View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Archivio istituzionale della ricerca - Università di Palermo

Granulocyte–colony stimulating factor plus plerixafor in patients with β-thalassemia major results in the effective mobilization of primitive CD34+ cells with specific gene expression profile

Elena Baiamonte,[1] Rita Barone,[1] Flavia Contino,[2] Rosalia Di Stefano,[1] Anna Marfia,[3] Aldo Filosa,[4] Emanuela D'Angelo,[5] Salvatore Feo,[2] Santina Acuto,[1] Aurelio Maggio[1]

[1]Campus of Hematology F. and P. Cutino, Villa Sofia-Cervello Hospital, Palermo; [2]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies and ATeN Center, University of Palermo; [3]Division of Hematology, Villa Sofia-Cervello Hospital, Palermo; [4]Center for Anemias, A. Cardarelli Hospital, Napoli; [5]Pediatric Clinic De Marchi, Policlinico Hospital, Milan, Italy

Abstract

Successful gene therapy for β-thalassemia requires optimal numbers of autologous gene-transduced hematopoietic stem and progenitor cells (HSPCs) with high repopulating capacity. Previous studies suggested superior mobilization in these patients by the combination of granulocyte–colony stimulating factor (G-CSF) plus plerixafor over single agents. We mobilized four adult patients using G-CSF+plerixafor to assess the intra-individual variation of the circulating CD34+ cells number and subtypes pre- and post-plerixafor administration. The procedure was well-tolerated and the target cell dose of $\geq 8\times10^6$ CD34+ cells/kg was achieved in three of them with one apheresis procedure. The addition of plerixafor unanimously increased the number of circulating CD34+ cells, and the frequency of the most primitive CD34+ subtypes: CD34+/38- and CD34+/133+/38- as well as the *in vitro* clonogenic potency. Microarray analyses of CD34+ cells purified from the leukapheresis of one patient mobilized twice, with G-CSF and with G-CSF+plerixafor, highlighted in G-CSF+plerixafor-mobilized CD34+ cells, higher levels of expression genes involved in HSPC motility, homing, and cell cycles. In conclusion, G-CSF+plerixafor in β-thalassemia patients mobilizes optimal numbers of HSPCs with characteristics that suggest high capacity of engraftment after transplantation.

Introduction

Autologous hematopoietic genetically modified stem and progenitor cells (HSPCs) represent an emerging therapeutic option for curing β-thalassemia, particularly in patients who lack a suitable donor for allogeneic bone marrow transplantation (BMT). In recent years, clinical trials of gene therapy for β-thalassemia are ongoing in multiple centers and proof-of-principle of efficacy and safety has already been obtained in a number of patients.[1]

The success of gene therapy for β-thalassemia, in which engrafted gene-corrected stem cells do not have a selective advantage over unmodified cells, greatly depends on the number and the quality of autologous HSPCs collected for genetic modification. Blood mobilization using granulocyte-colony-stimulating factor (G-CSF) and collection of HSPCs by apheresis, are relatively easier and lesser invasive procedures than BM withdrawal and yield high numbers of CD34+ cells. For these reasons HSPCs mobilization represents a preferred source for allogeneic and autologous BMT in malignancies[2] and, more recently, in stem cell gene therapies.[1,3-6]

It has been reported that patients with inherited hematological disorders responded differently to G-CSF stimulus. Indeed, significantly impaired HSPC-mobilization has been reported in patients with primary immunodeficiency disorders.[7,8] In addition, in patients with sickle cell disease, the use of G-CSF has been shown to precipitate severe sickle cell crises and, in one case, death.[9,10] In patients with β-thalassemia, G-CSF-induced hyper-leukocytosis and spleen enlargement were found to be the major dose-limiting factors ultimately resulting in poor CD34+ cell yields.[11-14]

Plerixafor, is a recently available mobilizing agent that promote HSC trafficking by a mechanism different than G-CSF. Plerixafor is an CXCR4 antagonist that mobilizes HSCs within a few hours by disrupting the engagement of the CXCR4 stem cell receptor with its ligand (SDF-1), which is expressed on marrow osteoblasts. In contrast, G-CSF indirectly mobilizes stem cells by down-regulating the expression of SDF-1 and by releasing proteolytic enzymes, which degrades important HSC trafficking and adhesion molecules.

Plerixafor promotes a more robust mobilization when used in combination with G-CSF in both healthy volunteers and patients with hematologic diseases.[15,16]

Plerixafor, alone or in combination with G-CSF, has also been used to improve the yield of HSPCs in β-thalassemia patients. Plerixafor, when used as single agent, was safer than G-CSF. However, a third of patients failed to achieve the pre-determined cell dose. Conversely, a combination including G-CSF and plerixafor was shown to be synergistic and to induce successfully mobilization in four patients in whom single agent-mobilization had previously failed.[14,17]

Here, we report a clinical study of mobilization via the use of G-CSF+Plerixafor in four adult transfusion-

Correspondence: Elena Baiamonte, Campus of Hematology F. and P. Cutino, Villa Sofia-Cervello Hospital, via Trabucco 180, 90146 Palermo, Italy.
Tel.: +39.91.6802433 - Fax: +39.91.6880828.
E-mail: elenabaiamonte77@gmail.com

Key words: β-thalassemia; CD34+ cells expression profiling; G-CSF+plerixafor mobilization; gene therapy.

Acknowledgments: we thank R. Messina and G. Lucania for their expert care of patients during apheresis, B. Spina for laboratory technical assistance and A. Vitrano and M. Sacco for statistical analysis. We thank I. Rivière for providing the purified CD34/G cells. We also thank M. Lo Iacono and G. Damiano for their comments on the manuscript. We would especially like to acknowledge the patients for their participation in this study.

