ELSEVIER

Experimental Hematology

Experimental Hematology 2010;38:292–300

Effects of high-dose chemotherapy on bone marrow multipotent mesenchymal stromal cells isolated from lymphoma patients

Karen de Lima Prata[a,b], Maristela Delgado Orellana[a], Gil Cunha De Santis[a], Simone Kashima[a], Aparecida Maria Fontes[a], Rita de Cássia Viu Carrara[a], Patricia Vianna Bonini Palma[a], Luciano Neder[b], and Dimas Tadeu Covas[a,b]

[a]*National Institute of Science and Technology in Stem Cell and Cell Therapy, Center for Cell Therapy and Regional Blood Center, Ribeirão Preto, Brazil;* [b]*Faculty of Medicine, University of São Paulo, Ribeirão Preto, Brazil*

(Received 17 September 2009; revised 26 January 2010; accepted 29 January 2010)

Objective. **High-dose chemotherapy (HDCT) followed by autologous stem cell transplantation is a widely applied treatment for hematological and autoimmune diseases. Little is known about the effects of this therapy on multipotent mesenchymal stromal cells (MSCs). We aimed to characterize, morphologically and functionally, MSCs isolated from bone marrow aspirates of patients after HDCT.**

Materials and Methods. **We studied 12 consecutive lymphoma patients submitted to BEAM conditioning regimen followed by autologous stem cell transplantation 28 to 1836 days before the sample collection. Thirteen normal donors were used as control. MSCs were isolated by adherence to plastic and expanded ex vivo by culture in flasks containing α−minimum essential medium plus 15% fetal bovine serum.**

Results. **The cell population isolated showed a typical MSC morphology, immunophenotype, and differentiation capacity into adipogenic, osteogenic, and chondrogenic lineages. The MSCs obtained from patients with Hodgkin's disease and non-Hodgkin's lymphoma showed decreased fibroblastoid colony-forming unit count ($p = 0.023$) and increased doubling time ($p = 0.031$) related to the control group. The total cell expansion of MSCs from normal subjects was marginally superior to the patient group ($p = 0.064$). There were no differences in gene expression profile, MSCs plasticity, or hematopoiesis support capability between control and patient group.**

Conclusions. **Results suggest that HDCT applied to lymphoma patients damaged MSCs, which was demonstrated by their reduced clonogenic potential, doubling time, and cell expansion rates when compared to controls.** © **2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

The multipotent mesenchymal stromal cells [1–3] (MSCs) represent an interesting choice for cell therapy because they can differentiate into multiple mesenchymal lineages [4,5], support hematopoiesis [4,5], and modulate the immune system [6]. In addition, they can be easily isolated, manipulated, and expanded ex vivo. Because of these properties, the MSCs have been used therapeutically to facilitate hematopoietic stem cell grafting [7–10] and to treat [11] or prevent graft-vs-host disease [12], among other conditions [13–17].

Despite their therapeutic potential, concern remains about the efficacy of using MSCs isolated from patients previously submitted to high-dose chemotherapy (HDCT) for clinical applications. Some studies have suggested that chemotherapy and radiotherapy cause quantitative [18,19] and qualitative [20–22] damage to the stromal microenvironment. However, most of them refer to bone marrow stroma and not specifically to MSCs [18–22]. Only recently, studies were published assessing in vitro behavior of MSCs isolated from bone marrow (BM) of patients receiving radio- or chemotherapy in vivo [23–26]. These studies, however, have reported contradictory results.

Offprint requests to: Dimas Tadeu Covas, M.D., Ph.D., Centro Regional de Hemoterapia, FMRP, USP, Rua Tenente Catão Roxo, 2501, 14051-140 Ribeirão Preto, SP, Brazil; E-mail: dimas@fmrp.usp.br

Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.exphem.2010.01.006.

0301-472X/10 $–see front matter.
doi: 10.1016/j.exphem.2010.01.006

Mueller et al. [23] analyzed MSCs isolated from patients with various hematologic diseases who were previously submitted to standard chemotherapy and, in some cases, to HDCT. The authors found heterogeneous results but concluded that BM-MSCs obtained from chemotherapy-exposed patients could be considered for clinical applications. In contrast, Cao et al. [26] reported a reduction in both fibroblastoid colony-forming unit (CFU-F) frequency and interleukin-6, stem cell factor, and Fms-related tyrosine kinase expression in the BM-MSCs isolated from patients with colorectal carcinoma previously submitted to standard chemotherapy. The conclusion of their study was that MSCs can be damaged by chemotherapy. In addition, Isaikina et al. [24,25], evaluating BM-MSCs isolated from children with oncohematological diseases who were previously submitted to radiotherapy and chemotherapy, observed a reduced frequency of CFU-F [24], although the isolated cells presented a replication potential [24] and hematopoiesis support similar to those of normal controls [25].

To clarify these contradictory results, in the present study we evaluated CFU-F counts, expansion, plasticity, and hematopoiesis supportive potential of MSCs isolated from lymphoma patients submitted to HDCT followed by autologous stem cell transplantation (ASCT).

Materials and methods

Characteristics of the patients and normal donors studied

This study was conducted on 12 lymphoma patients (8 males) aged 22 to 49 years (median: 37.5 years), 6 with Hodgkin's disease (HD) and 6 with non-Hodgkin's lymphoma (NHL), without BM infiltration by the disease at any time during its course. The morphologic analysis of the BM aspirate specimen showed cellularity and a myeloid-to-erythroid ratio within normal limits in all samples, except one, for which evaluation was impaired by the absence of the bone spicules. Ten of the 12 samples studied presented mild dyserythropoiesis. All patients had received two (n = 7) or three (n = 5) previous standard chemotherapy schemes (median: 11 cycles; range, 9–13), followed by a second-line salvage chemotherapy (dexamethasone, high-dose aracytin, and platinum in 9 patients). After that, they were submitted to the BEAM protocol (BCNU, etoposide, cytarabine, melfalan) as conditioning regimen for the ASCT. The main clinical characteristics of these patients are summarized in Table 1.

Samples of bone marrow aspirates were obtained from 13 normal donors and used as the control group. They were recruited from an allogeneic BM transplantation service. Patients median age was 31 years (range, 22–52 years).

The study was approved by the local Ethics Committee (University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo) and all subjects gave written informed consent to participate in the study.

MSC isolation from culture

MSCs were cultured as described previously [27,28]. Briefly, 4 to 5 mL BM were aspirated from posterior iliac crest. BM mononuclear cells (MNC) were isolated by centrifugation at 900*g* for 30 minutes in a density gradient of 1077 g/L (Ficoll-Paque Plus; Amersham Biosciences, Uppsala, Sweden) and cultured at an initial concentration of 10 to 50 $\times$ 10^4 cells/cm^2 in α–minimum essential medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 15% heat-inactivated standard fetal bovine serum (HyClone, Logan, UT, USA), L-glutamine (2 mM; Gibco), and 1% penicillin-streptomycin (Gibco). After 3 to 7 days, the culture medium was changed and nonadherent cells were removed. Cell culture was maintained with a weekly change of 50% of the medium until cell confluence. At this moment, trypsinization was performed (0.05% trypsin and 0.53 mM ethylenediamine tetraacetic acid; Gibco), followed by replating in plastic flasks at a concentration of 2 to 2.7 $\times$ 10^3 cells/cm^2. Cell cultures were incubated in a humidified atmosphere of 5% CO_2, at 37°C. Assays were performed on cells between the second and fifth passage.

