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ORIGINAL ARTICLE

Engraftment capacity of mesenchymal cells following hematopoietic stem cell transplantation in patients receiving reduced-intensity conditioning regimen

A Poloni[1], P Leoni[1], L Buscemi[2], F Balducci[1], R Pasquini[1], MC Masia[1], N Viola[3], E Costantino[3], G Discepoli[4], P Corradini[5], A Tagliabracci[2] and A Olivieri[1]

[1]Department of Hematology, University of Ancona, Ancona, Italy; [2]Department of Legal Medicine, University of Ancona, Ancona, Italy; [3]Department of Clinical Immunology, University of Ancona, Ancona, Italy; [4]Unit of Medical Genetic and Prenatal Diagnosis, Ospedale Salesi, Ancona, Italy and [5]Division of Hematology-Bone Marrow Transplantation, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy

The engraftment ability of mesenchymal cells was investigated in 26 patients receiving allogeneic transplantation from HLA-identical siblings with reduced-intensity conditioning (RIC). The stem cell source was bone marrow (BM) in eight patients and G-CSF-mobilized peripheral blood hematopoietic cells in 18 cases. A total of 32 patients engrafted very quickly and the chimerism evaluation (both on myeloid and on lymphoid subsets) showed that they were full donor by day 60. At the time of the study they were in complete hematological remission and displayed a full donor hematopoiesis. Two patients showed early disease progression while one did not engraft. Forty-eight out-marrow samples harvested from the 26 patients generated a marrow stromal layer adequate for the chimerism evaluation. Monocyte-macrophage contamination of marrow stromal layers was always reduced below 2% by repeated trypsinizations and treatment with the leucyl-leucine (leu-leu) methyl ester. The chimerism evaluation was performed by PCR analysis of STRs microsatellites and the amelogenin locus, by using capillary electrophoresis (CE) and by FISH analysis in case of the sex mismatch. In eight patients, a partial donor origin of stromal cells was shown (7–86% cells of donor). The source of hematopoietic cells was BM in three patients and mobilized peripheral blood in the other five.
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Introduction

In the bone marrow (BM) extravascular space, a dense network of cells constitutes the microenvironment that provides structural and functional support for the self-renewal and differentiation of hematopoietic progenitor cells.[1] Several *in vitro* and *in vivo* studies show the existence of mesenchymal stem cells residing within the BM microenvironment and having multilineage differentiation capacity.[2–4] Although in some animal models it has been shown that MSC can be transferred within the allogeneic transplantation setting, the transplantability of marrow stromal cells remains controversial in humans.[5–17] We investigated the engraftment ability of mesenchymal cells in patients receiving allogeneic transplantation from HLA-identical siblings with reduced-intensity (RIC) conditioning. The chimerism evaluation was performed by PCR analysis of STRs microsatellites and the amelogenin locus, by using capillary electrophoresis (CE), and by FISH analysis in case of a sex mismatch.

Correspondence: Dr A Poloni, Department of Hematology, University of Ancona, Via Tronto 10, 60020 Ancona, Italy.
E-mail: a.poloni@univpm.it
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Patients and methods

Patients

This study included 26 consenting patients who had received an allogeneic transplantation from an HLA-identical sibling: 10 male and 16 female subjects, whose age ranged from 23 to 70 years. Six patients were affected by non-Hodgkin's lymphoma (NHL), three by chronic lymphocytic leukemia (CLL), nine by acute leukemia, five by chronic myeloproliferative diseases (CMD) (three chronic phase and two blast crisis) and three by multiple myeloma. Five patients had received a prior autologous stem cell transplantation. Patient characteristics are shown in Table 1. Patients were conditioned with thiotepa-based, reduced-intensity regimens including fludarabine (60 mg/m^2), cyclofosphamide (60 mg/kg) and thiotepa (10 mg/kg); in two patients, melphalan (120 mg/m^2) was added; a patient who was affected by chronic myeloid leukemia (CML) was conditioned with thiotepa and fludarabine. The dosage of thiotepa was adjusted to 15 mg/kg for patients younger than 45 years and to 5 mg/kg for those older than 60 years. The stem cell source was BM in eight patients and G-CSF-mobilized peripheral blood hematopoietic cells in 18 cases. MNC reinfused ranged from 0.9×10^8/kg to 11.1×10^8/kg; CD34+ cells ranged from 1.8×10^6/kg to 11.6×10^8/kg. Graft-versus-host disease prophylaxis consisted of cyclosporine A and a short course methotrexate.