Funding: this work was mainly supported by a grant from PO FESR 4.1.1.1 RIMEDRI (B75f1200150004), funds to SA via the Franco and Piera Cutino Foundation, Palermo. The travel and accommodation expenses for patient 4 were funded by the De Marchi Foundation, Milan, Italy. The manuscript has been certified by AJE.

Contributions: EB carried out the laboratory experiments, SF, FC and RD contributed to the array analyses and interpretation of data; AM1 performed haematopoietic stem cells immunoselection and cryopreservation; RB and AM2 recruited the patients and are responsible for the clinical data; AF and ED recruited patients; SA designed and directed the research plane and wrote the manuscript. All authors approved the manuscript.

Conflict of interest: the authors declare no potential conflict of interest.

Received for publication: 15 November 2016.
Revision received: 2 March 2017.
Accepted for publication: 20 April 2017.

This work is licensed under a Creative Commons Attribution 4.0 License (by-nc 4.0).

©Copyright E. Baiamonte et al., 2017
Licensee PAGEPress, Italy
Thalassemia Reports 2017; 7:6392
doi:10.4081/thal.2017.6392

Non-commercial use only

dependent β-thalassemia patients. We aimed to achieve a yield of a minimum of 8×10^6 CD34+cells/kg in apheresis collection and to assess the effects of plerixafor addition on the quantity and the quality of HSPCs that were mobilized. In particular we were interested in an intraindividual comparison of counts, subtypes and clonogenic potency of peripheral blood (PB) CD34+ cells before and after plerixafor addition. Moreover, in one patient we compared gene expression analysis of purified CD34+ cells mobilized with G-CSF(CD34/G) and with G-CSF+Plerixafor combination (CD34/G+pl).

Materials and Methods

Study approval

The study was approved by the local Institutional Ethics Committee and conducted in the Division of Rare Diseases of Blood and Hematopoietic Organs-Campus of Hematology F. and P. Cutino at the Villa Sofia-Cervello, hospital in Palermo. Informed consent for privacy and research use was obtained from all eligible patients in accordance with the Declaration of Helsinki.

Patient characteristics and hematopoietic genetically modified stem and progenitor cells mobilization

Four patients with β-thalassemia major who were similar in age and weight were enrolled in the study based on the criteria listed in Table 1. Patient characteristics are listed in Table 2. The patients received subcutaneous injections of 10 µg/kg of G-CSF (Filgrastim, Amgen, Thousand Oaks, CA, USA) in the morning from day 1 to day 4 and 240 µg/kg of plerixafor (Mozobil, Sanofi-Genzyme, Cambridge, MA, USA) on day 4 at 12 hours after treatment with G-CSF. Leukapheresis was performed 10-12 h later, on day 5. If fewer than 8×10^6 CD34+ cells/kg, were collected during the first apheresis, an additional dose of plerixafor was administered on the evening of day 5, and a second apheresis procedure was performed 12 hours later (on day 6). The leukapheresis product was harvested using a cell separator (COM.TEC® multi-procedural apheresis platform, Fresenius KABI, Bad Homburg, Hessen, Germany) according to institutional standards. The volume of blood that was processed per leukapheresis was approximately three times the total blood volume. The entire process of mobilization and apheresis was performed in outpatient clinic. A minimum of 6×10^6 cells were immune-enriched and cryopreserved for future β-globin/lentiviral vector transduction and a volume of leukapheresis containing 2×10^6/kg CD34+ cells was stored as back-up in case of graft failure, according to JACIE guidelines, at the Bone Marrow Transplantation Unit of our hospital.

Efficacy outcomes and safety

Efficacy outcomes included the number of patients who reached the target dose, the number of apheresis that was required, and the fold increase in circulating CD34+ cells/µl after plerixafor administration.

Non commercial use only

Table 1. Eligibility criteria.

Inclusion criteria	Exclusion criteria
1. Diagnosis of ß-Thalassemia Major.	1. Hepatitis B and C, HIV 1 and HIV 2, HTLV 1, HTLV2 or other active infections.
2. Age > 18 years old	2. Mellitus diabetes.
3. Lack of an HLA-matched sibling to undergo an allo-trasplantation	3. History of thrombosis or known thrombophilia (Factor V Leiden, positive anti-phospholipidic-anticardiolipin antibodies).
4. Without hydroxyurea and erythropoietin administration for at least three months before the study.	4. Pregnancy or breast-feeding.
5. Performance of Karnofsky status > 70.	5. Severe pulmonary hypertension.
6. Optimal hepatic function with ALT- <3N, serum total bilirubin <2.0 (unless secondary to haemolysis), no evidence of cirrhosis in the according to abdomen US and fibroscan.	6. Neoplastic syndrome familiarity.
7. FEVS > 50%.	7. Splenectomized patients with a platelet count > 900.000/mmc.
8. Renal function: creatinine <1.5 mg/dl, when serum creatinine is outside of the normal range, creatinine clearance (CrCl)>60 ml/min/1.73 m².	
9. Splenectomized or non-palpable spleen.	

Table 2. Patient characteristics and side effects during mobilization.

	Age (yrs)	Gender	Weight (kg)	Genotype (mutation)	Chelation	Ferritin (ng/ml)	Spleen	Side effects
Pt 1	22	F	47	β0/+ Cod39/IVS1-110	DFP	1938	Not palpable	Bone pain, Warm feeling, Pulse-pounding
Pt 2	22	M	50	β+/+ IVS 1-110/IVS1-110	DFX	1143.9	Splenectomy	Bone pain, Warm feeling, Pulse-pounding, Leukocytosis
Pt 3	21	F	56	+/+ IVS1-6/IVS2-1	DFX	603.4	Not palpable	Bone pain, Fatigue, Anemia, Thrombocytopenia
Pt 4	23	F	51	+/+ IVS1-110/IVS2-745	DFX	485.4	Not palpable	Bone pain, Thrombocytopenia

Safety was evaluated as the incidence of adverse events in daily clinical and hematological follow-ups that were performed during and after mobilization, until the complete normalization of blood cell counts was achieved. In non-splenectomized patients, the degree of enlargement of the spleen was evaluated daily using ultrasonography. If the spleen volume increased by more than 80% from baseline, G-CSF was discontinued, and the patient was withdrawn from the study. Hyperleukocytosis (WBC $\geq 50\times10^6/\mu l$) led to a reduction in the G-CSF dosage or the discontinuation of treatment in cases of excessive leukocytosis (WBC $\geq 100\times10^6/\mu l$).