Quantification of CFU-F

The BM-MNCs were plated onto eight (3.5 cm^2) dishes with grid at concentrations of 3 or 14 $\times$ 10^4 cells/cm^2. After 7 days, the medium was changed and nonadherent cells were removed. Between the 12th and 14th day of culture, colonies with >50 fibroblastoid cells were counted as CFU-F [29] and quantified by dividing the number of colonies by the total number of BM-MNCs plated.

Growth kinetics

Population doubling was calculated at each passage by the formula logN/log2 as described by Stenderup et al. [30], where N is the number of cells counted at the time of trypsinization divided by the initial number of cells plated. The cumulative population doubling was obtained and considered to be the total cell expansion. The doubling time was calculated by dividing the number of hours between the first and second passage by the cell expansion during the same period.

Flow cytometry

Cells harvested were labeled with CD105 phycoerythrin (PE) (Serothec, Oxford, UK), CD73-PE, CD51/61–fluorescein isothiocyanate (FITC), HLA-ABC-PE (BD PharMingen, San Jose, CA, USA), CD45-FITC, CD14-PE, CD34-PE, CD54-PE, CD44-FITC, CD49e-PE, CD29-PE, HLA-Dr–FITC, CD90-PE, and CD13-PE (Becton Dickinson, San Jose, CA, USA), KDR-FITC (Sigma-Aldrich, St Louis, MO, USA), Stro-1 (kindly provided by B. Torok-Storb, Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and analyzed on a FACSort cytometer (Becton Dickinson) as described previously [31].

MSC differentiation into adipocytes, osteocytes, and chondrocytes

Adipogenic, osteogenic, and chondrogenic differentiation was evaluated as described previously [32]. Basically, after incubation with the specific differentiation medium, cells were fixed and stained by the von Kossa method (for calcium deposition), with Sudan II and Scarlet stains (for fat accumulation), or immunostained with anti-type II collagen. Cells were analyzed with a model Axioskop 2.0 Zeiss microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Axiocam camera (Zeiss). The Axiovision program (Zeiss) was used to quantify the percentage of differentiation in 20 different hotspot areas from stained slides with 400× magnification for adipocytes and 100× for osteocytes.

Table 1. Some characteristics of patients submitted to high-dose chemotherapy

	Age	Gender	Disease	D + BMT	CT schemes before BMT	Cycles	Previous radiotherapy
LP3	22	M	LGCBM	28	CHOP	8	Mediastinum
					DHAP	3	
LP4	35	F	NSHD	662	ABVD	6	Cervical
					ESHAP	3	
					MINE	2	
LP5	29	F	MLBCL	1551	CHOP	8	Mediastinum
					Cy 2 g/m^2	1	
LP6	34	F	NSHD	1836	ABVD	6	Cervical and Mediastinum
					DHAP	2	
					MIFAP[a]	2	
LP7	42	M	NSHD	104	ABVD	8	Mediastinum
					DHAP	3	
LP8	32	M	LPHD	1620	MOPP	8	Left lower limb
					DHAP	2	
					Cy 2g/m^2	1	
LP9	40	M	DLBCL	124	CHOP	8	Abdominal and pelvic
					DHAP	3	
					MINE	1	
LP10	48	M	NSHD	1822	C-MOPPABV	8	Cervical
					DHAP	3	
LP11	49	F	DLBCL	1305	CHOP-BLEO	8	No
					DHAP	3	
LP12	42	M	DLBCL	168	CHOP	8	No
					DHAP	3	
					MINE	2	
LP13	28	M	MLBCL	159	CHOP	8	Mediastinum
					Cy 2 g/m^2	1	
LP14	47	M	MCHD	770	ABVD	6	Supraclavicular and axillary
					DHAP	3	

ABVD = adriamycin, bleomycin, vinblastine and dacarbazine; BMT = bone marrow transplantation; CHOP = cyclophosphamide, hydroxydaunomycin, oncovin and prednisone; CHOP-Bleo = CHOP + bleomycin scheme; C-MOPPABV = cyclophosphamide, oncovin, procarbazine, prednisone, adriamycin, bleomycin and vinblastine; CT = chemotherapy; Cy = cyclophosphamide; D + BMT = number of days after bone marrow transplantation; DHAP = dexamethasone, high-dose aracytin and platinum; DLBCL = diffuse large B-cell lymphoma; ESHAP = etoposide, solumedrol, high-dose aracytin and platinum; F = female; intensified MINE = mesna, ifosfamide, novantrone and etoposide; LP = lymphoma patient; LPHD = nodular lymphocyte-predominant Hodgkin's disease; M = male; MCHD = mixed cellularity Hodgkin's disease; MIFAP = mitoxantrone, fludarabin, aracytin and platinum; MLBCL = primary mediastinal large B-cell lymphoma; MOPP = mechlorethamine, oncovin, procarbazine, and prednisone; NSHD = nodular sclerosis Hodgkin's disease.
[a]After transplantation due to a relapse of the disease, but before the collection of cells for the study.

Results for each patient were given in mean and these values were compared with the values obtained from the control group.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from undifferentiated MSCs from six patients and four controls using RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) according to manufacturer's instructions. Two micrograms total RNA were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene expression kinetics was also evaluated for MSCs cultures under differentiation into adipocytes and osteocytes on different days (7, 14, 21, 28, and 35) isolated from patients (n = 5) and from controls (n = 3). RNA extraction was performed by TRIzol method (Invitrogen Corp., Carlsbad, CA, USA). RNA (2 μg) was reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), according to manufacturer's instructions.

Real-time quantitative polymerase chain reaction amplification was performed for CD73, CD140b, CD146, stem cell factor (KIT ligand), leukemia inhibitory factor, Fms-related tyrosine kinase 3 ligand, peroxisome proliferative activated receptor−γ, and osteopontin. The probes and primers sequences for the genes evaluated are listed in Supplementary Table E1 (online only, available at www.exphem.org). Measurements were performed on an ABI Prism 7500 sequence detection system (Applied Biosystems) using SYBR green chemistry and TaqMan assays. Cycles were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Results were normalized using an endogenous gene glyceraldehyde-3-phosphate dehydrogenase. Single-product amplification was confirmed by postmelting curve. Duplicate samples were measured and averaged. Results are reported as expression relative unit.

Hematopoiesis support

In vitro hematopoiesis support was evaluated in duplicate by MSC coculture with $CD34^+$ cells obtained from umbilical cord blood by positive selection using the $CD34^+$ Progenitor Cell Isolation Kit (MACS; Milteny Biotec, Bergisch Gladbach, Germany) and LS Separation Column (MACS) according to manufacturer's instructions.

Six aliquots of 4×10^4 MSCs were obtained from each subject (four controls and six patients), cultured on 24-well plates until 80% to 90% confluence, irradiated, and cocultured with $CD34^+$ cells (median: 6.97×10^3; range, $6.59-9.23 \times 10^3$ cells; 66–76% purity). The $CD34^+$ cells were cultured in parallel (without the MSCs as feeder layer). On days 0, 7, and 28 of coculture, $CD34^+$ cells were collected from the wells, counted, seeded onto 3.5 cm^2 dishes with grid in duplicate, and cultured for 14 days in semisolid medium (Methocult without cytokines; Stem Cell Technologies Inc., Vancouver, Canada) previously enriched with stem cell factor (50 ng/mL; PeproTech, Rocky, Hill, NJ, USA), interleukin-3 (10 ng/mL; PeproTech), granulocyte-macrophage colony-stimulating factor (10 ng/mL; PeproTech), granulocyte colony-stimulating factor (10 ng/mL; Leucin Dong-A Pharmaceutical, Taegue, Korea), and erythropoietin (4 U/mL; Eprex VETTER Pharma Fertigung, Ravensburg, Germany).

