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► To cite this version:

Kevin Kemp, Ruth Morse, Sarah Wexler, Christine Cox, Elizabeth Mallam, et al.. Chemotherapy-induced mesenchymal stem cell damage in patients with hematological malignancy. *Annals of Hematology*, 2010, 89 (7), pp.701-713. 10.1007/s00277-009-0896-2 . hal-00535119

HAL Id: hal-00535119

<https://hal.science/hal-00535119>

Submitted on 11 Nov 2010

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Chemotherapy-induced mesenchymal stem cell damage in patients with hematological malignancy

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Received: 18 August 2009 / Accepted: 27 December 2009 / Published online: 30 January 2010
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Abstract Hematopoietic recovery after high-dose chemotherapy (HDC) in the treatment of hematological diseases may be slow and/or incomplete. This is generally attributed to progressive hematopoietic stem cell failure, although defective hematopoiesis may be in part due to poor stromal function. Chemotherapy is known to damage mature bone marrow stromal cells in vitro, but the extent to which marrow mesenchymal stem cells (MSCs) are damaged by HDC in vivo is largely unknown. To address this question, the phenotype and functional properties of marrow MSCs derived from untreated and chemotherapeutically treated patients with hematological malignancy were compared. This study demonstrates a significant reduction in MSC expansion and MSC CD44 expression by MSCs derived from patients receiving HDC regimens, thus implicating potential disadvantages in the use of autologous MSCs in chemotherapeutically pretreated patients for future therapeutic strategies. The clinical importance of these HDC-

induced defects we have observed could be determined through prospective randomized trials of the effects of MSC cotransplantation on hematopoietic recovery in the setting of HDC with and without hematopoietic stem cell rescue.

Keywords Mesenchymal stem cells · Transplantation · Chemotherapy · Hematopoietic stem cells · Bone marrow

Introduction

Human bone-marrow-derived mesenchymal stem cells contribute to the regeneration of mesenchymal tissues such as the bone, cartilage, muscle, ligament, tendon, adipose, and marrow stroma [1] and are seemingly essential in providing support for the growth and differentiation of primitive hematopoietic cells within the bone marrow microenvironment [1–4]. Recently, MSCs have generated a great deal of interest in many clinical settings including those of regenerative medicine, immune modulation, and tissue engineering where studies have demonstrated the feasibility of transplanted MSCs becoming a new mode of cellular therapy.

High-dose chemotherapy and irradiation used with or without hematopoietic stem cell (HSC) rescue in the treatment of hematological malignancies and other cancers may cause long-lasting damage to bone marrow stromal cells, thus impairing hematopoiesis. Various studies to date have evaluated the toxic effects of HDC and demonstrated severe and irreversible damage on mature bone marrow stromal compartments in vivo and in vitro [5–14]. These effects are relevant not only in patients treated with HDC without allogeneic stem cells but also in recipients of allogeneic stem cell transplants in whom bone marrow stroma remains recipient in origin after the transplant [15–17].

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Although HDC is known to damage the mature bone marrow stromal cells *in vitro* and *in vivo*, the extent to which marrow MSCs are damaged by HDC *in vivo* is largely unknown. If damage to marrow MSCs is substantial, it would be logical to use cultured MSCs therapeutically to assist or repair the marrow microenvironment after HDC. Evidence from animal experiments [18–21] and clinical trials [22] suggests that the cotransplantation of cultured MSCs may have a role in facilitating HSC engraftment after stem cell transplants, although the biological mechanisms involved are unclear. If treatment-induced damage to patients' MSCs is substantial, this could mitigate against the therapeutic use of autologous MSCs.

This *in vitro* study was therefore designed using cultured MSCs and long-term hematopoietic cell models to investigate the effects of HDC given clinically on marrow MSCs. To address this question, the phenotype and functional properties of marrow MSCs derived from patients who have received chemotherapeutic treatment for various hematological diseases were compared with those derived from patients who had received no prior treatment.

Materials and methods

Patients and sample collection

Bone marrow samples were obtained from diagnostic or staging posterior iliac crest marrow aspirates performed in the Department of Hematology, Royal United Hospital, Bath, with written informed consent and local hospital ethic committee approval. Forty-five consecutive consenting patients with hematological malignancy undergoing diagnostic and follow-up marrow sampling were studied. Briefly, the bone marrow aspirate was taken under local anesthetic. The aspirate needle was inserted into the marrow cavity and a syringe used to withdraw the liquid bone marrow. All samples collected were placed into sterile 50-ml tubes containing 1,000 IU heparin. Eighteen patient samples were obtained at diagnosis, six after low-dose chemotherapy and 21 after high-dose chemotherapy. Details of the diagnosis and treatment of the study patients are shown in Table 1. The average time from the last dose of chemotherapy to the point at which the bone marrow aspirate was taken for the high-dose chemotherapy-treated patient group was 501 days (SE±160).