CD34+ cell counts and immune-phenotyping

Absolute numbers of PB-CD34+ cells were measured daily using a flow cytometer (FC-500 Beckman-Coulter, Cassina de Pecchi, Milano, Italy) according to the ISHAGE protocol using a single-platform method (Stem-Kit, Beckman-Coulter).

CD34+ cell subtyping was performed after 4 doses of G-CSF (on the morning of day 4) and at 10-12 hours after plerixafor administration (on the morning of day 5). Subtyping was done using a four-colour method with antibody combinations in a Beckman Coulter cytometer according to the manufacturer's recommendations. Cells were stained using the following monoclonal antibodies or corresponding isotype controls: FITC-conjugated CD45, ECD-conjugated CD34, PC5-conjugated CD38 (Beckman Coulter) and PE-conjugated CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were analysed using the software (Cxp Cytometer analysis software and Kaluza; Beckman Coulter).

Colony forming unit assay

Blood samples, containing 3×10^3 CD34+ cells, collected after G-CSF or G-CSF+plerixafor mobilization, were centrifuged, and the cellular portion lysed to eliminate mature erythrocytes. The remaining cells were plated in triplicate in complete methylcellulose medium (MethoCult H4434 Enriched - StemCell Technology, Vancouver, British Columbia, Canada) and incubated at 37°C in 5% CO_2 for 14 days. Identification and counts of colony forming unit-granulocyte/macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) were assessed using an inverted microscope.

The erythroid colonies consisting of maximum 8 clusters were classified as small BFU-E, and those that consisted of 9 or more clusters were classified as large BFU-E.

Microarray hybridization and analysis

Whole gene expression profiling was performed on immuno-selected CD34+ cells (purity higher than 90%) using a CliniMacs CD34Kit (CliniMacs, Miltenyi Biotec, Bergisch Gladbach, Germany). CD34+ cells were derived from the leukapheresis of patient 1 that was mobilized twice, once with G-CSF+plerixafor (CD34/G+pl) and once, one year later, with G-CSF alone (CD34/G).

In particular, CD34/G cells were enriched from the combined leukapheresis products that were collected, at MSKCC, NY, USA, for gene therapy purposes on days 5 and 6 of mobilization using G-CSF (10 μg/kg/day).[6]

Total RNA was isolated using TRIZOL according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and dissolved to a final concentration of 0.2-0.5 μg/μl. The quality of the RNA was tested using capillary electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the samples exhibited common high quality RNA integrity numbers (RINs) ranging from 8 to 9.

Fluorescently labelled cRNA targets were generated from 150 ng of total RNA in each reaction using a Low RNA Input Linear Amplification Kit and Cyanine 3- or Cyanine 5-labelled CTP according to the manufacturer's instructions (Agilent Technologies). Labelled cRNAs were purified using an RNeasy Mini Kit (Qiagen) and applied to a 4×44 K Whole Human Genome Oligo Microarray Kit (G4112F, Agilent Technologies).

For each hybridization, 825 ng of cyanine-labelled cRNA from each sample was mixed with an equal amount of reverse-colour cyanine-labelled cRNA from Universal Human Reference RNA (Stratagene, La Jolla, CA), which was used as a common reference. Hybridization and washing were performed using an *in situ* hybridization kit according to the manufacturer's instructions (Agilent Technologies). The arrays were scanned using a dual-laser DNA microarray scanner (Sure Scan, Agilent Technologies). The data were then extracted from the images using Feature Extraction software (Agilent Technologies, ver. 9.5.3). All hybridization experiments were performed in duplicate using dye-swap.

GeneSpring software (GX software ver. 11, Agilent Technologies), and lists of selected genes were generated using different statistical and visualization methods. Intensity-dependent normalization (known as Lowess normalization) was used to correct for the artefacts that were caused by non-linear rates of dye incorporation and inconsistencies in the relative fluorescence intensities between some red and green dyes.

The data from the CD34/G+pl and CD34/G RNA samples were then normalized to the Universal Human Reference RNA (Stratagene, La Jolla, CA), and an initial gene list was created using confidence filtering at $P<0.01$ (t-test P-values were used as the measure of confidence, and the Benjamini-Hochberg false discovery rate was used for multiple testing correction) to eliminate genes with unreliable measurements. Consecutive lists of differentially expressed genes were generated assuming a 2-fold expression rate and using data from all independent experiments.

Gene ontology analysis was first performed using DAVID Bioinformatics Resource online software (ver. 6.7, NIAID, NIH: http://david.abcc.ncifcrf.gov/), and pathway analysis was subsequently performed using the same software.

The data reported here have been deposited in the National Centre for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70219)

Statistical analysis

The data are provided as the median, range or maximum values. Mean, standard deviation and p-value were performed with the STATA 12.0 software (StataCorp, USA) applying the Student t-test.

Results

Patients and safety

According to the Common Terminology Criteria for Adverse Events, version 4.0, the treatment was well tolerated with minimal side effects (Table 2). The splenectomized patient (Pt 2) developed hyperleukocytosis (WBC $60.2\times10^6/\mu l$) and required the G-CSF dosage dose to be halved from day 2 of mobilization (Table 3).

