Quantitatively different red cell/nucleated cell chimerism in patients with long-term, persistent hematopoietic mixed chimerism after bone marrow transplantation for thalassemia major or sickle cell disease

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ABSTRACT

Background
Persistent mixed chimerism represents a state in which recipient and donor cells stably co-exist after hematopoietic stem cell transplantation. However, since in most of the studies reported in literature the engraftment state was observed in the nucleated cells, in this study we determined the donor origin of the mature erythrocytes of patients with persistent mixed chimerism after transplantation for hemoglobinopathies. Results were compared with the engraftment state observed in singly picked out burst-forming unit – erythroid colonies and in the nucleated cells collected from the peripheral blood and from the bone marrow.

Design and Methods
The donor origin of the erythrocytes was determined analyzing differences on the surface antigens of the erythrocyte suspension after incubation with anti-ABO and/or anti-C, -c, -D, -E and -e monoclonal antibodies by a flow cytometer. Analysis of short tandem repeats was used to determine the donor origin of nucleated cells and burst-forming unit – erythroid colonies singly picked out after 14 days of incubation.

Results
The proportions of donor-derived nucleated cells in four transplanted patients affected by hemoglobinopathies were 71%, 46%, 15% and 25% at day 1364, 1385, 1314 and 932, respectively. Similar results were obtained for the erythroid precursors, analyzing the donor/recipient origin of the burst-forming unit – erythroid colonies. In contrast, on the same days of observation, the proportions of donor-derived erythrocytes in the four patients with persistent mixed chimerism were 100%, 100%, 73% and 90%.

Conclusions
Our results showed that most of the erythrocytes present in four long-term transplanted patients affected by hemoglobinopathies and characterized by the presence of few donor engrafted nucleated cells were of donor origin. The indication that small proportions of donor engrafted cells might be sufficient for clinical control of the disease in patients affected by hemoglobinopathies is relevant, although the biological mechanisms underlying these observations need further investigation.

Key words: hemoglobinopathies, persistent mixed chimerism, hematopoietic stem cell transplantation.

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Introduction

Notwithstanding the progress in supportive care,[1-3] hematopoietic stem cell transplantation (HSCT) is currently the only available curative treatment for patients affected by congenital hemoglobinopathies.[4-7] Mixed chimerism, the simultaneous presence of both host- and donor-derived cells in the recipient, is often observed in a large proportion of patients after HSCT for hemoglobinopathies.[8-11]

A state of mixed chimerism detected early after transplantation often moves towards complete chimerism, though it may evolve into graft rejection, especially if the proportion of donor cells is lower than 25%. On the other hand, some patients have stable mixed chimerism, which is defined as persistent when donor- and host-derived cells co-exist for periods longer than 2 years after HSCT.[12] Patients with persistent mixed chimerism do not require additional red blood cell (RBC) support and, regardless of the presence in some cases of an extremely low percentage of donor-derived nucleated cells, they are clinically controlled by an incomplete, but functional graft.[13-16] These findings have tremendous implications not only in the context of allogeneic HSCT, but also in the design of gene therapy trials based on the transplantation of genetically modified autologous $CD34^+$ cells.[17] The clinical control of the disease in the presence of persistent mixed chimerism indicates that the proportion of corrected nucleated stem/progenitor cells necessary to achieve a therapeutic level of circulating RBC is within the frequency of gene transfer by β-globin lentiviral vectors.[18]

Most of the studies in the literature have investigated the impact of donor engraftment on nucleated cells rather than mature erythrocytes, which are functionally crucial for patients affected by hemoglobinopathies. The evidence that the RBC in patients with persistent mixed chimerism might originate from the donor is indirectly supported by observations relative to hemoglobin β-globin chain synthesis[12,13,19] or, more recently, by the analysis of single nucleotide polymorphisms expressed by genes encoding RBC antigens and structural proteins.[20] We very recently demonstrated the presence of a large majority of donor-derived RBC in a transplanted thalassemic patient with persistent mixed chimerism of nucleated cells by analyzing the differences between the donor and recipient surface erythrocyte markers using cytofluorimetry.[21]

The aim of the present study was to determine the proportion of RBC and erythroid precursors of donor-recipient origin in long-term transplanted patients with persistent mixed chimerism, characterized by the presence of a low proportion of donor-derived nucleated cells in the peripheral blood and bone marrow.