CFU-F

The assay for colony-forming unit-fibroblast (CFU-F) was performed according to Castro-Malaspina *et al.*[18] Briefly, 4×10^5 marrow mononuclear cells (MNCs) obtained by centrifugation on a Ficoll-Hypaque gradient (density, 1.077 g/ml), were plated in 60-mm Petri dishes in 4 ml of complete medium (MyeloCult, Stem Cell Technologies, Vancouver, Canada BC, supplemented with 10^{-6} mol/l freshly dissolved hydrocortisone). Fibroblast colony growth was evaluated after incubation (37°C, 5% CO_2) for 14 days in a humidified atmosphere. For scoring aggregates of at least 50 cells of CFU-F, the dishes were stained with crystal violet and examined under an inverted microscope.

Table 1 Characteristics of the patients

Case no.	*Age/sex*	*Diagnosis*	*Conditioning regimen*	*Cell source*	*MNC reinfused, $\times 10^8$/kg*	*CD34+ reinfused, $\times 10^6$/kg*	*CD3+ reinfused, $\times 10^8$/kg*	*Acute GVHD, grade*	*Days to PMN, 1000/μl*	*Days to PLT, 20 000/μl*	*Hematopoietic chimerism, % donor cells*	*DLI*
1	50/F	NHL	TT+FLU+CTX	BM	5.4	3.7	0.35	0	15	18	100	No
2	70/F	MM	TT+FLU+CTX	PB	4.5	6.4	5.9	0	12	12	100	Yes
3	59/F	NHL	TT+FLU+CTX	BM	1.3	2.6	0.45	0	17	21	100	Yes
4	38/F	AML	TT+FLU+CTX	PB	3.6	4.6	1.18	1	15	15	100	Yes
5	56/F	AML	TT+FLU+CTX	BM	0.9	1.8	0.05	0	16	16	100	No
6	46/M	ALL	TT+FLU+CTX	PB	9.8	6.0	5.0	3	17	19	100	No
7	23/M	AML	TT+FLU+CTX+MEL	PB	9.4	3.8	3.98	3	16	19	100	No
8	57/F	NHL	TT+FLU+CTX	BM	4.0	2.4	0.4	0	16	15	100	No
9	50/M	AML	TT+CTX	PB	4.6	6.9	2.6	0	14	14	100	No
10	50/F	IMF	TT+CTX	BM	2.3	3	0.9	1	16	13	85	Yes
11	27/M	CML	TT+FLU	BM	1.1	4.4	0.44	3	11	14	100	No
12	44/M	NHL	TT+FLU+CTX	PB	7.3	6.6	3.4	1	16	18	100	No
13	37/F	NHL	TT+FLU+CTX	PB	11.1	7.8	4.9	0	14	13	100	No
14	49/F	CML	TT+CTX	PB	0.9	3.9	5.6	2	14	20	100	Yes
15	45/F	AML	TT+CTX	PB	8	4.1	4.7	0	16	15	100	No
16	58/F	CML	TT+CTX	PB	6.1	11.6	2.5	2	14	14	75	Yes
17	47/F	CML	TT+CTX	PB	0.9	7.3	1.8	1	13	13	100	No
18	46/F	AML	TT+CTX	PB	5.5	9.7	1.58	1	18	13	100	No
19	50/M	MM	TT+CTX+FLU+MEL	PB	7.4	5.1	4.2	2	11	10	100	No
20	70/F	AML	TT+CTX+	PB	9.8	2.9	2.69	0	15	19	82	Yes
21	68/F	CLL	TT+FLU+CTX	BM	0.4	1.2	0.16	1	19	21	92	No
22	63/M	CLL	TT+FLU+CTX	PB	10.4	4.3	3. 2	2	13	14	100	No
23	51/M	NHL	TT+FLU+CTX	BM	0.95	3.6	0.45	4	16	12	100	No
24	60/M	MM	TT+FLU+MEL	PB	7.3	5.1	2.74	2	12	12	90	No
25	49/F	AML	TT+FLU+CTX+MEL	PB	10.7	6.27	3.9	0	15	17	100	No
26	54/M	CLL	TT+FLU+CTX	PB	3.2	1.46	0.17	3	20	30	90	No

NHL indicates non-Hodgkin's lymphoma; MM, multiple myeloma; AML, acute myelogenous leukemia; APL, acute promyelocytic leukemia; ALL, acute lymphoblastic leukemia; IMF, idiopathic myelofibrosis; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; TT, thiotepa; FLU, fludarabine; CTX, cyclophosphamide; MEL, melphalan; BM, bone marrow; PB, peripheral blood; GVHD, graft-versus-host disease; PMN, polymorphonucleates; PLT, platelet count; DLI, donor lymphocyte infusion.