An average Hb reduction of 0.9 g/dl and an average reduction in platelet numbers were observed at the end of the mobilization period compared with baseline (354.5±187.8 *vs* 175±97.5). After the second apheresis, patient 3 developed intermediate grade thrombocytopenia (platelet count of 68.0/μl) that recovered completely within one week and a reduction in hemoglobin levels to 8.7 g/dl, which required a blood transfusion.

Other mild side effects lasted for 1-2 days after apheresis and resolved spontaneously. The patient's hematological

Non-commercial use only

parameters during mobilization are shown in Table 3.

G-CSF+plerixafor results in efficient CD34+ cell mobilization

8×10^6 CD34+ cells/kg was defined as target cell dose for gene therapy. Three out of four patients achieved the target cell dose in a single apheresis collection ($8.15\times10/^6$ cells/kg, median value), including the patient 2, despite the fact that G-CSF dose was reduced in this patient starting from day 2 to avoid hyperleukocytosis (Table 3).

In patient 3, the number of cells collected in the first apheresis was slightly below the established target and a second apheresis collection was performed on day 6, at 12 hours after an additional dose of plerixafor was administered. The total yield from the combined apheresis in this patient was 13.5 CD34+ cells/kg (Pt 3, Table 3). The kinetics of HSPC mobilization were analysed by counting the PB-CD34+ cell daily during treatment (Table 3). The addition of plerixafor mobilized a substantial portion of CD34+ cells in all of the patients, resulting in a 12-fold mean peak increase (range, 4.8- to 23-fold).

Addition of plerixafor increases the mobilization of CD34+ cell subsets with a more primitive phenotype and higher clonogenic capacity

To investigate whether plerixafor exposure causes the release of a subset of HSPCs distinct from GCSF-primed progenitor cells, we studied the co-expression of the surface antigens CD133 and CD38 in PB-CD34+ cells before and after plerixafor was administered.

The frequency of primitive CD34+ subtypes (CD34+/CD38- and CD34+/CD133+) consistently increased in all patients after plerixafor was added. CD34+/CD38- cells were increased by 2.4-fold, despite the high inter-individual variability that was observed at baseline, and CD34+/CD133+ cells were increased by 1.3-fold. A significant increase was also observed in the frequency of the most primitive CD34+ subtype, CD34+/CD133+/CD38- cells, after plerixafor addition. The same analysis performed in PB sample obtained from patient 3 at 11 hours after the second administration of plerixafor, showed no change in the proportion of CD34+ subtypes compared to that of the day before (data not shown). These data suggest that the difference in subset composition that was observed between days 4 and 5 can be attributed to plerixafor stimulation instead of a longer period of mobilization. In support of this hypothesis, we also did not detect substantial changes in the proportion of PB-CD34+ cell subtypes in the three healthy donors on days 4 and 5 during mobilization with G-CSF (10 μg/kg/day) used as single agent (data not shown). These data are in line with those reported by others regarding the frequency of CD34+/CD38- cells in PB of donors on days 4, 5 and 6 of G-CSF mobilization.[18]

Clonogenic capacity of PB CD34+ cells was investigated in short-term colony forming (CFC) assay based on the numbers and types of colonies that developed, before and after plerixafor.

The absolute number of CFU-GM and BFU-E that developed from PB-CD34+ cells plated at day 5 was unanimously higher than that developed from the same number of cells plated, before plerixafor addition. In addition, the proliferative capacity of the progenitors also increased after plerixafor was added, as observed comparing the sizes of the BFU-E colonies that devel-

Non-commercial use only

Table 3. Blood CD34+ cell counts and hematological parameters during G-CSF+plerixafor mobilization.

	Day	+1	+2	+3	+4	+5*	+6*	+7
Pt 1	WBC 10^3/uL	6.6	25.0	32.1	30.0	*60.3*	19.4	
	ANC 10^3/uL	3.9	21.4	27.1	24.3	*40.2*	14.1	
	HGB g/dL	11.5	10.9	10.7	10.6	*10.6*	10.0	
	PLT 10^3/uL	294	260	262	279	*318*	211	
	CD34+/uL	4	8	10	48	*361*	80	
	CD34+/Kg					*29×10^6*		
Pt 2°	WBC 10^3/uL	12.8	60.2	56.6	55.8	*92.2*	21.5	
	ANC 10^3/ /uL	5.9	NA	NA	NA	*61.6*	13.7	
	HGB g/dL	10.9	10.7	9.7	10.8	*10.1*	10.2	
	PLT 10^3/uL	633	587	503	588	*538*	292	
	CD34+/uL	6	6	6	19	*92*	NA	
	CD34+/Kg					*8.3×10^6*		
Pt 3	WBC 10^3/uL	4.2	24.3	27.5	30.0	*68.1*	65.0	38.0
	ANC 10^3/uL	2.1	19.8	22.8	25.7	*42.3*	NA	NA
	HGB g/dL	11.2	9.2	9.0	9.8	*9.5*	10.0	8.7
	PLT 10^3/uL	225	220	213	250	*239*	120	68
	CD34+/uL	1	1	1	5	*115*	76	NA
	CD34+/Kg					*7×10^6*	6.5×106	
Pt 4	WBC 10^3/uL	6.2	20.6	21	25.1	*53*	NA	
	ANC 10^3/uL	3.3	18.4	18.3	21.7	*42.9*	NA	
	HGB g/dL	10.7	10.7	11.5	10.6	*10.7*	10.4	
	PLT 10^3/uL	266	263	238	211	*217*	129	
	CD34+/uL	1	1	1	8	*102*	NA	
	CD34+/Kg					*8×10^6*		

Values obtained after administration of plerixafor to GCSF regimen are indicated in italics. WBC, white blood cell; ANC, absolute neutrophil count; HGB, hemoglobin; PLT, platelet. *Days of treatment when the phenotype and clonogenic ability were analyzed; °in this patient, the G-CSF dosage was halved beginning on day 2 of mobilization.