The number of CFUs was calculated by dividing the number of colonies obtained by the number of cells plated and multiplying by the number of cells collected. The clonogenic potential was calculated by dividing the number of CFUs obtained after coculture by the number of CFUs on day 0. Expansion of colony-forming unit granulocyte-macrophage, burst-forming unit erythroid, and $CD34^+$ cells was calculated by the logN/log2 formula, where N is the value referring to the clonogenic potential or the value corresponding to the division of the number of viable $CD34^+$ cells after coculture by the number of cells plated.

Statistical analysis

Data obtained, reported as median and range, were used to compare control vs patient groups, HD vs NHL patients groups, and controls vs HD vs NHL patients groups. Mann-Whitney and the Kruskal-Wallis test with Dunn's multiple comparisons test were applied using the GraphPad InStat software, version 3.0 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com), with the level of significance set at $p < 0.05$.

Results

Morphological characteristics of cultured MSCs

MSCs were isolated from all samples and cultured until they lost the capacity to expand. They exhibited a typical MSC morphology during the log growth phase of the culture period and showed a significant increase in cytoplasm size and loss of the fibroblastoid aspect at the final (plateau) phase.

CFU-F count

Counts of CFU-F (per 10^6 BM-MNC) for the normal group (n = 11) and all patients taken together (n = 12) were 13.6 (range, 1.3–92.5) and 9.4 (range, 0.0–37.5), respectively ($p = 0.079$). However, the normal group presented higher

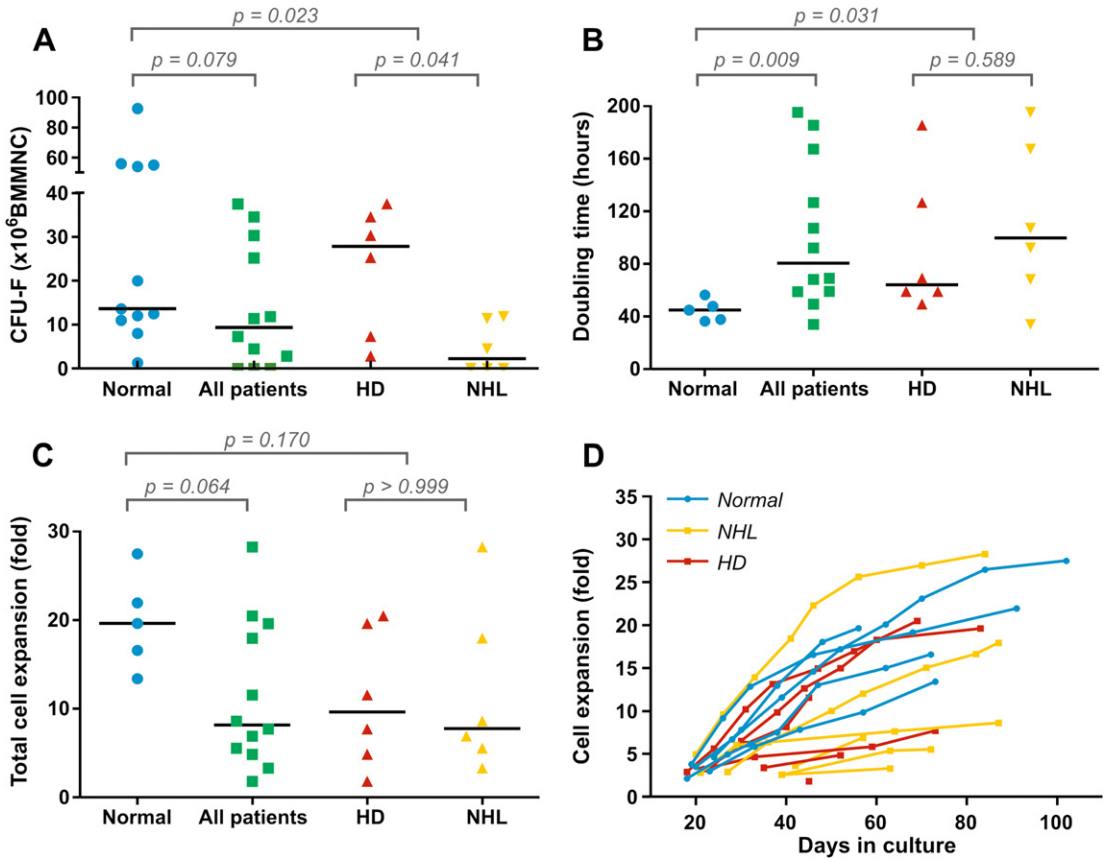


Figure 1. Quantification of fibroblastoid colony-forming units (CFU-F) and kinetics of mesenchymal stromal cell (MSC) expansion. (**A**) CFU-F counts ($\times 10^6$ bone marrow mononuclear cells). (**B**) Doubling time (hours). (**C**) Total cell expansion (fold). (**D**) Cell expansion kinetics curve. The short horizontal line corresponds to the median. HD = Hodgkin's disease; NHL = non-Hodgkin's lymphoma.

CFU-F counts than HD (n = 6) and NHL (n = 6) groups considered separately (p = 0.023). We also observed that patients with HD showed higher CFU-F counts than NHL patients (p = 0.041) (Fig. 1A, Supplementary Table E2, online only, available at www.exphem.org).

Growth kinetics

Doubling time for MSCs from the control (n = 5) and patient (n = 12) groups were 44.86 hours (range, 36.36–56.38 hours) and 80.66 hours (range, 34.08–195.35 hours) (p = 0.009), respectively (Fig. 1B). We also observed that the control group had faster doubling time than HD (n = 6) and NHL (n = 6) groups considered separately (p = 0.031). The three phases of cell expansion kinetics were clearly observed during culture. The plateau phase started in 7 of 12 (58.34%) of the patients by the fourth passage, whereas in all normal donors, it started after the sixth passage (data not shown).

Total cell expansion detected for controls (n = 5) and patients (n = 12) was 19.63× (range, 13.42–27.48×) and 8.17× (range, 1.81–28.27×), respectively (p = 0.064) (Figs. 1C and D). Eight of 12 patients (66.7%) presented values $<25^{th}$ percentile for controls (16.59×).

Immunophenotype of cultured MSCs

MSCs derived from patients and controls expressed similarly the markers CD73, CD105, CD90, CD13, CD29, CD49e, CD54, CD44, HLA-ABC, and Stro-1, and did not express HLA-DR, hematopoietic (CD34, CD14, CD45), or endothelial (CD51/61, KDR) markers (Table 2).

Quantitative gene expression profile of MSCs

Expression profile of genes related to MSC characterization and function were similar in control and patient groups. However, a large dispersion of the results was observed, mainly in the patient group (Fig. 2 and Supplementary Table E3, online only, available at www.exphem.org).