Marrow stromal cell culture

To generate marrow stromal layers, 5×10^5/ml MNCs resuspended in complete medium were inoculated into 25 cm^2 tissue culture flasks and incubated in a humidified atmosphere (37°C, 5% CO_2). On a weekly basis, the cultures were fed by complete replacement of the medium. Confluent or subconfluent stromal layers were trypsinized 4–5 times to achieve a substantial depletion of stromal layers by monocyte-macrophages. Stromal layers were further depleted of monocyte-macrophages by incubation (22°C, 40 min) with the antilysosomal compound leucyl-leucine (leu-leu) methyl ester (5 mmol/l) (ICN Biomedicals Inc., Aurora, OH 44202, USA), which induces a selective depletion of monocyte-macrophages related to the characteristically rich endowment of lysosomes within these cells.[19,16] At the end of the incubation period, the cells were washed twice and resuspended in phosphate-buffered saline (PBS). An aliquot of stromal cells was analyzed by flow cytometry to confirm the purity of cell population.

Hematopoietic progenitor cell assays

Assays for myeloid and erytroid progenitors (CFU-GM and BFU-E) were performed in a single-layer methylcellulose, using MethoCult (Stem Cell Technologies, Vancouver, Canada BC) 4434. Briefly, 1×10^5 cells were plated in 35 mm tissue culture dishes (Falcon) in duplicate, in 1 ml aliquots of Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% FBS, 1% BSA, 2 mM L-glutamine, 10^4 M β-mercaptoethanol and SCF (50 ng/ml), IL3 (10 ng/ml), Epo (3 U/ml) and GM-CSF (10 ng/ml). Cultures were incubated at 37°C in a fully humidified atmosphere and 5% CO_2. BFU-E and CFU-GM were scored on day 14, using an inverted microscope. Megakaryocyte progenitor cells (CFU-MK) were grown in serum-free assay using 'MegaCult™-C' kit (StemCell Technologies Inc, Vancouver, Canada). Briefly, cells (1×10^5/ml) were plated in double-chamber slides, in four replicate cultures, in IMDM supplemented with 1.1 mg/ml collagen, 1% BSA, 0.01 mg/ml bovine pancreatic insulin, 0.2 mg/ml human tranferrin and 50 ng/ml Thrombopoietin, 10 ng/ml IL6, 10 ng/ml IL3. Cultures were incubated at 37°C in a fully humidified atmosphere and 5% CO_2 and on day 12, cultures were dehydrated and fixed and then stored at 4°C until immunocytochemical staining. MK colonies were considered clusters, if they contained more than three cells. Primitive progenitor (LTC-IC) assays were performed on the murine feeder M2 B10 (kindly provided by Dr C Eaves, Vancouver, British Columbia, Canada); irradiated (80 Gy) cells were seeded at the concentration of 7×10^4/2 cm^2 in a final volume of 1 ml of the growth medium MyeloCult. Cultures were fed every week by half-medium change. The progenitor cell content of each well was assessed by sacrificing wells after 5 weeks in culture, and plating cells in methylcellulose colony assays and the total CFC content was estimated in LTC-IC assay culture. LTC-IC values were derived by dividing the total CFC number by 4; this calculation was based on data obtained by limiting dilution experiments.

Flow cytometry

Mesenchymal cells (1×10^5/ml) were incubated for 20 min with 10 μl CD45-FITC (fluorescein isothiocyanate) and CD14-PE (phycoerythrin), CD90-FITC, CD34-PE, CD105-FITC, CD13-PE. Each fluorescence analysis included a negative isotype control immunoglobulin G1-FITC and IgG1-PE; antibodies and isotype controls were purchased (Becton Dickinson, San Jose, CA, USA). The cells were analyzed in a FAC Scalibur flow cytometry (Becton Dickinson) using the Cell Quest software.

