Differential effects of the type of iron chelator on the absolute number of hematopoietic peripheral progenitors in patients with β-thalassemia major

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ABSTRACT

Several studies have established an association between iron chelation therapy with deferasirox and hematopoietic improvement in patients with myelodysplastic syndromes. There are no data from patients with β-thalassemia major. In a cross-sectional study, we evaluated the absolute number of several hematopoietic peripheral progenitors (colony-forming unit-granulocyte/macrophage, erythroid burst-forming units, colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte, and long-term culture-initiating cells) in 30 patients with β-thalassemia major (median age 29.5 years, 40% males) and 12 age-matched controls. For the β-thalassemia major patients, data on splenectomy status, the type of iron chelator used, and serum ferritin levels reflecting changes in iron status on the chelator were also retrieved. All patients had to be using the same iron chelator for at least 6 months with >80% compliance. The absolute number of all hematopoietic peripheral progenitors was higher in β-thalassemia major patients than in controls, and varied between splenectomized and non-splenectomized patients (lower number of erythroid burst-forming units and higher numbers of colony-forming unit-granulocyte/macrophage, colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte, and long-term culture-initiating cells). The number of erythroid burst-forming units was significantly higher in patients taking deferasirox (n=10) than in those taking either deferoxamine (n=10) or deferiprone (n=10) (P<0.05). After adjusting for age, sex, splenectomy status, and serum ferritin changes, the association between a higher absolute number of erythroid burst-forming units in deferasirox-treated patients than in patients taking deferoxamine or deferiprone remained statistically significant (P=0.011). In conclusion, in β-thalassemia major patients, compared with other iron chelators, deferasirox therapy is associated with higher levels of circulating erythroid burst-forming units. This variation is independent of iron status changes and is more likely to be due to the type of chelator.

Introduction

β-thalassemia major (TM) is an inherited disorder of hemoglobin synthesis characterized by ineffective erythropoiesis and compensatory erythroid hyperplasia.[1] Regular blood transfusions are effective in supplying normal erythrocytes and partially suppressing erythroid marrow expansion.[2-4] Iron overload is the main negative consequence of transfusion therapy and remains a major cause of morbidity and mortality in TM patients. This necessitates life-long iron chelation therapy to avoid/remove the toxicity of iron overload and improve survival.[5] A dynamic regulation between erythropiesis and iron overload in patients with β-thalassemia has been described, in which ineffective erythropoiesis leads to increased intestinal iron absorption through the hepatic hormone hepcidin.[6] Moreover, there is mounting evidence from animal studies that treatment of iron overload affects erythropoietic capacity and leads to improvements in hemoglobin level and red cell survival.[7-9] Similar evidence from clinical studies is limited and has been primarily reported for patients with myelodysplastic syndrome receiving the oral chelator deferasirox (DFX) and showing hematologic responses or decreased transfusion requirements, although some cases of different anemias, including thalassemia, and using other chelators were also reported.[10-17]

We undertook an evaluation of hematopoietic peripheral progenitors (HPP) in patients with TM with the initial aim of assessing whether enough HPP could be collected for a possible gene transfer therapy trial with β globin constructs. Because we found a great heterogeneity in the absolute number of HPP between patients, we conducted the current study with the aim of determining whether such variations are attributable to differences in the type of iron chelation therapy the patients are receiving.

Design and Methods

Patients

This was a cross-sectional study of 30 out of 112 TM patients attending the *Centro della Microcitemia e delle Anemie Congenite* at the

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Galliera Hospital in Genoa (Italy). Inclusion criteria were regular transfusion therapy to maintain a pre-transfusional hemoglobin level >9.5 g/dL[18] and iron chelation therapy with the same chelator for more than 6 months with a compliance rate evaluated by the treating physician as >80%. Exclusion criteria were concomitant therapy with hydroxycarbamide or human immunodeficiency virus infection. Twelve age- and sex- frequency matched healthy volunteers were also included as controls for *in vitro* clonogenic assays and for flow cytometry. The study was granted local Ethical Committee approval and all participants signed informed consent prior to recruitment.

For all TM patients, we retrieved data on splenectomy status, time from splenectomy, serum ferritin level at the time of study and at initiation of the current chelator, type of current chelator, and duration of its use.

Cell preparation

Peripheral blood samples were collected from all participants by aspiration into heparinized syringes. In TM patients, samples were collected around midway between two consecutive transfusion sessions. Mononuclear cells were isolated by density gradient centrifugation using a lymphocyte separation medium. After washing, cells were suspended in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum.