MSC differentiation into adipocytes, osteocytes, and chondrocytes

All samples tested were able to differentiate into adipogenic and osteogenic lineages. For the adipogenic lineage, the percentages of differentiation for controls (n = 5) and patients (n = 8) were 30.79% (range, 16.97–93.98) and 23.17% (range, 4.69–45.15), respectively (p = 0.7242; Figs. 3A and B). The specimens studied were obtained at different time points after the initiation of the stimulus: 24 days (range, 16–33 days) for controls and 25 days (range, 17–28 days) days for patients. For the osteogenic lineage, the percentage of differentiation for controls (n = 5) and patients (n = 6) were 4.23% (range, 1.47–26.06%) and 8.5% (range, 1.08–25.85%), respectively ($p > 0.999$; Fig. 3C and D). The specimens studied were obtained at different time points after the initiation of the stimulus: 55 days (range, 18–69 days) for controls and 39 days (range, 32–69 days) for patients. MSCs from patients cultured as internal control of this experiment (α–minimum essential medium 7.5% fetal bovine serum) for >40 days, differentiated spontaneously into osteocytes (data not shown). Spontaneous differentiation into osteocytes was not evaluated in normal donors.

Table 2. Immunophenotyping of mesenchymal stromal cells from controls and patients

	Controls (n = 5), median %	Patients (n = 12), median %
CD45	0.06	0.10
CD14	0.00	0.21
CD34	0.76	0.40
KDR	1.80	0.59
HLA-DR	0.79	0.80
CD51/61	1.74	1.58
STRO-1	14.6	15.0
CD73	90.9[a]	82.2
CD105	80.6[b]	51.6[c]
CD90	98.8	98.2
CD13	97.5	95.2
CD29	91.0	87.1
CD49e	87.4	83.6
HLA-ABC	71.9	73.6
CD44	54.5	63.4
CD54	32.9	35.8

[a] n = 4.
[b] n = 3.
[c] n = 5.

Differentiation into chondrocytes was demonstrated by production of collagen II, as revealed by immunohistochemistry with a specific antibody (Fig. 3E–F) and by the rounded aspect of the cells associated with the presence of regions similar to chondrocyte gaps.

MSCs samples from three controls and five patients were assessed by real-time polymerase chain reaction for expression of peroxisome proliferative activated receptor–γ and osteopontin genes, respectively. Data show high variability in both normal and patient specimens (Supplementary Fig. E1 and Supplementary Table E4, online only, available at www.exphem.org).

Hematopoiesis support

In the absence of MSCs as feeder layer, expansion of $CD34^+$ cells was for controls and patients 1.32× (range, 0.97–1.67×) and 1.95× (range, 1.05–2.00×) during culture for 7 and 28 days, respectively. The expansion of $CD34^+$ cells during coculture with MSCs from control and patient groups were 3.24× (range, 2.15–4.91×) and 3.13× (range, 2.42–4.29×) after 7 days (p = 0.914) and 4.12× (range, 3.84–4.68×) and 4.46× (range, 3.42–5.09×) after 28 days (p = 0.476), respectively. Median expansion values were similar for control and patient group, but were approximately 2× higher during coculture for 7 or 28 days compared to cell culture in the absence of MSCs as feeder layer (Fig. 4A, Supplementary Table E5, online only, available at www.exphem.org).

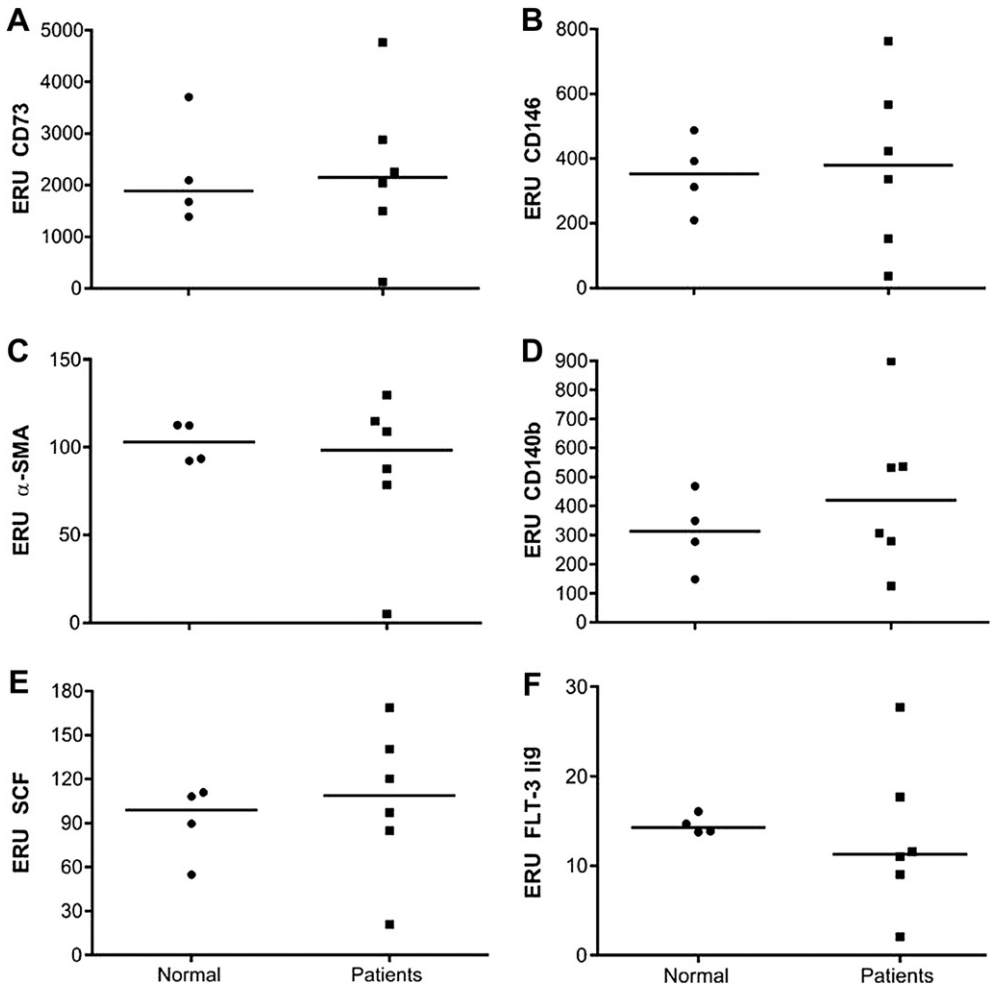


Figure 2. Quantification of selected transcripts by real-time polymerase chain reaction. (**A**−**F**) Transcript levels of CD73, CD146, CD 140b, stem cell factor (SCF), leukemia inhibitory factor (LIF), and Fms-related tyrosine kinase 3 (FLT-3) were evaluated in mesenchymal stromal cells (MSCs) isolated from controls and patients. Data are reported as expression relative units (ERU). The short horizontal line corresponds to the median.