OPEN ACCESS

oped from PB-CD34+ cells at these two time points. On day 5 of mobilization, we observed a clear increase in the portion of intermediate/large BFU-E colonies that consisted of more than 9 clusters over the total number of BFU-E colonies.

G-CSF+Plerixafor combination mobilizes of CD34+ cells with gene expression pattern distinct from that of G-CSF-primed cells

We performed a microarray analysis to analyse global gene expression in CD34+ cells that were purified from the leukapheresis of patient 1 by two independent mobilization regimens: one with G-CSF+plerixafor (CD34/G+pl) and one, with G-CSF used as a single agent (CD34/G).

The CD34/G and CD34/G+pl samples were compared after normalization using Universal Human Reference RNA (Stratagene) as a common reference.

A subset of differentially expressed genes was selected from all of the microarray data after they were first filtered according to confidence at P<0.01 and then by expression level (≥2-fold). Using these selection criteria, we found that 873 genes were commonly up-regulated and that 1691 genes were down-regulated in the CD34/G+pl cells than in the CD34/G cells.

The genes were classified according to their function using the Gene Ontology Classification System (Figure 1A and Supplementary Table 1). As shown in Figure 1B a substantial number of genes associated with homing and engraftment processes (*e.g.*, CXCR4, CD82, DPP4 and ROBO4) and genes known to be linked to stress resistance (*e.g.*, CXCL4, SOD2, IL8 and PPBP) were up-regulated. Several genes, encoding chemokines that have been shown to be involved in cell mobility (*e.g.*, CXCL2, CXC3 and CXCR2) were also up-regulated, and genes involved in transcriptional regulation (*e.g.*, GATA-2, BTG2, DUSP2, MS4A3 and TNF) were either up- or down-regulated. The array analysis also indicated in both CD34/G+pl and CD34/G cells the substantial over-expression (more than 16-fold compared to the Universal Reference RNA) of several genes (RGS18, MPO, TNFSF4, EGR1) that are involved in mobilization, homeostasis, homing and engraftment (data not shown).

Discussion

The outcome of gene therapy for β-thalassemia, in addition to requiring sufficient genetic modification of CD34+ cells, and efficient myeloablative conditioning, largely depends on the quantity and the quality of the autologous hematopoietic stem cell collected. Indeed, to reduce morbidity resulting from ineffective erythropoiesis and to achieve transfusion independence, it is necessary to infuse an adequate number of HSPCs that are capable of generating long-term gene-corrected erythroid precursors.

In this study, G-CSF+plerixafor mobilization was shown to be safe and efficacious for collecting high numbers of HSPCs. The target cell dose was achieved in all patients, and in three of them, it was achieved in a single apheresis collection. This result was similar to that previously reported by Yannaki and co-workers, in other 4 patients mobilized using the same combination;[17] conversely, in other studies in which G-CSF was used as a single agent, similar cell yields were achieved after two apheresis collections in the majority of the patients.[14,6]

It has been shown that G-CSF and plerixafor promote HSPCs trafficking via different mechanisms, that may explain different HSPC subsets.[19]

Papers describing differences in G-CSF and G-CSF+plerixafor grafts in healthy donors and onco-hematological patients have concluded that CD34/G+pl cells show more immature phenotypes than CD34/G cells.[15,20-24]

