Relationship between mixed chimerism and rejection after bone marrow transplantation in thalassaemia

Marco Andreani[1], Manuela Testi[1], Mariarosa Battarra[1], Paola Indigeno[1], Annalisa Guagnano[1], Paola Polchi[1], Giorgio Federici[2], Guido Lucarelli[1]

[1]Laboratorio di Immunogenetica e Biologia dei Trapianti, Fondazione IME –Istituto Mediterraneo di Ematologia, Roma; [2]Dipartimento di Medicina di Laboratorio, Policlinico di Tor Vergata, Roma, Italy

Background. Thalassaemia is a genetic disease that requires a hypertransfusion regimen to treat the anaemia caused by enhanced red blood cell destruction. The only radical cure for thalassaemia is to correct the genetic defect by bone marrow transplantation from an HLA-identical donor capable of producing and maintaining a normal haemoglobin level in the recipient. Complete donor haematopoiesis is not essential for sustained engraftment and the simultaneous presence of haematopoietic cells of both donor and recipient origin is not a rare event after a transplant.

Patients and methods. The evolution of marrow engraftment of 93 transplanted thalassaemic patients, all from Middle East or Asian countries, was monitored by analysis of short tandem repeats.

Results. Forty-three of 93 (46%) patients experienced a status of mixed chimerism early after bone marrow transplantation. Results of further engraftment analysis in these patients showed in 27 complete donor engraftment; rejection occurred in seven, while eight maintained the presence of both host and donor-derived cells. Interestingly, five out of the seven patients who rejected their transplant showed more than 25% residual host cells early after transplantation.

Discussion and conclusion. Our study confirmed that the presence of large amounts of residual host cells within the first 2 months after a transplant is a risk factor for graft rejection also in a group of patients with wide ethnic heterogeneity, irregular transfusion regimens and/or poor chelation treatment. Ten percent of the transplanted thalassaemic patients maintained coexistence of donor and recipient cells, showing a stable functional graft, characterized by normal production of beta globin chains and high levels of haemoglobin. A mechanism responsible for peripheral tolerance induction, such as the production of specific regulatory T-cell clones, seems to play a key role in the induction of long-term tolerance after the transplant.

Key words: bone marrow transplantation, mixed chimerism, rejection, thalassaemia.

Introduction

Thalassaemia is a genetic disease that requires a hypertransfusion regimen to treat the anaemia caused by enhanced red blood cell destruction. Such a regimen does, however, lead to progressive iron overload and consequent organ deterioration. The only radical cure of thalassaemia is to correct the genetic defect by a bone marrow transplant from an HLA-identical donor who is normal or heterozygous for thalassaemia; the transplant must be capable of producing and maintaining a normal haemoglobin level in the recipient[1-3]. Engraftment of donor-derived cells is a crucial event in order to obtain a successful transplant. It has, however, been demonstrated that complete donor haematopoiesis is not essential for sustained engraftment and that the simultaneous presence of haematopoietic cells of both donor and recipient origin is not a rare event after a bone marrow transplant (BMT) in thalassaemic patients[4-6]. Donor and recipient cells may

coexist and produce a functional graft with a status identified as persistent mixed chimerism. As already reported, approximately 10% of transplanted thalassaemic patients with a follow-up longer than 2 years shows persistent mixed chimerism. These patients, in some cases with a low amount of donor engrafted cells, no longer require red blood cell transfusions and are cured from their disease. On the other hand, mixed chimerism (MC) observed in the early phase after a transplant is a risk factor for graft rejection, particularly when there are more than 25% residual host cells[7-9]. In order to confirm these observations, in this study we monitored the evolution of bone marrow engraftment in a series of 97 consecutive transplanted thalassaemic patients who came from foreign countries, mostly from the Middle East, and who were treated in Rome in the Mediterranean Institute of Haematology (IME) Foundation.

Patients and methods

Patients

Between July 2004 and June 2007, 97 consecutive patients transplanted from an HLA-identical sibling in the BMT unit of the IME Foundation located in the Polyclinic of Tor Vergata in Rome were studied to evaluate engraftment kinetics. Forty patients were treated with busulphan 14 mg/kg and cyclophosphamide 200 mg/kg (protocol 6)[3] and 57 patients were treated with busulphan 14 mg/kg and cyclophosphamide 160 mg/kg after fludarabine 20 mg/m^2, hydroxyurea 30 mg/kg, and azathioprine 3 mg/Kg (protocol 26)[10]. Patients received cyclosporine-A as an immunosuppressant. There was a sex mismatch between 52 of the patients and their donors (24 female/male; 28 male/female). The median age of the patients was 9.2 years (range 2 –24). The patients' characteristics are reported in Table I.