There was no difference between controls and patients regarding clonogenic potential or CFU expansion after coculture of $CD34^+$ cells with MSCs (Supplementary Table E5, online only, available at www.exphem.org). The clonogenic potential of $CD34^+$ for CFU granulocyte-macrophage after coculture with MSCs from control and patient groups were 12.62× (range, 6.10−18.58×) and 10.58× (range, 8.27−16.37×) for 7 days ($p = 0.914$) and 0.92× (0.24 and 1.75×) and 0.49× (0.0 and 2.24×) for 28 days ($p = 0.762$), respectively. In the absence of MSCs as feeder layer, the clonogenic potential was 2.90× (range, 2.28−3.51×) and 0.11× (range, 0.0−0.22×) for 7 and 28 days, respectively (Fig. 4B). For burst-forming unit erythroid, the clonogenic potential of $CD34^+$ cells after coculture with MSCs was 2.94× (range, 1.49−9.67×) and 2.47× (range, 0.88−4.87×) for 7 days ($p = 0.610$), and 0.08× (range, 0.0−3.98×) and 0.0× (range, 0.0−0.0×) for 28 days for controls and patients, respectively, and in the absence of MSCs as feeder layer the potential was 0.38× (range, 0.32−0.44×) and 0.0× (range, 0.0−0.03×) for 7 and 28 days, respectively (Fig. 4C).

Discussion

In this study, we have shown that the CFU-F counts detected for normal subjects were within the limits reported previously in the literature [33–37]. We could not demonstrate a difference in CFU-F counts between the control and patient groups ($p = 0.079$), perhaps because of the small number of subjects included. However, when the patient group was divided into HD and NHL subgroups, we observed a decreased count of CFU-F for the NHL patients ($p < 0.05$), but not for HD patients ($p > 0.05$). We believe that this finding could be explained by the fact that NHL patients were more exposed to alkylating agent−containing chemotherapy. This reduction in the counts of CFU-F is consistent with studies that

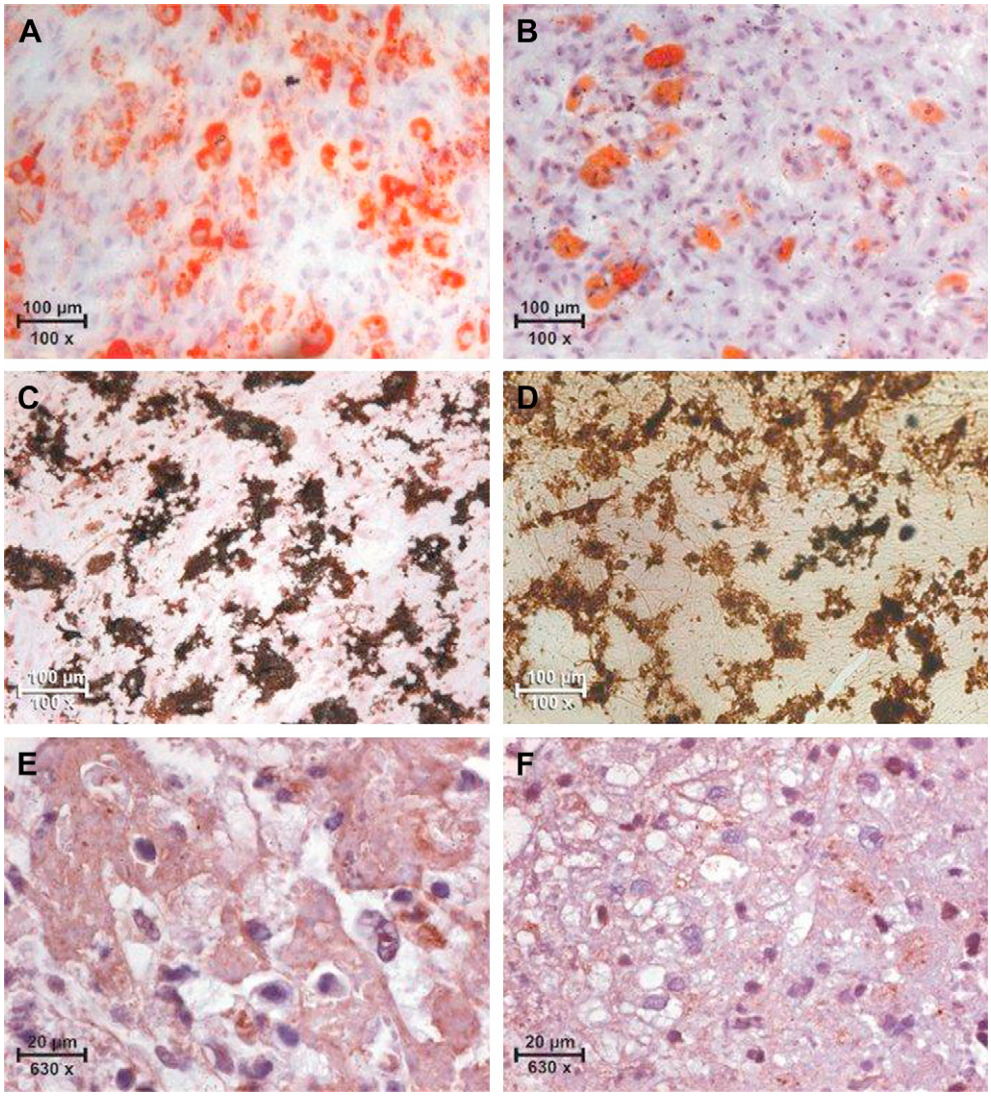


Figure 3. Morphological characterization of mesenchymal stromal cells (MSCs) induced to differentiation into adipocytes, osteocytes, and chondrocytes: (**A**,**B**) Differentiation into adipocytes (Sudan II scarlet and Harris hematoxylin staining)**:** (**A**) normal (ND08) on the 11th day of culture; (**B**) patient (LP08) on the 21st day of culture. (**C,D**) Differentiation into osteocytes: Von Kossa and Harris hematoxylin staining; (**C**) normal (ND11) on the 18th day of culture; (**D**) patient (LP05) on the 39th day of culture. (**E,F**) Differentiation into chondrocytes**:** imunohistochemistry for collagen II showing cells deeply stained brown by the specific antibody (**E**) normal (ND13) on the 17th day of culture; (**F**) patient (LP10) on the 14th day of culture.

demonstrated quantitative damage to the stromal [18,19] and mesenchymal cells [24] of patients previously submitted to chemotherapy alone or associated with radiotherapy.

We also observed that the patient group presented a longer doubling time than the control group ($p = 0.009$), which reinforces the hypothesis that chemotherapy can damage MSCs. It is interesting to emphasize that doubling time was evaluated between the first and second passages, when cells are in exponential expansion, a phase in which it is reasonable to expect subtle differences to become clearer. Results obtained for MSCs isolated from patients presented a wide dispersion. In contrast, the pattern of the doubling time of MSC from normal subjects was homogeneous, but superior to those reported previously [38,39]. The total cell expansion of MSCs from normal subjects was marginally superior to the patient group ($p = 0.064$). Despite the fact that we could not demonstrate a statistical difference between the results of the two groups, we believe that it is reasonable to assume that this alteration has a biological significance.