Flow cytometry

Circulating $CD34^+$ cells in the peripheral blood of TM patients and controls were assessed according to the International Society of Hematotherapy and Graft Engineering guidelines.[19] Briefly, 100 μL of EDTA-anticoagulated blood were incubated with directly conjugated monoclonal antibodies CD45-FITC and CD34-PE and isotype controls [immunoglobulin G1 (IgG1)-FITC/IgG1-PE] (Becton Dickinson Immunocytometry System, San Jose, CA, USA). A minimum of 100 $CD34^+$ events and 10^5 $CD45^+$ events were collected for the $CD34^+$ cell quantification by flow cytometry.

Clonogenic assays

Colony-forming cells [colony-forming unit-granulocyte/macrophage (CFU-GM), erythroid burst-forming units (BFU-E), colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM)] measurements were performed under standard conditions. Briefly, 10^5 peripheral blood mononuclear cells were plated in semi-solid medium (Methocult H 4434; Stem Cell Techologies Inc., Vancouver, BC, Canada). The absolute number of colony-forming cells per milliliter was assessed as follows: absolute number of colony-forming cells/mL: colony number/mononuclear cells plated x total mononuclear cells/blood. Assays for long-term culture-initiating cells (LTC-IC) were performed by seeding an aliquot of light density peripheral blood and bone marrow cells in dishes over a feeder layer of irradiated (1500 cGy) murine stromal cell line (MS-5).[20] After 5 weeks, adherent cells were trypsinized and combined with the non-adherent fraction. These harvested cells were washed and aliquots were assayed for clonogenic precursors in standard methylcellulose culture. The value provided a relative measure of the number of LTC-IC present in the original sample input. The absolute number of LTC-IC/mL was calculated as for colony-forming cells.

Statistical analysis

Descriptive statistics are presented as medians and interquartile ranges (IQR) or percentages. Median HPP values between different variable categories were compared using the Mann-Whitney U test. Correlations between HPP values and continuous variables were assessed using Spearman's correlation coefficients (r_s). A multivariate linear regression model was used to adjust the association between chelator type and HPP values of interest for selected potential confounders. All *P*-values are two-sided with the level of statistical significance set at 0.05.

Results

The median age of TM patients was 29.5 years (IQR: 23.4-35.0; min: 8.1, max: 51.1): there were 12 (40.0%) males and 18 (60.0%) females. The median age of the healthy volunteers was 30.3 years (IQR: 26.5-32.4) and five (41.7%) were males. Nineteen (63.3%) TM patients were splenectomized with the median time since splenectomy being 20.1 years (IQR: 16.5-29.0; min: 1.1, max: 39.3). Ten (33.3%) patients were receiving deferoxamine (DFO) therapy at a median dose of 45 mg/kg/day, 10 (33.3%) patients were receiving deferiprone (DFP) at a median dose of 75 mg/kg/day, and the remaining 10 (33.3%) patients were receiving DFX at a median dose of 22.5 mg/kg/day. Table 1 summarizes the patients' characteristics and iron overload indices in the three chelator groups.

Hematopoietic peripheral progenitors in patients and controls

The median absolute number of circulating $CD34^+$ cells in TM patients was 8.7/μL (IQR: 6.1-19.0) compared with 2.5/μL (IQR: 0.9-5.3) in controls (*P*=0.003). The median absolute numbers of all of CFU-GM, CFU-GEMM, BFU-E, and LTC-IC were higher in TM patients than in controls (Figure 1).

Hematopoietic peripheral progenitors and splenectomy status

Splenectomized patients had a lower median absolute number of BFU-E, but higher numbers of CFU-GM and CFU-GEMM than non-splenectomized patients, although

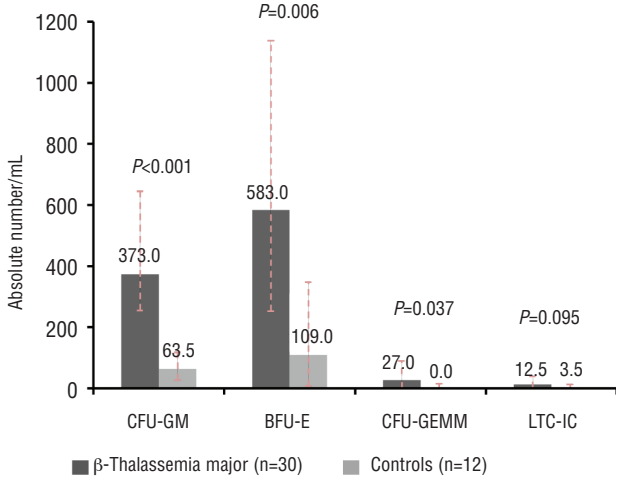

Figure 1. Absolute numbers of hematopoietic peripheral progenitors in β-thalassemia major (TM) patients and controls. Bars represent medians while whiskers represent interquartile ranges. Data on absolute number of LTC-IC were missing for 11 TM patients and four controls. CFU-GM: colony forming unit-granulocyte/macrophage; BFU-E: erythroid burst-forming unit; CFU-GEMM: colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; LTC-IC: long term culture-initiating cells.