Design and methods

Patients

The present study included 44 consecutive patients who underwent bone marrow transplantation between June 2004 and February 2008 from an HLA identical 12/12 allele-level matched sibling, and who were checked at least once 2 years after the marrow infusion to determine the state of engraftment (Table 1). Patients treated during the same period without a minimum follow-up of 2 years or who rejected their graft were not included in the study. Forty patients were affected by β-thalassemia major (hereafter referred to simply as β-thalassemia) and four by sickle cell disease. All of the patients received a myeloablative conditioning regimen followed by the infusion of unmanipulated bone marrow cells (median dose, 4.5×10^8/kg; range, $1.3\text{-}8.7\times10^8$/kg). Patients with β-thalassemia in class 1 or 2 (according to the Pesaro classification) were given a conditioning regimen based on 14 mg/kg busulfan and 200 mg/kg cyclophosphamide. In addition, patients aged less than 4 years were treated with 10 mg/kg thiotepa. β-thalassemia patients in class 3 were conditioned with 14 mg/kg busulfan and a reduced dose of cyclophosphamide (160 mg/kg). Patients with sickle cell disease were conditioned with 14 mg/kg busulfan, 200 mg/kg cyclophosphamide, and 10 mg/kg anti-thymocyte globulin from day -6 to day -3 relative to infusion of the graft. Busulfan was administered orally to 23 patients and intravenously to 21 patients. Post-HSCT graft-*versus*-host disease prophylaxis consisted of cyclosporine A, methylprednisolone, and a short course of methotrexate. Intravenous cyclosporine A was started at a dose of 5 mg/kg from day -2 to day +5, and later reduced to 3 mg/kg until day 60 post-transplant when it was tapered off by 5% per week and discontinued at day 365. The desired plasma range was 150-250 ng/mL. All patients discontinued immunosuppressive therapy with cyclosporine A at 1 year after transplantation if no signs of chronic graft-*versus*-host disease were present. Intravenous methylprednisolone was started at 0.5 mg/kg on day –1 and stopped 30 days post-transplant. A short course of methotrexate was given intravenously at a dose of 10 mg/m^2 on days 1, 3 and 6 post-transplant with folic acid rescue. Table 1 shows the characteristics of the population studied.

Chimerism analysis of nucleated cells and burst-forming unit – erythroid colonies

Peripheral blood and bone marrow samples were collected in EDTA at least once from donors and patients between days 20 and 60, on day 180, and thereafter during the annual routine follow-up examinations. Recipient and donor DNA samples were extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) or an automatic DNA extractor (Promega, Madison, WI, USA), and the DNA typed by short tandem repeats (STR) and the amelogenin locus using the AmpFISTR Profiler Plus kit (Applera, Foster City, CA, USA). Amplification reactions were carried out using 1-2 ng of input DNA following the manufacturer's recommendations. Polymerase chain reaction products were run on an ABI Prism 3130xl Genetic Analyzer (Applera, Foster City, CA, USA). Informative loci in post-transplant samples were screened to quantify the percentage of donor cells in mixed chimeras. HSCT engraftment was quantified using fluorescent polymerase chain reaction primers for human identity markers based on the ratio between the peak areas of donor and recipient alleles. The mean value obtained after performing calculations for each informative STR was taken as the percentage of mixed chimerism. Burst-forming unit – erythroid (BFU-E) colonies were grown in agar and picked out singly for STR evaluation.

Table 1. Clinical characteristics of the 44 patients in the present study.

Disease	Number of patients
Beta thalassemia major	40
Sickle cell disease	4
Gender	
Male	28
Female	16
Age (median)	8.5 years (range, 2 - 24 years)
HLA compatibility	related - 12/12 alleles matched
Follow-up (median)	1010 days (range, 720 to 1673 days)

Clonogenic assay

Assays for clonogenic hematopoietic progenitors were performed in methylcellulose semisolid cultures. Briefly, 1-2×10^5 low-density bone marrow/peripheral blood cells were plated in duplicate in 35 mm tissue culture dishes, suspended in 1 mL methylcellulose medium supplemented with stem cell factor, granulocyte/macrophage colony-stimulating factor, interleukin-3, and erythropoietin (Methocult GFH4434, Stem Cell Technologies, Vancouver, British Columbia, Canada). Cultures were incubated at 37°C in a fully humidified atmosphere containing 5% CO_2. Plates were scored for BFU-E growth after 14 days of incubation. Using an inverted microscope, individual colonies were picked up from the Petri dishes and dispersed to single cell suspensions in 100 μL saline to assess the donor/recipient origin of the individual colonies using STR.