No statistically significant differences between MSC from patient and control specimens were observed in the patterns of gene expression, differentiation potential, or capacity to support in vitro hematopoiesis. On the other hand, it is important to emphasize that we have not obtained enough cells for induction to differentiation and to evaluate the hematopoiesis support capability or RNA for analysis of the gene expression profile from the three HDCT patients who presented the lowest cell expansion. New samples

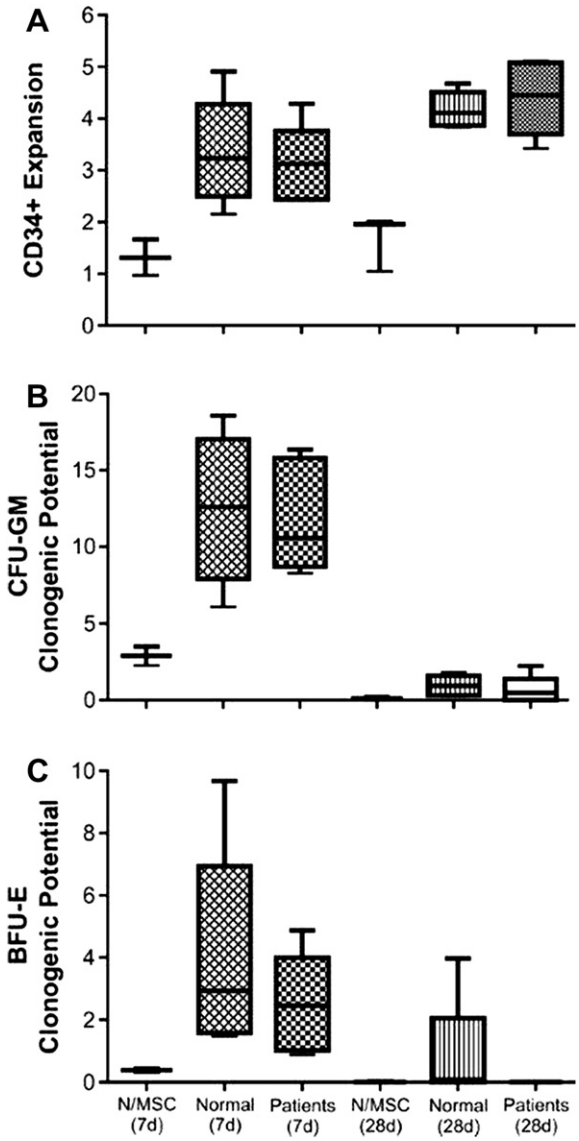


Figure 4. Hematopoiesis support. The ability of mesenchymal stromal cells (MSCs) to support hematopoiesis was evaluated in vitro by coculture of these cells isolated from four normal donors and from six patients with umbilical cord blood $CD34^+$ cells. (**A**) $CD34^+$ cell expansion quantified by flow cytometry. (**B**,**C**) Clonogenic potential of $CD34^+$ cells after coculture with MSCs for 7 and 28 days. (**B**) Colony-forming unit granulocyte-macrophage (CFU-GM); (**C**) burst-forming unit erythroid (BFU-E). N/MSC = No MSC.

were collected from these patients and again we could not obtain enough cells to conduct these experiments. We believe that the scarcity of this material could have limited the statistical power of these experiments, which were carried out with cells that presented a growth kinetics behavior similar to controls. Another explanation for the lack of difference between the groups concerning the MSCs gene expression profile, plasticity, and supportive role on hematopoiesis could be related to essential aspects from MSC biological nature, which are less likely to be influenced by external factors such as chemotherapy. Particularly in the hematopoiesis support capability, its effect was indirectly evaluated through another cell lineage, in a way that subtler alterations could not be detected by this assay. These facts could possibly explain the findings mentioned here, which means that the chemotherapy treatment has a more evident influence on proliferative aspects from the MSCs, but less or none, on their gene expression, plasticity, or hematopoiesis support capability. Subtler functional alterations could perhaps be revealed by assays with a larger number of subjects.

Expression of osteopontin by undifferentiated MSCs, as well as their spontaneous differentiation into osteocytes, supports the hypothesis that the osteogenic pathway is the preferential one for MSCs isolated from bone marrow [40,41].

There was no obvious influence of age or gender on CFU-F counts, growth kinetics, multipotential differentiation, or hematopoietic stem cell supportive capacity. The different time points after ASCT were not considered for analysis because five of six HD patients were submitted to ASCT >660 days before collection of the BM sample for this study, which could influence the results and conclusions.

Our results suggest that MSCs of lymphoma patients submitted to HDCT present quantitative and proliferative differences compared to normal subjects regarding CFU-F counts and kinetics of cell expansion probably secondary to the use of HDCT.

Acknowledgments

The authors would like to thank Abel Dorigan Neto, Ana Flávia Gembre, Ana Maria A. Dorigan, Ane Rose L. da Silva, Fabiana R. de Morais, Fernanda U.F. Melo, Karina R. Solano, Luciene Medeiros, Luiz Alberto M. de Andrade, and Valéria M. Motta for the laboratory assistance, Antônio D. Campos, M.D., Ph.D. and his assistant Sidney Porcincula for helping with the statistical analysis, Prof. Dr. Ademilson E. S. Egea and his assistant Jairo de Souza for helping with cell irradiation and Alessandra Almeida for the language review. The authors are also grateful to the patients and to Luciana C.O. de Oliveira, M.D., M.S. responsible for the ambulatory where patients are assisted. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (São Paulo, SP, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Brasília, DF, Brazil), and Financiadora de Estudos e Projetos (FINEP) (Rio de Janeiro, RJ, Brazil).

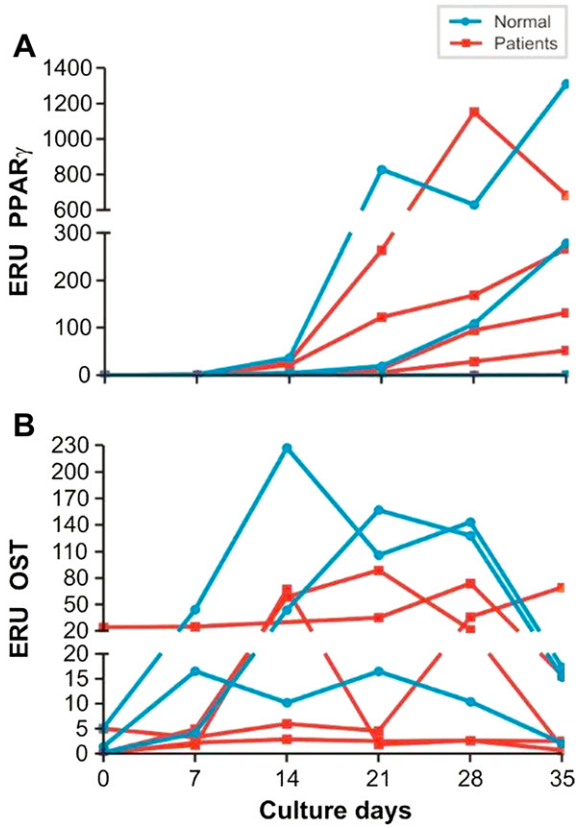
Conflict of Interest Disclosure

None of the authors has a commercial interest related to the contents of the study to declare.

References

1. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006; 8:315–317.