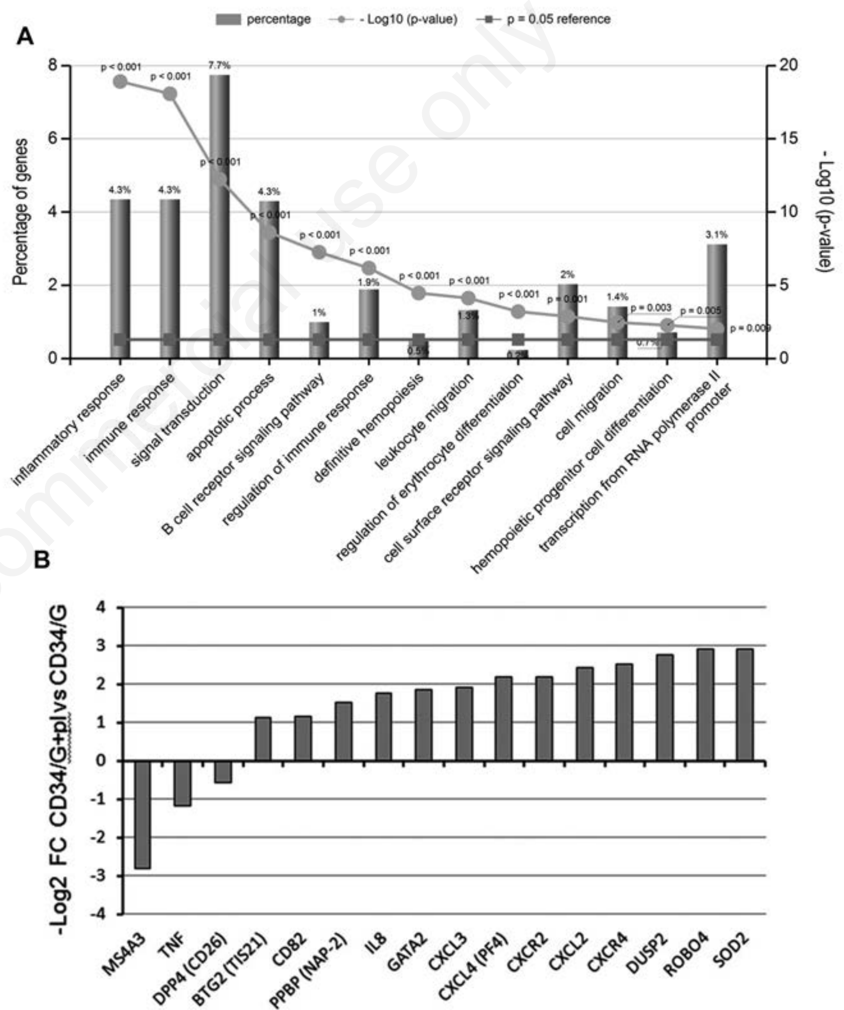

Figure 1. Bar graph representations of the differential expression profiles of genes that were analysed in the microarray analysis. A) Bar graph of biological process overrepresented in CD34/G+pl cells compared to CD34/G cells. B) Log changes are shown to represent the difference in the expression level of genes differentially expressed between CD34/G+pl cells and CD34/G cells. -Log2-fold changes (FC) were calculated after normalization to Universal Reference RNA (P<0.01, FC cut-off ±2). Values greater than or equal to 1 represent gene up-regulation, and values less than 1 indicate gene down-regulation.

Non-commercial use only

pagepress

However, the hematopoietic cell repertoire in thalassemia patients may differ from that of other subjects because they endure chronic anaemia, prolonged transfusion regimens, and iron overload may alter the balance that regulates the retention/release of these cells. We were interested in characterizing intra-individual variations in CD34+ cells that were mobilized using G-CSF or G-CSF+plerixafor.

We found that the addition of plerixafor uniformly resulted in a substantial increase in the proportion of primitive CD34+ subtypes that have previously been shown to possess extensive proliferation and repopulation capacities.[25,26] Moreover, CD34/G+pl cells displayed a higher capacity for clonogenicity and proliferation than CD34/G cells from the same patients. A trend towards higher frequencies of primitive cells has been previously observed in G-CSF+plerixafor grafts (compared to single-agent-mobilized grafts) obtained from thalassemia patients and from mouse model of thalassemia.[14,17,27]

Our results corroborate and extend on this finding at an intra-patient level that allowed us to abolish variability in cellular composition between individuals.

Expression profiling of CD34/G and CD34/G+pl cells, derived from patients with hematologic malignancies,[28] and primates[29] mobilized with both modalities, revealed that CD34/G+pl cells expressed significantly higher levels of genes that promote superior engraftment after myeloablation. We found that also in thalassemia patients, gene expression profiles depended on the mobilization protocol. This result may explain published functional data that have shown that G-CSF+Plerixafor grafts obtained from thalassemia patients are more competitive than G-CSF grafts at engrafting murine recipients.[30]

In particular, according to the mechanism of plerixafor mobilization, the expression level of CXCR4 was 5-fold higher in CD34/G+pl cells than in CD34/G cells. This result was analogous to that reported by Fruehauf and co-workers in patients with multiple myeloma and non-Hodgkin lymphoma.[28]

CXCR4 is expressed on the surface of HSPCs, especially more primitive CD34+ cells with long-term repopulating potential, and it plays a central role in regulating the adhesion of HSPCs to their native niche in BM.[31] Conversely, genes involved in the cleavage of VCAM-1 and SDF-1 (CXCL12) antigens, such as elastase (ELANE) and cathepsin G (CTSG), were overexpressed in both CD34/G+pl and CD34/G cells. These data are in line with a mechanism involving G-CSF mobilization.[32]

The chemokines CXCL2, CXC3 and CXCR2 , involved in a rapid HSPCs mobilization, were also up-regulated in CD34/G+pl cells.[33] Among the genes that were found to be differentially expressed in the two samples, there was a group of antigen surface genes that regulate adhesion to the microenvironment. In particular, CD82 and Robo4 were overexpressed and CD26 was down-regulated in CD34/G+pl cells. It has been reported both CD82 and Robo 4 play a key role in anchoring HSPC to BM niches and in long-term reconstitution,[34,35] while inhibiting CD26 may increase the success of HSPC transplantation.[36]

Several transcription regulators have also been shown to be up-regulated in CD34/G+pl cells. Among these, GATA-2, BTG2/TIS21 and DUSP2 are of particular relevance. GATA-2 acts as a regulator of hematopoiesis, during which it inhibits differentiation by preventing the activation of lineage-specific genes.[37] Furthermore, BTG2/TIS21 and DUSP2 are negative cell-cycle regulators of cell proliferation.[38]

In particular DUSP2-mediated suppression of ERK activity is required to maintain a pluripotent state in murine stem cells.[39]

A group of genes, superoxide dismutase 2 (SOD2), CXC-type chemokine PF4 (CXCL4), IL-8 and NAP-2 (PPBP) that improve cell survival and reduce cell sensitivity to cytotoxic agents limiting the harmful effects of cellular stress caused by the mobilization process, was also significantly expressed in CD34/G+pl cells.[40,41]

Interestingly, the expression levels of MS4A3 and tumour necrosis factor (TNF), which influence the fate of HSPCs by directing them towards differentiation instead of self-renewal,[42-44] were down-regulated in CD34/G+pl cells. Several other genes (RSGS18, MPO, TNFSF4 and EGR1) involved in mobilization, homeostasis, homing, engraftment, cell survival and self-renewal were also substantially overexpressed in both samples.

Conclusions

Overall, the mobilization study reported here, in thalassemia patients, highlighted how the addiction of plerixafor to G-CSF-based mobilization not only enhanced cell yields, but also promoted the egress from BM of CD34+ cells with phenotypic and functional characteristics and gene expression profiles that suggest high capacity of engraftment after transplantation.