FISH

FISH analysis to identify male and female cells using Vysis dual-color probe CEP X/Y (specific for human X and Y centromere regions: alpha satellite DXZ1 Spectrum Green and alpha satellite DYZ3 Spectrum Orange) was performed as recommended by the manufacturer (Vysis, Inc., Downers Grove, IL, USA).

In brief, cytocentrifuged slides were fixed in metanol/acetic acid solution for 10 min at room temperature and then air-dried. Slides were denatured in 70% Formamide/2 × SSC solution at 70°C for 5 min, then dehydrated in an ethanol series at room temperature and air-dried. The probe mixture was denatured at 70°C for 5 min, cooled at 37°C and applied to the slide. After overnight hybridization in a humified incubator at 37°C, the slides were washed in 0.4 × SSC/0.3% NP40 solution at 70°C for 2 min, then in 2 × SSC/0.1% NP40 solution at room temperature for 1 min and air-dried. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (Vector Laboratories).

Slides were examined using a Zeiss Axioplan 2 (Carl Zeiss AG, Göttingen, Germany) epifluorescent microscope with single and dual bandpass filter sets for visualization of Spectrum Green, Spectrum Orange and DAPI. For each patient sample, 500 nuclei with two signals were evaluated (two green signals as XX female; one green and one orange signal as XY male, Figure 1).

PCR analysis

DNA was extracted using the QIAamp Blood Kit (Qiagen, Valencia, CA, USA) with minor modifications and quantified by agarose mini-gel (1%). Samples were amplified in multiplex at the loci amelogenin, D3S1358, VWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317 and D7S820, using the AmpFlSTR Profiler Plus kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and the loci amelogenin, D3S1358, D16S539, TH01,

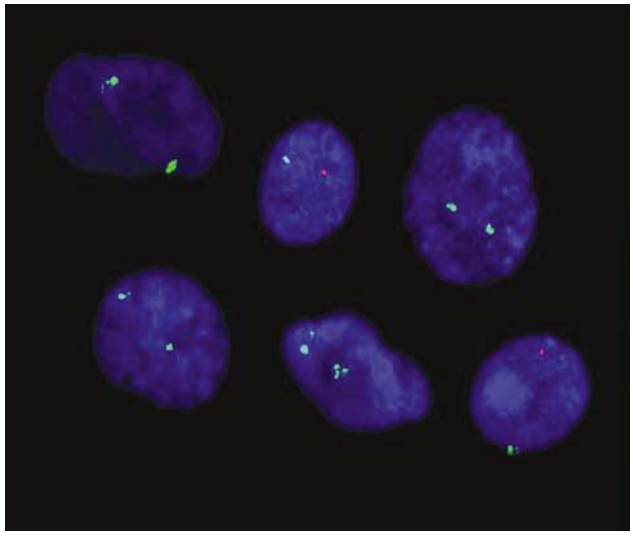

Figure 1 FISH analysis of mesenchymal cells. Two male donor cells (one green signal for X chromosome and one red signal for Y chromosome) and four female recipient cells (two green signals for X chromosome) are shown.

TPOX, CSF1PO and D7S820, using the AmpFlSTR Cofiler kit (PE/AB); SE33 locus was excluded from two commercial multiplex kits and was amplified in singlepex. PCR amplifications were carried out in a Perkin-Elmer 9700 thermal cycler; PCR products were electrophoresed on an ABI Prism 310 Genetic Analyzer (PE/AB) in Performance Optimized Polymer 4 (PE/AB). GeneScan Analysis 2.0.2 software was employed to establish the size of the amplified fragments. An arbitrary cutoff peak height threshold of 150 RFU (Relative Fluorescence Units) was used for scoring alleles; in some cases, this value was decreased to explore carefully the region occupied by all chimeric alleles. Allele designations were determined using Genotyper Software (PE/AB). Pretransplant recipient and donor DNA samples were typed by STRs and amelogenin locus. Informative loci for post-transplant samples were screened for quantification of the donor cell percentage in mixed chimerism (Figure 2). For quantitative assessment, the method previously described by Scharf *et al*.[20] was applied, 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 mixed chimerism.