Chimerism analysis

Recipient and donor DNA samples, extracted using a QIAamp DNA Blood mini kit (Qiagen, Valencia, CA, USA) were typed by short tandem repeats (STR) and 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 manufacturers' recommendations. Polymerase chain reaction (PCR) products were run on an ABI Prism 3130xl Genetic Analyzer (Applera, Foster City, CA, USA)[11]. Informative loci for post-transplant samples were screened for quantification of the donor cell percentage in MC. Quantitative determination of BMT engraftment was performed using fluorescent PCR primers for human identity markers based on the ratio between peak areas of donor and recipient alleles. The mean value obtained after performing calculations for each informative STR was taken as the percentage of MC.

Table I - Characteristics of the consecutive patients analysed

	Characteristics of the patients
No. of consecutive patients	97
Age (years)	9.2 (2-24)
Follow-up (months)	6-40
Sex Match	
M/M	24
M/F	28
F/F	21
F/M	24
Class of risk*	
1	17
2	33
3	47

*According to the Pesaro classification

Cell samples

Peripheral blood and bone marrow samples were collected in EDTA from patients and donors before transplantation and from all patients at least once between day 20 and day 60, and on days 180 and 365 after transplantation. Patients who rejected their transplant were not further investigated. Patients who achieved persistent engraftment (either complete or with MC) were studied thereafter during their routine annual follow-up examinations.

Specific analysis of peripheral blood cell subpopulations

Peripheral blood cell subpopulations were analysed in six patients who had MC and a follow-up of at least 1 year. CD3, CD19 and CD56 positive cells were obtained by rosette centrisep (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocols. The purity of the lymphoid cell subpopulations obtained was assayed by cytofluorometry analysis and estimated to be higher than 95%. DNA obtained from the different subpopulations was subjected to chimerism analysis.

Statistical analysis

The comparison between the different groups of patients was performed by means of the two-sided Fisher's exact test with the aid of MedCalc statistical software.

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Results

Engraftment analysis

We analysed the kinetics of donor cell engraftment in 93 out of 97 consecutive thalassaemic patients who received a BMT from an HLA-identical sibling. Two patients had no sign of marrow engraftment, with the reappearance of the disease after autologous reconstitution, while two patients died early after their transplant from causes related to the transplant. These four patients were excluded from the study. Among the remaining 93 patients, early after transplant, 50 had full donor engraftment while 43 showed the presence of MC. As already described[7], MC was classified, according to the proportion of residual host cells present in the recipient, into MC level 1 (residual host cells <10%), MC level 2 (residual host cells between 10% and 25%) and MC level 3 (residual host cells >25%). Based on this classification, we studied the incidence of graft rejection in patients with MC and in the patients showing full donor engraftment within the first 60 days after BMT. Eight of the 93 patients, after initial engraftment, rejected their grafts (8.6%). As reported in Table IIA, rejection occurred in one out of 50 (2.0%) patients with complete chimerism, while seven of the 43 (16.2%) patients with MC observed within the first 2 months after BMT lost the engraftment (p=0.0172). Within the patients with MC, however, rejection occurred in five out of ten patients with MC level 3 (50%) but in only one of 30 (3.3%) patients with MC level 1 (p=0.00205) (Table IIB). The number of patients with MC level 2 was too low to be included in any statistical analysis. As shown in Table III, four patients evolved to graft reaction within the first 2 months after the transplant, while in four patients this process occurred later, with a gradual increase in the proportion of host haematopoiesis, culminating in complete rejection between 6 and 11 months after transplantation.