Chimerism analysis of red blood cells

For cytofluorimetric analysis, RBC were washed and diluted in saline (0.5% final dilution). Five microliters of cell suspension were incubated with anti-ABO and anti-C, c, D, E and e monoclonal antibodies, following the manufacturer's instructions (ABH- and RH- Erythrokit, Institute Jacque Boy SA, Reims, France). After the incubation, cells were washed with phosphate-buffered saline and incubated with fluorescein isothiocyanate-conjugated anti-human immunoglobulin. After incubation and two additional washes, the analysis was performed using a FC500 flow cytometer and transferred to the CXP analysis program (Beckman-Coulter Hialeh, FL, USA).

Ethical approval

The study was approved by the Local Ethical Committee (July 2008, N.109/08) and samples were taken after obtaining written informed consent. Informed consent was obtained from all the patients before the standardized clinical treatment.

Results

Analysis of engraftment in nucleated cells

The presence of recipient- and donor-derived nucleated cells in the peripheral blood and/or bone marrow of the recipient was observed in seven of the 44 (15.9%) transplanted patients affected by hemoglobinopathies on the day of their last clinical contact (follow-up: range, 720-1673 days post-transplant; median, 1010 days post-transplant). Analysis of engraftment showed that three patients had more than 96% donor-derived nucleated cells in the peripheral blood (*data not shown*), whereas in patients UPN 31, UPN 35, UPN 41, and UPN 57, the proportions of donor-derived nucleated cells were 71%, 46%, 15%, and 25%, respectively (Table 2). Figure 1 summarizes the chimerism kinetics determined by analyzing the nucleated cells in the peripheral blood and bone marrow of these patients. None of these four patients suffered acute or chronic graft-*versus*-host disease. On the day of their last clinical control all the patients were in good condition and transfusion-independent, with blood count values within the normal ranges (Table 3).

Analysis of engraftment in red blood cells

We determined the proportion of donor-derived RBC by analyzing differences in erythrocyte surface markers between the donor and recipient RBC, using cytofluorimetric analysis in the four patients with low donor engrafted nucleated cells described above. We found a very high proportion of donor-derived RBC, indicating the presence of quantitatively different red cell/nucleated cell chimerism. In the four patients, the detection of erythrocytes to determine donor-recipient origin was performed more than 1 year after the last RBC transfusion therapy. Studying the erythrocyte group markers "O" *versus* "B" in patient UPN 31 (Figure 1A), it was found that RBC were consistently 100% of donor origin on days 635, 836, 1007, and 1364 after transplantation. Similar results were detected in patient UPN 35 on days 575, 940, and 1371 post-transplant (Figure 1B) for differences in Rh system surface markers "e" *versus* "E". The presence of quantitatively different red cell/nucleated cell chimerism was also observed in patients UPN 41 and UPN 57 (Figure 1C,D), although the proportion of donor-derived erythrocytes in these cases was high, but not complete. In patient UPN 41 (analyzing the ABO difference "O" *versus* "B"), the proportion of donor-derived RBC was 80% on day 935 and 73% on day 1314, whereas in UPN57, the proportion was 90% on day 935 (ABO difference "A" *versus* "O"). The proportion of donor-derived RBC was also examined in the three patients with persistent mixed chimerism characterized by a large proportion of donor-derived nucleated cells and the four patients with full donor engraftment in the nucleated cells. As expected in all of these patients, the proportion of donor-derived RBC was 100% (*data not shown*).

Analysis of engraftment in burst-forming unit – erythroid colonies

The ability of individual early progenitors to give rise to colonies of erythroid cells was assayed by plating the bone marrow or peripheral blood cells isolated from the patients with persistent mixed chimerism in semisolid medium. Single BFU-E colonies were picked out from the bone marrow/peripheral blood cultures of patients UPN 31, 35, and 41 to determine the origin of the erythroid precursors and compare it with the characteristics of the RBC. In patient UPN 31, the proportions of donor-derived BFU-

Table 2. Engraftment state in RBC, nucleated cells and BFU-E at the last clinical control in patients with persistent mixed chimerism.

UPN	Disease	Day of last clinical contact	% of donor nucleated cells in PB	% of donor nucleated cells in BM	% of donor RBCs in PB	% of donor BFU-E in PB	% of donor BFU-E in BM
31	β-thalassemia	1364	71	80	100	96	96
35	β-thalassemia	1371	46	51	100	55	46
41	β-thalassemia	1314	15	20	73	11	35
57	Sickle cell disease	932	25	ND	90	ND	ND

UPN: unique patient number; PB: peripheral blood; BM: bone marrow; RBC: red blood cells; BFU-E: burst-forming unit-erythroid; ND: not determined; β-thalassemia: β-thalassemia major.