these associations did not reach statistical significance. However, the median absolute number of LTC-IC was significantly higher in splenectomized patients than in non-splenectomized ones (P=0.043) (Figure 2). There were no statistically significant correlations between the time from splenectomy and the absolute number of any of the evaluated HPP.

Hematopoietic peripheral progenitors, iron overload, and chelation therapy

The absolute number of CFU-GM was statistically comparable between patients receiving DFO, DFP, or DFX, although DFP-treated patients showed the lowest values. A similar observation was noted for CFU-GEMM. A significant trend was noted in the absolute number of BFU-E, with DFX-treated patients having higher values than those receiving DFP (P=0.046) or DFO (P=0.008), and DFP-treated patients having higher values than those receiving DFO (P=0.034). There were no statistically significant differences in the absolute number of LTC-IC between the three chelator groups (Figure 3).

There was no significant correlation between the absolute number of HPP and: (i) the duration of use of the current chelator, (ii) serum ferritin level at the start of taking the current chelator, (iii) serum ferritin level at the time of study, or (iv) the variation in serum ferritin level from the beginning of chelation therapy (Table 2).

We constructed a multivariate linear regression model with the absolute number of BFU-E as the dependent variable. After adjusting for age, sex, splenectomy status, serum ferritin at the start of chelation therapy, and change in serum ferritin level while on the current chelator, the association between higher absolute number of BFU-E in patients taking DFX compared to those taking DFO or DFP remained statistically significant (β: 627.3, 95% CI: 160.7-1093.9; P=0.011).

Discussion

Our study provides evidence that TM patients have higher levels of circulating HPP than have normal individ-

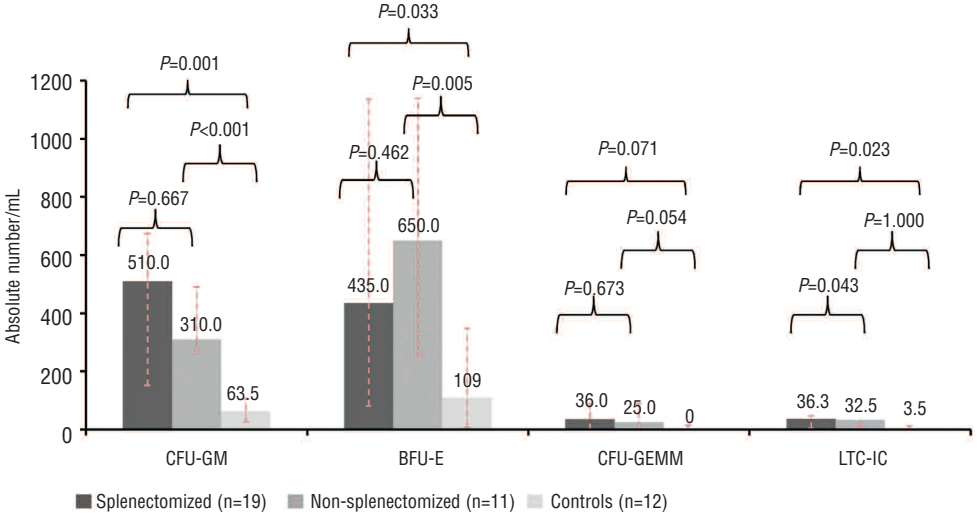

Figure 2. Absolute numbers of hematopoietic peripheral progenitors in splenectomized and non-splenectomized β-thalassemia major patients. Bars represent medians while whiskers represent interquartile ranges. Data on absolute number of LTC-IC were missing for six splenectomized and five non-splenectomized patients. CFU-GM: colony-forming unit-granulocyte/macrophage; BFU-E: erythroid burst-forming unit; CFU-GEMM: colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; LTC-IC: long-term culture-initiating cells.