Evolution of engraftment

Analysing the kinetics of engraftment in patients who did not reject their transplants, we found that initial donor complete chimerism was maintained in 46 patients, while three patients evolved towards a status of MC (one to MC level 1 and two to MC level 3). On the other hand, MC evolved into full donor engraftment in 23 of the 30 patients who, early after the transplant, were classified as having MC level 1 (74.2%) while six remained mixed chimeras (20%). A similar evolution from a status of MC towards full donor engraftment was observed in one patient with initial MC level 2, and three patients with initial MC level 3, while the

Table IIA - Evolution of chimerism based on early post-transplant engraftment status

Engraftment status within 60 days	Number of patients	Evolution to		
		Rejection*	MC	CC
CC	50	1	3	46
MC	43	7	9	27

MC: presence of residual host cells; CC: complete chimerism; *Fisher's exact test between the two groups: p = 0.0172

Table IIB - Evolution of chimerism based on the levels of MC early post-transplant

Engraftment status within 60 days	Number of patients	Evolution to		
		Rejection*	MC	CC
MC 1	30	1	6	23
MC 2	3	1	1	1
MC 3	10	5	2	3

MC: presence of residual host cells (RHC); CC: complete chimerism; MC1: <10% RHC; MC2: 10%-25% RHC; MC3: >25% RHC. *Fisher's exact test between groups MC1 and MC3: p = 0.0020

Table III - Engraftment status within 60 days after BMT in eight patients who rejected their grafts

UPN	% of donor cells within the first 60 days	MC level	Rejection (days after BMT)
1	80	2	340
15	35	3	180
26	60	3	89
40	90	1	161
53	65	3	64
73	18	3	25
90	100	CC	285
94	70	3	30

UPN: unique patient number; MC: mixed chimerism; CC: complete chimerism

Table IV - Evolution of engraftment in six patients with persistent mixed chimerism

UPN	% of donor cells within the first 60 days	MC level	Last contact (months)	% of donor cells on day of last observation	MC level	Alpha/non-alpha chains	% of beta globin chain synthesis
8	100	CC	36	95	1	1.37	100
25	100	CC	13	40	3	1.09	100
31	95	1	21	75	3	1.0	100
35	100	CC	19	52	3	1.02	100
41	36	3	19	10	3	NT	NT
71	96	1	12	98	1	1.04	100

UPN: unique patient number; MC: mixed chimerism; CC: complete chimerism; NT: not tested

remaining three patients retained their status of MC without further evolution (Table IIB).

Persistent mixed chimerism

The condition of MC (Table IV) was maintained for a period of at least 12 months in six patients. Although the risk of graft rejection in these patients is not totally excluded, since MC is defined as persistent when present 2 years after the transplant, it is very likely that the engraftment status of this group of patients will not change over the future follow-up. Moreover, the ratio of alpha/non-alpha chains in these patients was between 1 and 1.37, while the percentage of beta globin chain synthesis was never lower than 100%, indicating that these patients had functional grafts. To define the condition of MC better, we analysed the proportion of donor engraftment in the different lymphoid subsets at different times after BMT (Table V), since the presence of less than 50% CD3+ cells was reported to be a risk factor for graft rejection in the early phase after BMT. Patients UPN 8, 25 and 35, who at 60 days after BMT were complete chimeras, when studied at day +1095, +365 and +575, respectively, showed a status of MC in all the different lymphoid cell subsets analysed. Interestingly, at the last analysis, both patients 25 and 35 showed a slightly higher proportion of donor CD3+ cells with respect to the percentage of donor cells present in the peripheral blood

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Table V - Analysis of lymphoid subsets in six patients with persistent mixed chimerism

UPN	Days of observation	Percentage of donor cells (%)				
		PB	BM	CD3	CD19	CD56
8	60	100	100	NT	NT	NT
	1.095	95	NT	90	100	100
25	60	100	100	100	100	100
	365	45	40	67	25	30
31	60	98	95	100	100	100
	635	75	83	58	65	86
35	100	100	100	100	100	100
	575	52	55	60	30	40
41	60	44	36	58	45	70
	575	20	10	NT	NT	NT
71	60	96	97	90	97	100
	305	100	98	90	97	100

UPN: unique patient number; PB: peripheral blood; BM: bone marrow; NT: not tested.

and a much higher proportion in comparison to the B-cell compartment. Unfortunately, data on lymphoid cell subsets in patient 41 were not available. It will be interesting to establish whether the proportion of donor CD3+ cells in this patient is equivalent to or higher than the proportion of donor cells present in the peripheral blood, since the proportion of donor engrafted cells reported at day +575 was extremely low.