E colonies cultured from the peripheral blood and bone marrow were 93% on day 1007 post-transplant, and 96% on days 1182 and 1364. In patient UPN 35, the proportions of donor-derived BFU-E colonies were 63% and 55% in the peripheral blood and 41% and 46% in the bone marrow on post-transplant days 940 and 1371, respectively. A similar composition was obtained for patient UPN 41 on post-transplant day 1314 as the proportions of donor-derived BFU-E colonies obtained from the peripheral blood and bone marrow were 11% and 35%, respectively. Due to technical problems, we could not analyze the proportion of BFU-E on the day of last contact in patient UPN 57. Table 2 summarizes the data relative to the proportion of donor-derived BFU-E on the day of last clinical contact, compared to the percentage of donor-derived RBC and nucleated cells.

Discussion

The presence of mixed chimerism early after transplantation is associated with an increased risk of graft rejection in patients treated with HSCT for hemoglobinopathies, mostly when the proportion of donor-derived cells in the peripheral blood or bone marrow early after the transplant is less than 75%.[10-13] On the other hand, patients who move into a state of stable mixed chimerism, which becomes persistent when the recipient overcomes a minimum post-transplant follow-up period of 2 years, are no longer exposed to the risk of graft failure. The potential role of T regulatory cells in establishing persistent mixed chimerism has been recently pointed out.[21-24] However, independently of the biological mechanisms involved, data from several studies have shown that patients with persistent mixed chimerism have clinical control of the disease, despite the presence of an extremely low proportion of donor nucleated cells.[10-13,25] The majority of studies concerning the assessment of persistent mixed chimerism have, however, almost exclusively focused attention on the percentage of donor engraftment in the nucleated cells rather than in the mature erythrocytes, cells functionally crucial for patients affected by hemoglobinopathies.

Our results showed that in the long-term, in transplanted patients with persistent mixed chimerism, despite the presence of a few donor engrafted nucleated cells in the peripheral blood and bone marrow, the erythrocytes were almost completely of donor origin. In three patients, in particular, the proportions of donor-derived nucleated cells in the peripheral blood and bone marrow at their last follow-up were, respectively, 46% and 51%, 15% and 20%, and 25% and not determined, whereas the percentages of donor-derived RBC were 100%, 73%, and 90%, respectively (Table 2). We further investigated the proportion of erythroid precursors in the bone marrow to assess whether the distribution was similar to that of the nucleated cells or whether it overlapped with the RBC picture. Interestingly, the donor proportion of the BFU-E colonies examined was equivalent to that of the nucleated cells,

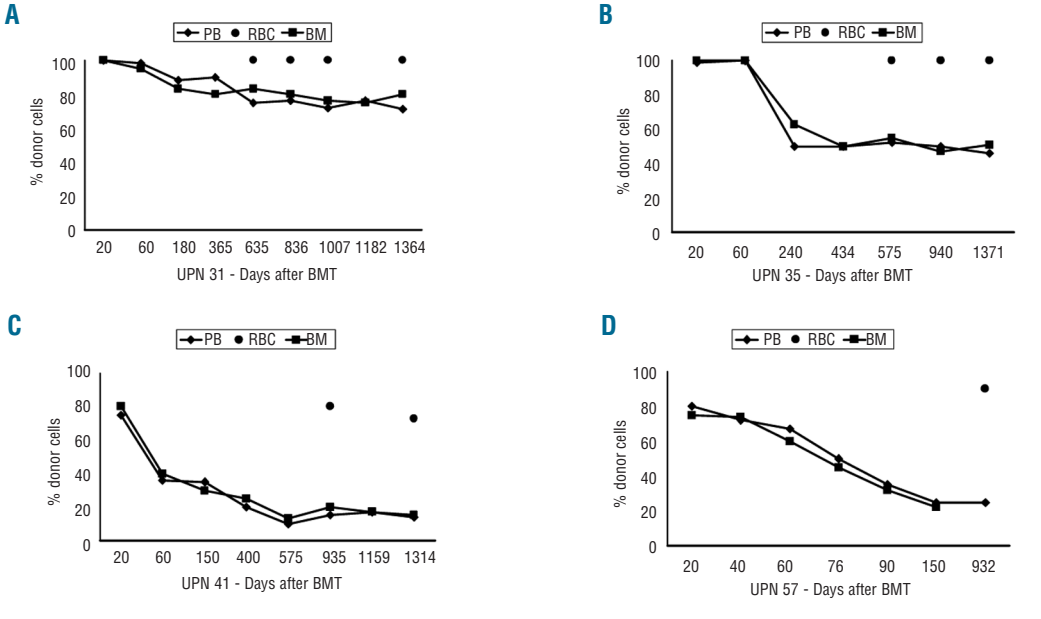

Figure 1. Engraftment evolution over time of patients with persistent mixed chimerism. UPN: unique patient number; PB: peripheral blood; BM: bone marrow; RBC: red blood cells; BMT: bone marrow transplantation.