2. Horwitz EM, Le Blanc K, Dominici M, et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy. 2005;7:393–395.
3. da Silva Meirelles L, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. Stem Cells. 2008;26:2287–2299.
4. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–147.
5. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. Exp Biol Med (Maywood). 2001;226:507–520.
6. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007;110:3499–3506.
7. Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol. 2000;18:307–316.
8. Lazarus HM, Koc ON, Devine SM, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant. 2005;11:389–398.
9. Le Blanc K, Samuelsson H, Gustafsson B, et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia. 2007;21:1733–1738.
10. Fouillard L, Chapel A, Bories D, et al. Infusion of allogeneic-related HLA mismatched mesenchymal stem cells for the treatment of incomplete engraftment following autologous haematopoietic stem cell transplantation. Leukemia. 2007;21:568–570.
11. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008;371:1579–1586.
12. Lee ST, Jang JH, Cheong JW, et al. Treatment of high-risk acute myelogenous leukaemia by myeloablative chemoradiotherapy followed by co-infusion of T cell-depleted haematopoietic stem cells and culture-expanded marrow mesenchymal stem cells from a related donor with one fully mismatched human leucocyte antigen haplotype. Br J Haematol. 2002;118:1128–1131.
13. Arthur A, Zannettino A, Gronthos S. The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. J Cell Physiol. 2009;218:237–245.
14. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. Proc Natl Acad Sci U S A. 2002;99:8932–8937.
15. Koc ON, Day J, Nieder M, Gerson SL, Lazarus HM, Krivit W. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant. 2002;30:215–222.
16. Fouillard L, Bensidhoum M, Bories D, et al. Engraftment of allogeneic mesenchymal stem cells in the bone marrow of a patient with severe idiopathic aplastic anemia improves stroma. Leukemia. 2003; 17:474–476.
17. Ringden O, Uzunel M, Sundberg B, et al. Tissue repair using allogeneic mesenchymal stem cells for hemorrhagic cystitis, pneumomediastinum and perforated colon. Leukemia. 2007;21:2271–2276.
18. Galotto M, Berisso G, Delfino L, et al. Stromal damage as consequence of high-dose chemo/radiotherapy in bone marrow transplant recipients. Exp Hematol. 1999;27:1460–1466.
19. Banfi A, Bianchi G, Galotto M, Cancedda R, Quarto R. Bone marrow stromal damage after chemo/radiotherapy: occurrence, consequences and possibilities of treatment. Leuk Lymphoma. 2001;42:863–870.
20. Carlo-Stella C, Tabilio A, Regazzi E, et al. Effect of chemotherapy for acute myelogenous leukemia on hematopoietic and fibroblast marrow progenitors. Bone Marrow Transplant. 1997;20:465–471.
21. Domenech J, Roingeard F, Herault O, et al. Changes in the functional capacity of marrow stromal cells after autologous bone marrow transplantation. Leuk Lymphoma. 1998;29:533–546.
22. Schwartz GN, Warren MK, Rothwell SW, et al. Post-chemotherapy and cytokine pretreated marrow stromal cell layers suppress hematopoiesis from normal donor CD34+ cells. Bone Marrow Transplant. 1998;22:457–468.
23. Mueller LP, Luetzkendorf J, Mueller T, Reichelt K, Simon H, Schmoll HJ. Presence of mesenchymal stem cells in human bone marrow after exposure to chemotherapy: evidence of resistance to apoptosis induction. Stem Cells. 2006;24:2753–2765.
24. Isaikina Y, Kustanovich A, Svirnovski A. Growth kinetics and self-renewal of human mesenchymal stem cells derived from bone marrow of children with oncohematological diseases during expansion in vitro. Exp Oncol. 2006;28:146–151.
25. Isaikina Y, Shman T. Influence of mesenchymal stem cells derived from bone marrow of children with oncohematological diseases on proliferation and self-renewal of hematopoietic progenitor cells in vitro. Exp Oncol. 2008;30:121–128.
26. Cao J, Tan MH, Yang P, et al. Effects of adjuvant chemotherapy on bone marrow mesenchymal stem cells of colorectal cancer patients. Cancer Lett. 2008;263:197–203.
27. Carrara RC, Orellana MD, Fontes AM, et al. Mesenchymal stem cells from patients with chronic myeloid leukemia do not express BCR-ABL and have absence of chimerism after allogeneic bone marrow transplant. Braz J Med Biol Res. 2007;40:57–67.
28. Covas DT, Panepucci RA, Fontes AM, et al. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol. 2008;36:642–654.
29. Tondreau T, Lagneaux L, Dejeneffe M, et al. Isolation of BM mesenchymal stem cells by plastic adhesion or negative selection: phenotype, proliferation kinetics and differentiation potential. Cytotherapy. 2004;6:372–379.
30. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone. 2003;33:919–926.
31. Silva WA Jr, Covas DT, Panepucci RA, et al. The profile of gene expression of human marrow mesenchymal stem cells. Stem Cells. 2003;21:661–669.
32. Covas DT, Siufi JL, Silva AR, Orellana MD. Isolation and culture of umbilical vein mesenchymal stem cells. Braz J Med Biol Res. 2003; 36:1179–1183.
33. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000;28:875–884.
34. Gutierrez-Rodriguez M, Reyes-Maldonado E, Mayani H. Characterization of the adherent cells developed in Dexter-type long-term cultures from human umbilical cord blood. Stem Cells. 2000;18:46–52.
35. Short B, Brouard N, Occhiodoro-Scott T, Ramakrishnan A, Simmons PJ. Mesenchymal stem cells. Arch Med Res. 2003;34:565–571.
36. Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol. 2003;121:368–374.
37. Dazzi F, Ramasamy R, Glennie S, Jones SP, Roberts I. The role of mesenchymal stem cells in haemopoiesis. Blood Rev. 2006;20:161–171.
38. Stute N, Holtz K, Bubenheim M, Lange C, Blake F, Zander AR. Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol. 2004;32:1212–1225.
39. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol. 1999;181:67–73.
40. Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. Exp Hematol. 2000;28:707–715.
41. Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. Exp Hematol. 2004;32:414–425.



Supplementary Figure E1. Kinetics of the expression of genes involved in the differentiation into adipocytes and osteocytes. Total RNA was obtained from undifferentiated mesenchymal stromal cells (MSCs) (day 0) and from cultures for the differentiation into adipocytes and osteocytes on days 7, 14, 21, 28, and 35 of culture and analyzed by reverse transcription polymerase chain reaction for the genes peroxisome proliferative activated receptor−γ (PPARγ) and osteopontin (OST), respectively. Data are reported as expression relative units (ERU).

Supplementary Table E1. Primer supplemental sequences and probe number for the genes evaluated by real-time reverse transcription polymerase chain reaction

Title	SYBR green primers and Taq man probes
PPAR89 sense	5′ GTTATGGGTGAAACTCT GGGAG 3′
PPAR429 antisense	5′ GGAGATGCAGGCTCCACTTTG 3′
OST66 sense	5′ CCATGAGAATTGCAGTGATTTGC 3′
OST388 antisense	5′ GTCGTTCGAGTCAATGGAGTCC 3′
CD140b sense	5′ AACATCATCTGGTCTGCCTGC 3′
CD140b antisense	5′ TCAAACTCCTGCTCCTCCTC 3′
GAPDH sense	5′ GCCTCAAGATCATCAGCAATGC 3′
GAPDH antisense	5′ CATGGACTGTGGTCATGAGTCCT 3′
NTSE (CD73)	Hs00159686_m1
MCAM (CD146)	Hs00174838_m1
Stem cell factor (KIT ligand)	Hs00241497_m1
Leukemia inhibitory factor	Hs00171455_m1
Fms-related tyrosine kinase 3 ligand)	Hs00181740_m1

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; OST = osteopontin; PPAR = peroxisome proliferative activated receptor.