Discussion

The aim of this study was to determine the evolution of donor marrow engraftment in 93 thalassaemic patients by molecular genetic techniques that enable a sensitive assessment of host haematopoiesis after marrow transplantation. It is well known that complete chimerism of the marrow donor cells is required in order to obtain a successful transplant; however, the presence of MC, i.e. the coexistence of donor and host cells in the recipient, is not a rare event [3]. There is evidence that MC in the marrow is associated with an increased risk of subsequent graft failure, usually accompanied by a reappearance of the patient's defective marrow and a return of thalassaemia [7-8]. While the Pesaro experience was predominantly gained in a population of Italian patients, in the present study we investigated ethnically heterogeneous patients from countries with limited economic resources. Specifically for this reason most of them received an irregular transfusion regimen and poor chelation treatment, which could have increased the incidence of MC and, consequently, the risk of graft rejection, as a result of sensitisation to HLA antigens due to non-leucodepleted red blood cell transfusions. In this study we confirmed our previous results indicating that the probability of graft rejection is higher among thalassaemic patients with MC than among those with complete chimerism, as detected within the first months after the transplant, and is proportional to the amount of donor type haematopoiesis present in the recipient. In fact when we correlated graft failure with MC, establishing a grading of the amount of residual host cells present early after BMT, we showed that the incidence of rejection was significantly higher in the group of patients with MC level 3 than in the group of patients with MC level 1 (Table IIB). These data confirm that in thalassaemic patients with transient MC, the probability of rejection is related to the number of residual host cells present in the recipient in the early stage after BMT [7,8,12]. It is likely that in patients with MC level 3 there are so many recipient cells that a prompt immunological reaction is induced against the donor cells.

In contrast, when the level of residual host cells is low, as in the case of MC level 1 or 2, donor and recipient cells have time to trigger a mechanism that may induce a status of tolerance allowing their co-existence[13-15]. A similar explanation may be extended to the patients who showed complete engraftment in the first months after BMT and subsequently developed a status of persistent MC. It is evident that in this situation there were too few residual host cells in the early phase to be detected, but that such cells were, nevertheless, present. Our data have shown that the occurrence of transient MC does not necessarily lead to graft rejection. In fact, almost 10% of the patients studied achieved a state of stable MC with enough donor marrow cells to produce sufficient beta-globin chain synthesis and normal levels of haemoglobin in the peripheral blood, so that transfusions to correct the anaemia were no longer required. In some cases, the number of donor engrafted cells in the recipient was very low and yet a functional graft was maintained. In these patients MC was also observed in the different peripheral lymphoid subsets, equally distributed in the different cell lineages.

The reasons why MC is transient in some patients, and persistent in others, are still unknown. Unfortunately, we have no evidence about the precise time after BMT at which peripheral chimerism is established; it would be very interesting to understand when this does happen in order to characterize the cellular mechanisms involved in the development of long-term tolerance better[16,17]. The induction and maintenance of tolerance are strategic issues for improving the results of BMT. Ongoing studies in patients with persistent MC have shown that T regulatory cell clones are functional *in vitro*, inhibiting cytokine production of both donor and host responder cells, capable of suppressing specific alloreactivity and, therefore, responsible for the induction of tolerance (*Serafini, in press*). The intensive transfusion regimens that thalassaemic patients undergo before transplantation might further contribute to the induction of tolerance. $CD4^+CD25^+$ regulatory T and/or T regulatory type 1 cells have been identified as crucial components of tolerance in clinical gene transfer trials and may represent a new tool to achieve tolerance and avoid immune-mediated rejection. With regards to the feasibility of gene therapy in thalassaemia, it is possible that in the future the gene defect might be corrected by introducing the normal gene into the patient's stem cells, although it is very difficult to imagine that all the thalassaemic cells will be repaired.

The report that, in the case of persistent MC, few donor-derived cells were able to sustain a functional graft and were, therefore, sufficient to cure the disease might be a strategic observation for gene therapy.

When transplantation with genetically modified stem cells becomes a possible curative option for thalassaemia, an expected scenario will be the co-existence of the repaired cells with those still carrying the thalassaemic genetic defect, not in an allogeneic, but in an autologous environment. In this context a better understanding of the mechanisms underlying the occurrence of persistent MC will be particularly useful.

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Correspondence: Dr. Manuela Testi
Laboratorio di Immunogenetica e Biologia dei Trapianti, Fondazione IME
Policlinico di Tor Vergata
Viale Oxford 81
00133 Roma, Italy
e-mail: m.testi@fondazioneime.org