Results

In total, 23 patients engrafted quickly and achieved a clinicohematological CR; two patients showed an early disease progression (CML in BC and AML); the last patient (AML) did not engraft (cases no. 10, 16 and 20). In these patients, the hematopoietic chimerism in the BM at day +30 was 85, 75 and 82%, respectively. The chimerism evaluation (both on myeloid and on lymphoid subsets) showed that all 23 were full donor by day +60. Acute severe GVH (grade 3–4) was observed in four patients; samples for chimerism evaluation of hemopoietic cells have been obtained during the CR phase in all patients. A total of 74 samples from the 26 consenting patients were plated under long-term culture conditions, in order to generate marrow stromal layers. In four patients, molecular analysis was prevented: in one AML patient (case no. 18), a persistently defective stromal cell growth was observed (at 1, 2, 3 months post-SCT). In the other three patients (case nos. 14, 16 and 17, all CML), a normal (confluent or subconfluent) stromal cell growth was observed, although adherent cells, after the first or the second passage, moved in the medium supernatant (seven samples). Marrow stromal layers reached confluence or subconfluence after 2–5 weeks of culture. Compared to normal donors, a reduction of the mean incidence of CFU-F progenitors was evident (45 ± 6 vs 26 ± 3; $P = 0.003$). A total of 48 samples from 22 patients generated a marrow stromal layer adequate for PCR analysis at fixed time points after transplantation. Six samples of six patients were also analyzed by FISH. Chimerism analysis could be detected 1–3 months after transplantation in 10 patients; the remaining patients were not studied soon after allografting; two patients were studied only after DLI. Among the 22 patients analysed, eight patients showed a partial donor origin of stromal cells (Table 2). Four patients (three NHL and

Table 2 Analysis of marrow stromal cell origin

Case no.	*Assay time (months post-SCT)*	*MSC*	*Chimerism*
		PCR analysis (% positive cells)	*FISH analysis (% positive cells)*
2	18	Donor 7%	ND
	30	Donor 20%	ND
	34	Host	ND
3	30	Donor 24%	ND
	34	Host	ND
5	9	Donor 86%	ND
	24	Host	ND
8	3	Donor 52%	ND
	18	Donor 12%	ND
12	1	Donor 27%	ND
	30	Donor 18%	Donor 15%
13	1	Donor 23%	ND
15	12	Donor 7%	ND
	18	Donor 11%	ND
	30	Donor 15%	Donor 16%
19	3	Donor 27%	ND
	6	Host	ND

Eight of 22 patients showed a partial donor origin of mesenchymal cells.

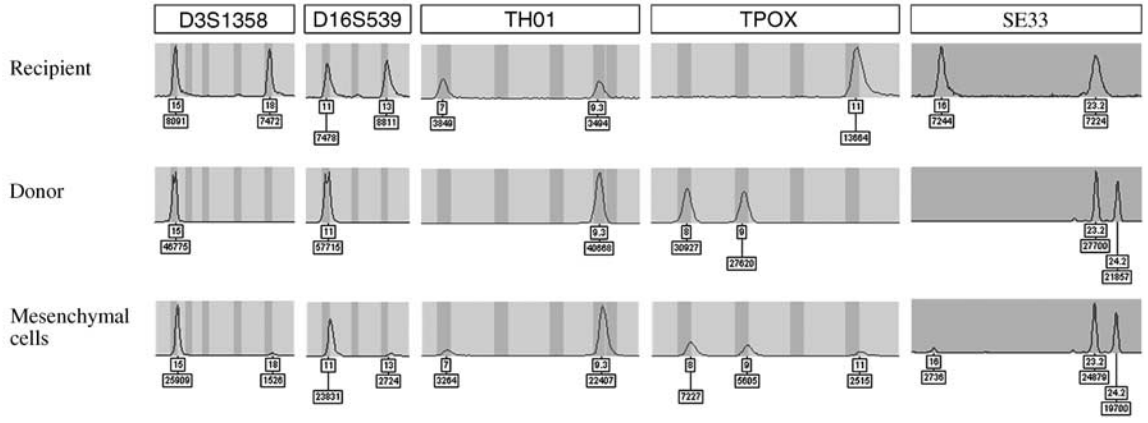

Figure 2 Representative results of mesenchymal cell analysis by PCR-based capillary electrophoresis analysis. Pretransplant recipient and donor DNA samples were typed by STRs and amelogenin locus. Informative loci (in this case, D3S1358, D16S539, TH01, TPOX, SE33) were screened for quantification of the donor cell percentage in mixed chimerism.