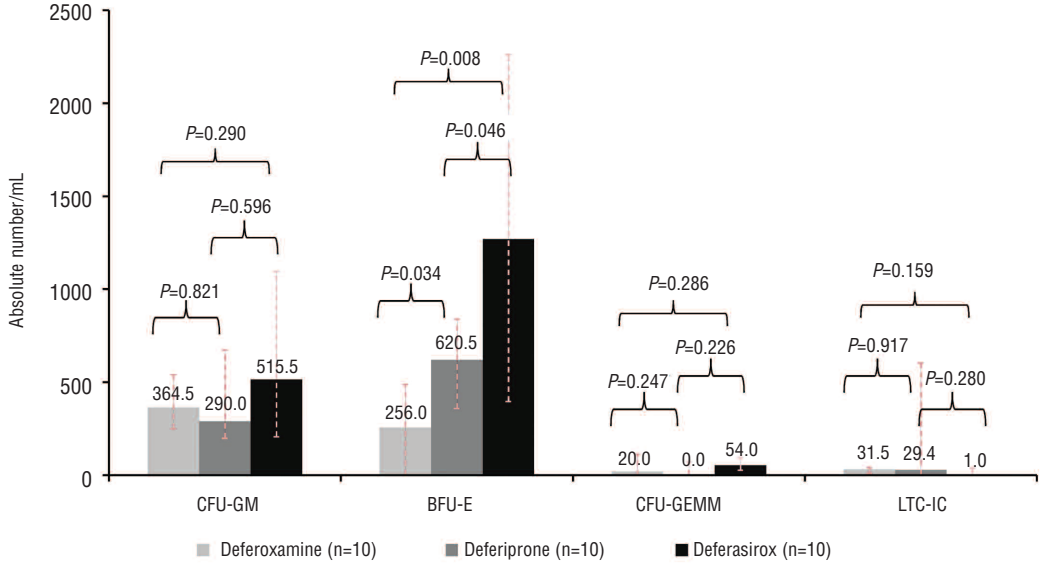

Figure 3. Absolute numbers of hematopoietic peripheral progenitors in β-thalassemia major patients according to current chelation therapy. Bars represent medians while whiskers represent interquartile ranges. Data on absolute number of LTC-IC were missing for five patients on deferoxamine, five on deferiprone, and one on deferasirox. CFU-GM: colony-forming unit-granulocyte/macrophage; BFU-E: erythroid burst-forming unit; CFU-GEMM: colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; LTC-IC: long-term culture-initiating cells.

Table 1. Patients' characteristics.

Parameter	All patients (n=30)	DFO (n=10)	DFP (n=10)	DFX (n=10)
Age in years, median (IQR)	29.5 (23.4-35.1)	26.3 (22.1-31.9)	31.7 (28.5-33.7)	30.1 (19.7-42.3)
Male, n. (%)	12 (40.0)	5 (50.0)	5 (50.0)	2 (20.0)
Splenectomized, n. (%)	19 (63.3)	8 (80.0)	6 (60.0)	5 (50.0)
Pretransfusion hemoglobin level in g/dL, median (IQR)	9.6 (8.9-10.3)	10.2 (9.2-10.6)	9.0 (8.7-10.1)	9.6 (8.9-10.2)
Duration on current chelator in years, median (IQR)	2.3 (1.1-17.9)	20.7 (14.7-21.7)	1.2 (0.5-2.3)	1.9 (1.5-2.8)
SF at study in ng/mL, median (IQR)	1222.5 (804.3-2559.0)	1131.0 (694.0-2821.3)	905.5 (625.5-1388.5)	2139.0 (1304.3-3042.8)
SF at start of current chelator in ng/mL, median (IQR)	1147.6 (821.5-2175.9)	1084.8 (824.9-1658.2)	787.7 (489.9-1268.2)	2472.4 (1099.9-4081.2)
Change in SF while on current chelator in ng/mL*, median (IQR)	16.4 (-310.4-578.3)	106.9 (-383.9-819.6)	-10.1 (-106.5-227.8)	60.9 (-920.6-882.7)

**Serum ferritin level at study minus serum ferritin level at start of current chelator. SF: serum ferritin level; DFO: deferoxamine; DFP: deferiprone; DFX: deferasirox; IQR: interquartile range.*

Table 2. Correlation between iron overload and chelation parameters with the absolute number of hematopoietic peripheral progenitors.

