifiers of HbF production include a number of α genes,[3,4] the β cluster haplotype[28] or heterocellular HPFH, a common condition associated with an increased number of F-cells[3] and not linked to the β cluster.[4,7,29,30] Preferential survival of F-cells is an acquired mechanism of HbF increase that operates maximally in severe β+ or β°-thalassemia.[2,3,8] Genetically determined high HbF is expected to decrease ineffective and total erythropoiesis, thus ameliorating the α/non-α ratio. This would not occur when high HbF is the result of F-cell selection. On this basis, total erythropoietic expansion in thalassemia would be not only erythropoietin-related, but also dependent on the genetic capability of the erythroid cell to produce HbF. However, it is difficult to separate the contribution of *genetic* and *acquired* mechanisms in single cases. The effect of different genotypes in thalassemia intermedia needs to be investigated in large groups of patients characterized according to their genotypes.

The relationships between anemia, HbF and total erythropoiesis in thalassemia are more complex than expected. Further studies of subjects with high HbF and benign conditions such as HPFH could be of help in clarifying this point, with the aim of safely increasing HbF in thalassemia intermedia.[9]

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