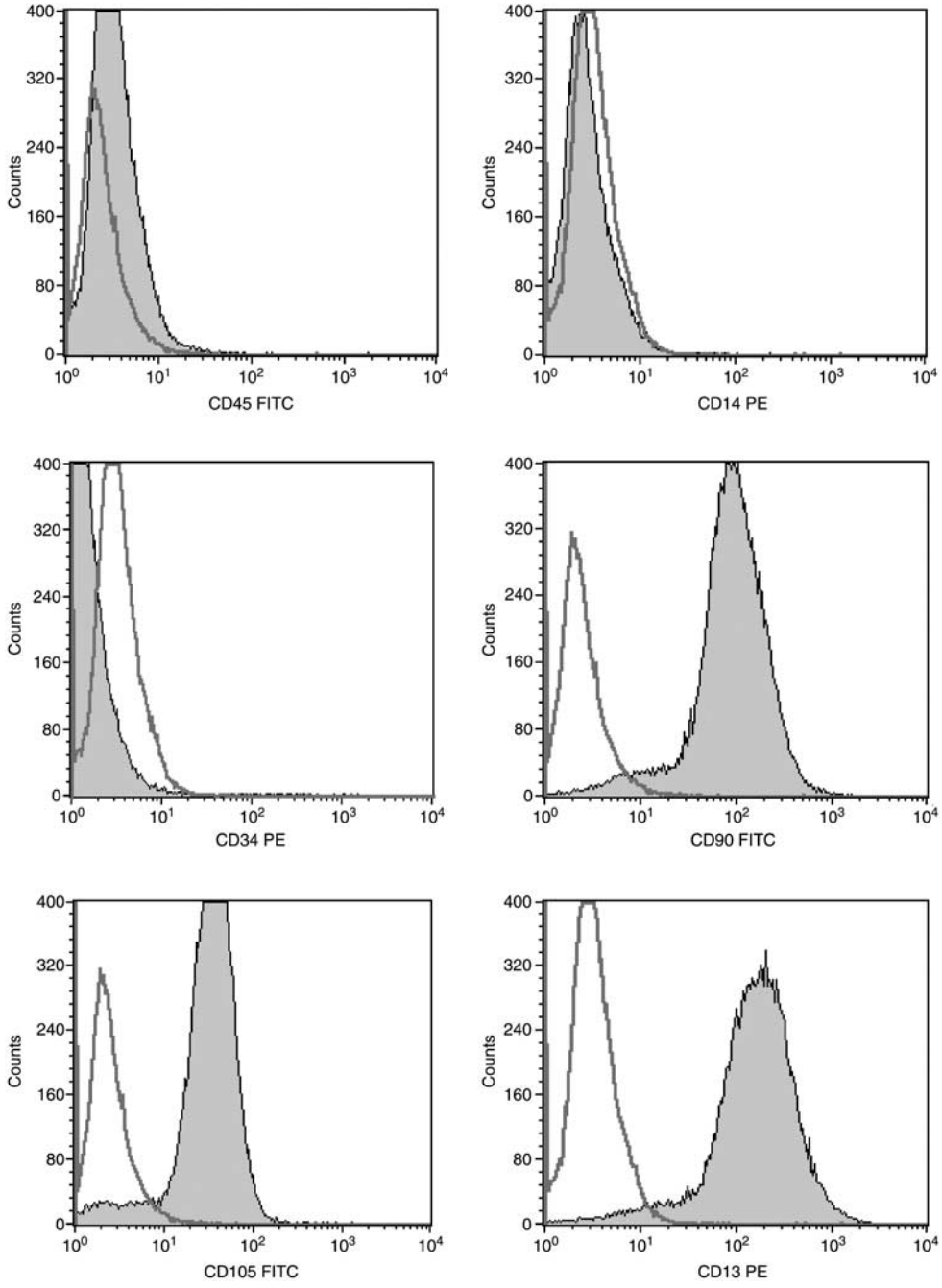

Figure 3 Representative immunophenotype of *in vitro* expanded mesenchymal cells. Open histogram indicates background signal; shaded histogram, positive reactivity with the indicated antibody.