Supplementary Table E2. Fibroblastoid colony-forming unit counts and mesenchymal stromal cells growth kinetics by samples

	Disease	D + BMT	CFU-F ($\times 10^6$ BM-MNC)	Doubling time (h)	Total cell expansion (fold)
Patients					
LP-03	LGCBM	28	11.40	34.08	28.27
LP-04	NSHD	662	2.80	185.64	1.81
LP-05	MLBCL	1551	4.50	167.44	5.53
LP-06	NSHD	1836	25.20	49.48	20.48
LP-07	NSHD	104	7.30	58.9	19.61
LP-08	LPHD	1620	37.50	59.08	11.55
LP-09	DLBCL	124	11.80	92.31	6.91
LP-10	NSHD	1822	34.50	69.01	7.71
LP-11	DLBCL	1305	0.00	195.35	3.32
LP-12	DLBCL	168	0.00	68.33	8.62
LP-13	MLBCL	159	0.00	107.22	17.95
LP-14	MCHD	770	30.30	126.69	4.87
Controls					
ND-01	—	—	—	44.86	16.59
ND-02	—	—	12.50	36.36	19.63
ND-03	—	—	13.60	37.8	21.95
ND-04	—	—	—	47.73	27.48
ND-05	—	—	1.30	56.38	13.42
ND-06	—	—	92.50	—	—
ND-07	—	—	8.00	—	—
ND-08	—	—	56.00	—	—
ND-09	—	—	54.00	—	—
ND-10	—	—	20.00	—	—
ND-11	—	—	55.00	—	—
ND-12	—	—	11.00	—	—
ND-13	—	—	12.00	—	—

	CFU-F ($\times 10^6$ BM-MNC)	Doubling time (h)	Total cell expansion (fold)	Statistical test
Controls × patients	0.079	0.009	0.064	Mann-Whitney
HD × NHL patients	0.041	0.589	>0.999	Mann-Whitney
Controls × HD × NHL patients	0.023	0.031	0.170	Kruskal-Wallis
Controls × HD × NHL patients	<0.05 NHL	<0.05 NHL	—	Dunn's Multiple Comparisons
Controls × HD × NHL patients	>0.05 HD	<0.05 HD	—	Dunn's Multiple Comparisons

CFU-F = fibroblastoid colony-forming units; D + BMT = number of days after bone marrow transplantation; DLBCL = diffuse large B-cell lymphoma; HD = Hodgkin's disease; LP = lymphoma patient; LPHD = nodular lymphocyte-predominant Hodgkin's disease; MCHD = mixed cellularity Hodgkin's disease; MLBCL = primary mediastinal large B-cell lymphoma; ND = normal donor; NHL = non-Hodgkin's lymphoma; NSHD = nodular sclerosis Hodgkin's disease.

Supplementary Table E3. Gene expression analysis by reverse transcription polymerase chain reaction

	CD140b	CD146	CD 73	LIF	SCF	FLT-3
ND 14	149.00	313.04	1390.81	16.67	89.46	13.75
ND 13	277.09	392.41	2094.97	75.59	54.82	13.87
ND 09	468.92	487.99	3707.45	32.86	110.75	16.06
ND 11	349.88	210.00	1677.63	69.75	108.14	14.70
Median	313.48	352.73	1886.30	51.305	98.80	14.29
Minimum	149.00	210.00	1390.81	16.67	54.82	13.75
Maximum	468.92	487.99	3707.45	75.59	110.75	16.06
LP 05	279.31	37.10	127.53	66.31	20.95	2.10
LP 10	536.98	336.44	2879.72	142.69	97.15	9.03
LP 12	899.33	763.09	4764.84	45.37	140.28	11.03
LP 13	533.08	423.35	2034.86	38.19	168.51	27.74
LP 06	125.47	567.59	2257.82	185.81	120.19	11.59
LP 03	307.03	152.88	1498.92	253.13	84.67	17.72
Median	420.05	379.90	2146.34	104.5	108.67	11.31
Minimum	125.47	37.10	127.53	38.19	20.95	2.10
Maximum	899.33	763.09	4764.84	253.13	168.51	27.74
Mann-Whitney test	0.4762	0.9143	0.9143	0.2571	0.6095	0.2381

Data are reported as expression relative units.
Flt-3 = Fms-related tyrosine kinase 3; LIF = leukemia inhibitory factor; LP = lymphoma patient; ND = normal donor; SCF = stem cell factor.

Supplementary Table E4. Curve for differentiation into adipocytes and osteocytes: RT-PCR analysis

A) Differentiation into adipocytes: PPARγ gene expression analysis

Culture (days)	ND 09	ND 13	ND 11	LP 05	LP 03	LP 06	LP 13	LP 12
0	0.08	0.00	0.02	0.05	0.04	0.00	0.00	0.01
7	0.38	1.55	0.08	0.33	0.14	0.01	0.43	0.05
14	1.21	36.44	3.94	0.03	21.93	1.10	28.88	5.09
21	0.13	827.79	18.74	0.06	122.56	5.43	263.85	13.03
28	0.14	629.35	108.60	0.24	169.10	29.16	1153.16	94.69
35	0.56	1311.47	278.49	0.46	266.56	52.12	684.92	131.64

B) Differentiation into osteocytes: Osteopontin gene expression analysis

Culture (days)	ND 09	ND 13	ND 11	LP 05	LP 03	LP 06	LP 13	LP 12
0	0.24	1.41	5.13	24.25	0.24	0.13	0.34	5.03
7	4.11	16.57	44.23	25.17	4.94	2.21	1.73	3.30
14	44.10	10.22	227.12	-	58.82	2.87	67.35	6.01
21	156.68	16.48	105.88	35.18	88.67	2.54	1.87	4.58
28	127.82	10.46	143.21	74.08	21.65	2.65	2.64	35.99
35	15.38	2.09	17.30	15.70	1.27	2.55	0.67	69.39

LP = lymphoma patient; ND = normal donor; PPARγ = peroxisome proliferative activated receptor−γ.

Supplementary Table E5. Hematopoiesis support

No MSC (7 d)	Controls (7 d)	Patients (7 d)	No MSC(28 d)	Controls (28 d)	Patients (28 d)
$CD34^+$ expansion					
0.97	2.83	2.42	1.95	3.88	5.07
1.67	4.91	2.46	2.00	4.68	4.40
	2.15	3.09	1.05	3.84	5.09
	3.64	3.16		4.35	3.42
		3.22			4.51
		4.29			3.97
Mann-Whitney test		$p = 0.9143$			$p = 0.4762$
CFU-GM clonogenic potential					
2.28	15.51	10.06	0.22	0.39	0.48
3.51	18.58	8.27	0.11	1.45	0.00
	6.10	9.16	0.00	1.75	0.49
	9.72	15.25		0.24	2.24
		11.09			0.00
		16.37			0.56
Mann-Whitney test		$p = 0.9143$			$p = 0.7619$
BFU-E clonogenic potential					
0.32	1.66	4.87	0.00	0.00	0.00
0.44	9.67	2.61	0.03	3.98	0.00
	4.22	2.32	0.00	0.00	0.00
	1.49	1.17		0.15	0.00
		3.11			0.00
		0.88			0.00
Mann-Whitney test		$p = 0.6096$			—

BFU-E = burst-forming unit erythroid; CFU-GM, colony-forming unit granulocyte-macrophage; MSC, mesenchymal stromal cell.