Parameter	Duration on current chelator in years	SF at study in ng/mL	SF at start of current chelator in ng/mL	Change in SF while on current chelator in ng/mL
CFU-GM Absolute number /mL	r_s=0.118 *P*=0.535	r_s=-0.048 *P*=0.802	r_s=-0.155 *P*=0.415	r_s=0.010 *P*=0.958
BFU-E Absolute number /mL	r_s=-1.30 *P*=0.493	r_s=0.108 *P*=0.572	r_s=0.137 *P*=0.471	r_s=-0.032 *P*=0.866
CFU-GEMM Absolute number /mL	r_s=0.284 *P*=0.129	r_s=0.175 *P*=0.354	r_s=0.161 *P*=0.396	r_s=0.114 *P*=0.548
LTC-IC Absolute number /mL*	r_s=0.147 *P*=0.548	r_s=-0.369 *P*=0.120	r_s=-0.138 *P*=0.572	r_s=-0.158 *P*=0.519

*SF: serum ferritin; CFU-GM: colony forming unit-granulocyte/macrophage; BFU-E: erythroid burst-forming unit; CFU-GEMM: colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; LTC-IC: long-term culture-initiating cells; MNC: mononuclear cells; r_s: Spearman's correlation coefficient. *Data were missing for 11 patients.*

uals. Importantly, the type of chelator used also affected the absolute number of HPP in the circulation.

Our findings echo those recently reported by Yannaki *et al.* who found that the baseline content of $CD34^+$ cells in peripheral blood of TM patients was already slightly higher than normal values.[21] This suggests that in TM, characterized by an expansion of bone marrow cellularity, there is also an exodus of HPP into the circulation. The levels of several cytokines can be altered in TM patients. Serum erythropoietin levels in TM patients are higher than in healthy individuals.[22-24] Similarly, there are reports of elevated levels of interleukin-3,[25] vascular endothelial growth factor,[26] macrophage-colony stimulating factor,[27] and interleukin-6.[28] All such cytokines have a broad range of hematopoietic progenitors as target cells. However, an association between cytokine levels and HPP levels in the circulation has not been established so far. An abundance of $CD34^+$ and early progenitor cells in TM patients, as noted in our study, may turn out to be important since they could be a source of hematopoietic cells to be collected for a possible autologous transplant with gene therapy-modified progenitors without mobilization with granulocyte colony-stimulating factor or plerixafor.[21,29-30]

More importantly, we also determined differential effects of three available iron chelators on the absolute number of HPP. We found a difference that depended on the type of chelator rather than the extent of iron depletion. It is also relevant that the greatest difference in HPP in the peripheral blood concerned BFU-E which were particularly abundant in patients treated with DFX compared to the amounts in patients receiving other chelators. These findings suggest the potential of DFX therapy to induce hematologic responses and decrease transfusion demands. It has recently been shown that DFX, but not other iron chelators, inhibits nuclear factor-κB in patients with myelodysplastic syndromes and in leukemic cell lines, possibly explaining the improved erythropoiesis observed in such patients while on DFX.[31] Whether the same mechanism applies in TM patients warrants further study. In this respect, it may be relevant to emphasize that the determination of BFU-E content is based on the number of "bursts" that one counts in the culture dish after 15 days of culture. There are, therefore, two possible explanations for a higher BFU-E score: (i) there is indeed a higher number of BFU-E moving from the bone marrow to peripheral blood with DFX therapy; (ii) the DFX may render erythropoiesis more efficient and, as a consequence, more BFU-E may produce a discrete "burst" which, in turn, leads to a higher score at the end of the culture period.

We also noted a reduction of BFU-E in splenectomized TM patients compared to non-splenectomized ones, consistent with a previous observation.[32] It could be hypothesized that the spleen may be acting as a reservoir of progenitor cells or as a site where progenitor cell filtration occurs, rather than as a source of extramedullary hematopoiesis.[33] However, a more recent study found no association

between splenectomy status and the level of $CD4^+$ cells in the peripheral blood of adult thalassemia patients.[21]

The main limitation of our work is that it was an observational study of the effects of a therapeutic intervention, which means that confounding elements such as indications and patients' characteristics cannot be fully ruled out despite statistical adjustment. A residual confounding may persist. Moreover, we were unable to evaluate HPP in a group of thalassemic patients off iron chelation therapy. Such an investigation would help to delineate the effects of the disease from those of the iron chelation intervention on HPP levels in the circulation.

In conclusion, our results suggest that the type of chelation therapy in TM patients influences the level of circulating HPP, especially BFU-E. Our observations should be confirmed through larger, randomized trials and should stimulate further research into the role of DFX therapy in improving erythropoiesis and associated clinical endpoints in patients with TM.

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Authorship and Disclosures

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