one MM) were studied soon after transplantation (1–3 months). Two patients (case nos. 2 and 3) showed a mixed chimerism at 18- and 30-month evaluation. They received DLI for relapse. Patients no. 8, no. 12 and no. 15 were also evaluated 15, 29 and 6–18 months later, also showing a mixed chimerism. Flow cytometry analysis of stromal cells always demonstrated less than 2% of CD45+ cells, CD14+ cells and CD34+ cells (Figure 3). The source of hematopoietic cells was BM in three patients and mobilized peripheral blood in the other five. *In vitro* culture of hematopoietic progenitors revealed a significant reduction of the hematopoietic stem cell compartment and of the committed progenitors (Table 3). Compared to healthy controls, allografted patients showed a statistically significant reduction of the mean (mean$\pm$s.e.m.) number of LTC-IC progenitors (683 ± 198 vs 53 ± 8; $P<0.001$), CFU-GM (151 ± 12 vs 59 ± 5; $P<0.001$), BFU-E (139 ± 20 vs 81 ± 9; $P=0.01$) and CFU-MK (6 ± 2 vs 2.3 ± 0.7; $P=0.038$).

Discussion

In the present work, we demonstrate that following allogeneic transplantation from HLA-identical siblings with RIC, stromal progenitors reinfused with hemopoietic stem cells induced a

Table 3 *In vitro* growth of marrow hematopoietic progenitors

	CFU-GM	*BFU-E*	*CFU-MK*	*LTC-IC*
Cases	59±5 ($n=62$)	81±9 ($n=62$)	2.3±0.7 ($n=49$)	53±8 ($n=45$)
Controls	151±12 ($n=12$)	139±20 ($n=12$)	6±2 ($n=7$)	683±198 ($n=10$)
P-value	<0.001	0.01	0.038	<0.001

Values of CFU-GM, BFU-E and CFU-MK are given per 1×10^5 cells, values of LTC-IC are given per 2×10^6 cells.

mixed chimerism at the stromal cell level in eight of 22 evaluable patients (36%). In agreement with other authors,[15,16] we observed that CFU-F frequency was below the limits of the normal range in these patients receiving RIC allogeneic transplantation. In this respect, we could not find any correlation according to the kind of treatment before RIC: indeed, in the five patients who had received a previous autotransplant, a lower CFU-F frequency was already present, even many years after transplant; moreover, the interval between allografting and the time of study does not seem to be predictive for the CFU-F output (data not shown). In our patients, the detection of donor-derived stromal cells did not seem to be correlated with any peculiar clinical features or any graft-related parameters, such as the dose of reinfused cells, or the source of allografted stem cells or previous autotransplantation. It should be noted that all eight patients showing chimeric stroma did not develop acute severe (grade 2–4) GVHD; it is well known that acute GVHD is characterized by a cytokine-storm that might negatively influence the marrow homing and/or proliferation of stromal cells; therefore, the reduced intensity of conditioning regimen could decrease tissue damage, the release of inflammatory cytokines and the occurrence of severe acute GVHD. Some previous experiences have demonstrated that these stromal cells can engraft when infused in appropriate numbers also after myeloablative transplant, although conflicting results are still a cause of controversy.[5–17] It is not easy to interpret the controversial results so far reported by different groups; perhaps they could be explained by several methodological differences. However, we think that the large range of the doses of reinfused stromal cells could have played an important role. In addition, the heterogeneity of conditioning regimens, the kind of immunosuppression after transplantation, the graft manipulation and patient-related biological differences might account for such conflicting results. As five of our patients who showed a mixed chimerism have been transplanted with mobilized peripheral blood, this clearly suggests the presence of stromal cells or their precursors in peripheral blood. The mesenchymal cell content of the graft was analyzed by assaying the clonogenic growth of CFU-F progenitors; there was no evident correlation between the number of reinfused CFU-F progenitors and the post-transplantation detection of donor-derived stromal cells; CFU-Fs were never detected in PBPCs (data not shown). However, this finding may reflect intrinsic limitations of the CFU-F assay in detecting a population of primitive stromal cells with reconstitutive capacity. Some authors have shown that MSC can be obtained and expanded from mobilized peripheral blood progenitor cells,[21–24] but other studies[25] have failed to confirm this and the isolation of these cells is clearly difficult.

In summary, we have demonstrated the capacity of stromal progenitors to circulate among peripheral blood progenitor cells and to graft after reinfusion with HSCs, like MSCs derived from BM, in patients receiving an allogeneic transplantation with an RIC regimen.

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