Table 3. Hemocytometric blood tests at last clinical control in patients with persistent mixed chimerism.

UPN	Day of last clinical control	RBC ($x10^{12}$/L)	WBC ($x10^{9}$/L)	Hb g/dL	% of reticulocytes	% of β-globin chain synthesis in PB	α : non-α ratio
31	1364	5.50	6.5	10.9	1.5	100	1.48
35	1371	5.40	8.8	10.8	1.4	100	1.45
41	1314	4.40	12.0	10.1	3.7	100	1.9
57	932	3.70	6.0	9.	1.9	100	1.1

UPN: unique patient number; RBC: red blood cells; WBC: white blood cells; Hb: hemoglobin.

showing a quantitatively different erythroid precursor/erythrocyte chimerism. β-thalassemia is characterized by ineffective erythropoiesis leading to severe anemia and extensive erythroid expansion, caused in part by accelerated apoptosis of the thalassemic erythroid precursors, roughly 15-fold above that of healthy controls.[28-30] The evidence for ineffective erythropoiesis in patients affected by severe sickle cell disease, developing mixed chimerism early after allogeneic non-myeloablative stem cell transplantation was described by Wu *et al.*[26] A possible explanation for the presence of a greater proportion of donor-derived erythrocytes with respect to the BFU-E observed in the persistent mixed chimeras may be the improved survival of donor RBC precursors compared to their host counterparts, which might be destroyed during ineffective erythropoiesis. However, because the results of chimerism on BFU-E are comparable to those observed in bone marrow and nucleated cells, the disappearance of the recipient erythroid cells must take place at the level of more mature bone marrow erythroid precursors.

Recently, Serafini *et al.* determined the presence of quantitatively different red cell/nucleated cell chimerism between nucleated cells and RBC by analyzing erythrocyte surface markers in a long-term transplanted patient using cytometry, and showed that the majority of the patient's erythrocytes were of donor origin, whereas the nucleated cells were mostly derived from the recipient.[21] Similar results were obtained by Felfly and Trudel in thalassemic mutant mice which were transplanted in order to determine the minimal percentage of normal bone marrow cells necessary to correct the thalassemic phenotype in a competitive re-population transplantation assay.[27] They found a 2- to 2.5-fold amplification of normal RBC compared to white blood cells in the peripheral blood of mice with 19-24% bone marrow chimerism, indicating an *in vivo* selective advantage for the normal RBC. Recently, Miccio *et al.* demonstrated, in a gene therapy preclinical model, that there is a selective advantage for the genetically-corrected erythroid component, leading to correction of thalassemia in mice engrafted with as low as 30% of transduced hematopoietic stem cells.[28] Armistead *et al.* constructed a panel of ten different genes uniquely expressed in RBC and characterized by polymorphisms with high minor-allele frequencies to investigate RBC engraftment in hemoglobinopathies.[20] The panel was increased in donor-derived reticulocyte RNA compared to the recipient's baseline endogenous erythropoietic capacity, which manifested as recipient-derived nucleated RBC progenitors and reticulocyte RNA.

Although these data were obtained in a limited number of patients and further studies in larger cohorts are needed to corroborate our preliminary findings, these results support the evidence that low levels of donor engraftment can result in significant functional improvement for patients with hemoglobinopathies. Moreover, in the future, evaluation of RBC chimerism might provide relevant clinical information in the routine monitoring of engraftment. The observation that a few engrafted cells are sufficient to clinically control patients with a hemoglobinopathy is particularly interesting in the light of a possible gene therapy approach, as the gene defect might be corrected by introducing the normal gene into the patient's stem cells. In fact, previous experience in clinical trials, for both hemoglobinopathies and other genetic diseases, has shown that not all cells carrying the genetic defect can be repaired. When genetically modified stem cell transplantation becomes a possible option for treating β-thalassemia, the co-existence of repaired cells with those still expressing the genetic defect will be an expected scenario, not in an allogeneic environment, but in an autologous one.[27,31-36] Also in this light, a better understanding of the mechanisms underlying the establishment of quantitatively different red cell/nucleated cell chimerism will be particular relevant.

Authorship and Disclosures

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