Non-commercial use only

References

1. Negre O, Eggimann AV, Beuzard Y, et al. Gene therapy of the β-hemoglobinopathies by lentiviral transfer of the β(A(T87Q))-globin gene. Hum Gene Ther 2016;27:148-65.
2. Champlin RE, Schmitz N, Horowitz MM, et al. Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. IBMTR Histocompatibility and Stem Cell Sources Working Committee and the European Group for Blood and Marrow Transplantation (EBMT). Blood 2000;95:3702-09.
3. Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nat Med 2006;12:401-9.
4. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science 2009;326:818-23.
5. Boztug K, Schmidt M, Schwarzer A, et al. Stem-cell gene therapy for the Wiskott-Aldrich syndrome. N Engl J Med 2010;20:1918-27.
6. Boulad F, Wang X, Qu J, et al. Safe mobilization of CD34+ cells in adults with β-thalassemia and validation of effective globin gene transfer for clinical investigation. Blood 2014;123: 1483-86.
7. Sekhsaria S, Fleisher TA, Vowells S, et al. Granulocyte colony-stimulating factor recruitment of CD34+ progenitors to peripheral blood: impaired mobilization in chronic granulomatous disease and adenosine deaminase--deficient severe combined immunodeficiency disease patients. Blood 1996;88:1104-12.
8. Panch SR, Yau YY, Kang EM, et al. Mobilization characteristics and strategies to improve hematopoietic progenitor cell mobilization and collection in patients with chronic granulomatous disease and severe combined immunodeficiency. Transfusion 2015;55:265-74.
9. Abboud M, Laver J, Blau CA. Granulocytosis causing sickle-cell crisis. Lancet 1998;351:959.
10. Adler BK, Salzman DE, Carabasi MH, et al. Fatal sickle cell crisis after granulocyte colony-stimulating factor administration. Blood 2001;97:3313-14.
11. Taher AT, Otrock ZK, Uthman I, et al. Thalassemia and hypercoagulability. Blood Reviews 2008;22:283-92.
12. Eldor A, Rachmilewitz EA. The hypercoagulable state in thalassemia. Blood 2002;99:36-43.

OPEN ACCESS

13. Falzetti F, Aversa F, Minelli O, et al. Spontaneous rupture of spleen during peripheral blood stem-cell mobilisation in a healthy donor. Lancet 1999;353:55.
14. Yannaki E, Papayannopoulou T, Jonlin E, et al. Hematopoietic stem cell mobilization for gene therapy of adult patients with severe β-thalassemia: results of clinical trials using G-CSF or plerixafor in splenectomized and nonsplenectomized subjects. Mol Ther 2012;20:230-8.
15. Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med 2005;201:1307-18.
16. Di Persio JF, Stadtmauer EA, Nademanee A, et al. Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. Blood 2009;113:5720-6.
17. Yannaki E, Karponi G, Zervou F, et al. Hematopoietic stem cell mobilization for gene therapy: superior mobilization by the combination of granulocyte-colony stimulating factor plus plerixafor in patients with β-thalassemia major. Hum Gene Ther 2013;24:852-60.
18. Bellucci R, De Propris MS, Buccisano F, et al. Modulation of VLA-4 and L-selectin expression on normal CD34+ cells during mobilization with G-CSF. Bone Marrow Transplant 1999;23:1-8.
19. Nervi B, Link DC, Dipersio JF. Cytokines and hematopoietic stem cell mobilization. J Cell Biochem 2006;99:690-705.
20. Fruehauf S, Veldwijk MR, Seeger T, et al. A combination of granulocyte-colony-stimulating factor (G-CSF) and plerixafor mobilizes more primitive peripheral blood progenitor cells than G-CSF alone: results of a European phase II study. Cytotherapy 2009;11:992-1001.
21. Taubert I, Saffrich R, Zepeda-Moreno A, et al. Characterization of hematopoietic stem cell subsets from patientswith multiple myeloma after mobilization with plerixafor. Cytotherapy 2011;13:459-66.
22. Varmavuo V, Mäntymaa P, Silvennoinen R, et al. CD34+ cell subclasses and lymphocyte subsets in blood grafts collected after various mobilization methods in myeloma patients. Transfusion 2013;53:1024-32.
23. Roug AS, Hokland LB, Segel E, et al. Unraveling stem cell and progenitor subsets in autologous grafts according to methods of mobilization: implications for prediction of hematopoietic recovery. Cytotherapy 2014;16:392-401.
24. Girbl T, Lunzer V, Greil R, et al. The CXCR4 and adhesion molecule expression of CD34+hematopoietic cells mobilized by "on-demand" addition of plerixafor to granulocyte–colony-stimulating factor. Transfusion 2014;54: 2325-35.
25. Larochelle A, Vormoor J, Hanenberg H, et al. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nat Med 1996;2:1329-37.
26. de Wynter EA, Buck D, Hart C, et al. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. Stem Cells 1998;16:387-96.
27. Psatha N, Sgouramali E, Gkountis A, et al. Superior long-term repopulating capacity of G-CSF+plerixafor-mobilized blood: implications for stem cell gene therapy by studies in the Hbb(th-3) mouse model. Hum Gene Ther Methods 2014;25:317-27.
28. Fruehauf S, Seeger T, Maier P, et al. The CXCR4 antagonist AMD3100 releases a subset of G-CSF-primed peripheral blood progenitor cells with specific gene expression characteristics. Exp Hematol 2006;34:1052-59.
29. Donahue RE, Jin P, Bonifacino AC, et al. Plerixafor (AMD3100) and granulocyte colony-stimulating factor (G-CSF) mobilize different CD34 cell populations based on global gene and microRNA expression signatures. Blood 2009;17:2530-41.
30. Karponi G, Psatha N, Werner C, et al. Plerixafor+G-CSF-mobilized CD34+ cells represent an optimal graft source for thalassemia gene therapy. Blood 2015;126:616-19.
31. Hicks C, Isaacs A, Wong R, et al. CXCR4 expression on transplanted peripheral blood CD34+ cells: relationship to engraftment after autologous transplantation in a cohort of multiple myeloma patients. Ann Hematol 2011;90:547-55.
32. Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol 2002;30:973-81.
33. Pelus LM , Horowitz D, Cooper SC, et al. Peripheral blood stem cell mobilization. A role for CXC chemokines. Crit Rev Oncol Hematol 2002;43:257-75.
34. Larochelle A, Gillette JM, Desmond R, et al. Bone marrow homing and engraftment of human hematopoietic stem and progenitor cells is mediated by a polarized membrane domain. Blood 2012;119:1848-55.
35. Smith-Berdan S, Nguyen A, Hassanein D, et al. Robo4 cooperates with CXCR4 to specify hematopoietic stem cell localization to bone marrow niches. Cell Stem Cell 2011;8:72-83.
36. Yoo E, Paganessi LA, Alikhan WA, et al. Loss of CD26 protease activity in recipient mice during hematopoietic stem cell transplantation results in improved transplant efficiency. Transfusion 2013;53:878-87.
37. Pan X, Minegishi N, Harigae H, et al. Identification of human GATA-2 gene distal IS exon and its expression in hematopoietic stem cell fractions. J Biochem 2000;127:105-12.
38. Duriez C, Falette N, Audoynaud C, et al. The human BTG2/TIS21/PC3 gene: genomic structure, transcriptional regulation and evaluation as a candidate tumor suppressor gene. Gene 2002;28:2207-14.
39. Chappell J, Sun Y, Singh A, et al. MYC/MAX control ERK signaling and pluripotency by regulation of dual-specificity phosphatases 2 and 7. Genes Dev 2013;27:725-33.
40. Miao W, Xufeng R, Park MR, et al. Hematopoietic stem cell regeneration enhanced by ectopic expression of ROS-detoxifying enzymes in transplant mice. Mol Ther 2013;21:423-32.
41. Han ZC, Lu M, Li J, et al. Platelet factor 4 and other CXC chemokines support the survival of normal hematopoietic cells and reduce the chemosensitivity of cells to cytotoxic agents. Blood 1997;89:2328-35.
42. Dybedal I, Bryder D, Fossum A, et al. Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. Blood 2001;98:1782-91.
43. Zhang Y, Harada A, Bluethmann H, et al. Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vitro. Blood 1995;86:2930-7.
44. Rusten LS, Jacobsen FW, Lesslauer W, et al. Bifunctional effects of tumor necrosis factor α (TNF α) on the growth of mature and primitive human hematopoietic progenitor cells: involvement of p55 and p75 TNF receptors. Blood 1994;83:3152-9.

Non commercial use only

Supplementary Table 1

Gene Onthology classification of CD34/G+pl vs CD34/G differentially expressed genes

ID	Biological Process Description	Fold enrichment	Percentage of genes	p-value	p-value FDR
GO:0002819	regulation of adaptive immune response	12.50	0.28	2.60E-07	2.96E-03
GO:0045646	regulation of erythrocyte differentiation	12.50	0.24	3.26E-06	3.71E-02
GO:0045577	regulation of B cell differentiation	10.72	0.28	1.70E-06	1.93E-02
GO:0060216	definitive hemopoiesis	7.36	0.47	1.21E-07	1.38E-03
GO:0048535	lymph node development	6.26	0.38	1.19E-05	1.35E-01
GO:0030225	macrophage differentiation	5.56	0.38	3.48E-05	3.97E-01
GO:0050853	B cell receptor signaling pathway	5.15	0.99	9.10E-11	1.04E-06
GO:0071345	cellular response to cytokine stimulus	5.12	0.42	2.50E-05	2.85E-01
GO:0042102	positive regulation of T cell proliferation	4.74	1.04	2.20E-10	2.51E-06
GO:0006952	defense response	4.24	0.94	1.40E-08	1.59E-04
GO:0031295	T cell costimulation	4.17	1.23	1.49E-10	1.70E-06
GO:0050727	regulation of inflammatory response	3.82	0.85	4.47E-07	5.10E-03
GO:0030097	hemopoiesis	3.47	0.71	1.44E-05	1.64E-01
GO:0002250	adaptive immune response	3.35	1.93	2.97E-12	3.38E-08
GO:0002244	hemopoietic progenitor cell differentiation	3.18	0.71	4.54E-05	5.17E-01
GO:0006954	inflammatory response	3.11	4.34	3.18E-23	3.62E-19
GO:0006955	immune response	3.02	4.34	2.91E-22	3.31E-18
GO:0042127	regulation of cell proliferation	2.90	1.84	1.03E-09	1.17E-05
GO:0050900	leukocyte migration	2.87	1.32	2.88E-07	3.28E-03
GO:0050776	regulation of immune response	2.81	1.89	1.74E-09	1.99E-05
GO:0045087	innate immune response	2.70	4.11	8.39E-18	9.56E-14
GO:0019221	cytokine-mediated signaling pathway	2.44	1.13	3.68E-05	4.19E-01
GO:0035556	intracellular signal transduction	2.33	3.35	1.57E-11	1.79E-07
GO:0016477	cell migration	2.23	1.42	2.56E-05	2.92E-01
GO:0006915	apoptotic process	2.15	4.29	2.84E-12	3.23E-08
GO:0001525	angiogenesis	2.04	1.65	4.39E-05	5.00E-01
GO:0007166	cell surface receptor signaling pathway	2.01	2.03	8.30E-06	9.45E-02
GO:0007165	signal transduction	1.91	7.74	3.45E-16	3.93E-12
GO:0006355	transcription, DNA-dependent	1.90	12.84	2.86E-26	3.26E-22
GO:0006366	transcription from RNA polymerase II promoter	1.61	3.11	8.71E-05	9.93E-01

Non-commercial use only