Umbilical cord blood sample collection

Umbilical cord bloods (CBs) were obtained by midwives in the Central Delivery Suite, Southmead Hospital, Bristol, with maternal written informed consent and local hospital ethic committee approval. Cord blood samples were

collected by gravity into sterile 50-ml tubes containing 1,000 IU heparin after the umbilical cord had been clamped and cut. All samples were from normal full-term deliveries, and collection was entirely at the discretion of the midwife in charge.

Cell cryopreservation and thawing

Cell counts were obtained and cell density was adjusted to $<1 \times 10^7$ cells per milliliter with Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS), to which an equal volume of DMEM/20% DMSO (Sigma-Aldrich, UK) was added. The vials were cooled until they had reached -80°C using a 5100 Cryo 1°C freezing container (Nalgene, DK). Tubes were then transferred to a liquid nitrogen container for permanent storage until use.

Vials containing cells were thawed in a 37°C water bath with constant agitation. Cells were washed with DMEM/20% FCS and centrifuged, and a cell count was taken using Trypan blue (Sigma-Aldrich, UK) to determine live cell numbers. The cells were then resuspended in an appropriate prewarmed medium for use.

Assessment of peripheral blood contamination of marrow MNC harvests

Peripheral blood contamination of the marrow mononuclear cell (MNC) harvests was assessed using fluorescein-isothiocyanate-conjugated anti-CD3 (Dako, High Wycombe, UK). This was to ensure that any apparent alteration in MSC proliferation between groups was not due to differences in total MSC numbers within the initial bone marrow sample caused by peripheral blood contamination. MNCs harvested from bone marrow aspirates were resuspended in DMEM/10% FCS at 10^6 cells per milliliter. The cell suspension was incubated in the dark at 4°C for 30 min with the specific monoclonal antibody. Cells were then washed with DMEM, centrifuged at 400 g for 5 mins, and resuspended in DMEM/10% FCS for analysis. At least 10,000 events were analyzed on a BD FACS vantage SE and analyzed with CellQuest™ software (BD Biosciences, Oxford, UK). Gates were set on the analysis to remove cellular debris.

Establishment of mesenchymal culture

Marrow aspirates were overlaid onto an equal volume of Lymphoprep™ (Axis-Shield, Dundee, UK; density 1.077 ± 0.001 g/ml) and centrifuged at 600 g for 35 min at room temperature to separate the MNCs from neutrophils and red cells. The MNC layer was harvested and washed twice in DMEM (Sigma-Aldrich, Gillingham, UK).

Table 1 Classification of patients' chemotherapy protocol intensity

ID number	M/F	age	Diagnosis	Chemotherapy (Y/N)	Regimen	Myelotoxicity classification
02	M	68	High-grade B cell lymphoma	N		
13	M	57	NHL	N		
14	F	68	Myeloma	N		
15	F	83	MGUS	N		
19	M	93	High-grade NHL	N		
28	M	72	Myeloma	N		
33	M	45	MPD	N		
34	F	45	Primary thrombocytosis	N		
35	F	81	Plasma cell dyscrasia	N		
37	M	67	NHL	N		
38	F	74	MDS	N		
43	F	58	LG NHL	N		
44	M	65	MDS	N		
46	M	31	Lymphoma	N		
47	M	81	MDS	N		
48	F	82	Myeloma	N		
50	M	79	Myeloma	N		
52	F	72	Thrombocythemia	N		
Mean		68 (SE=4)				
05	M	58	AML M5	Y	DA ×1, FLAG with ATRA ×1, Ara-C high dose ×1, sibling PB-SCT	High
07	F	53	AML M3	Y	H-DAT3+10, H-DAT3+8	High
08	F	55	AML	Y	ADE, ADE, MACE, MIDAC, ICE	High
09	M	51	Myeloma	Y	VAD ×6, cyclophosphamide, fludarabine, and mini allo-SCT	High
10	M	49	AML	Y	Da×2, Ara-C ×2	High
17	M	62	Chronic lymphocytic leukemia	Y	CHOP ×3, chlorambucil ×6, fludarabine ×1	High
18	M	51	Myeloma	Y	VAD ×6, cyclophosphamide, fludarabine	High
22	F	54	APML	Y	HDAT+ATRA ×2, MACE, ATRA + arsenic trioxide, cyclophosphamide	High
31	M	66	AML	Y	ADE, ADE, MACE,	High
49	M	62	AML	Y	S.DAT, fludarabine, melphalan, and mini allo-SCT	High
64	M	43	Lymphoma	Y	Autologous transplant 6 years prev, chemotherapy?	High
86	F	34	Burkitt's lymphoma	Y	IVAC rituximab + Codox	High
101	M	46	MALT lymphoma	Y	COP ×2, CHOP × 8, R-ESHAP ×2	High
41.2	M	67	Myeloma	Y	VAD ×8, high-dose melphalan, auto-SCT	High
82	M	63	Myeloma	Y	VAD, high-dose melphalan, thalidomide, auto-SCT	High
01	M	37	AML	Y	ADE	High
04	M	54	Stage IVB mantle cell lymphoma	Y	CHOP ×3, fludarabine and cyclophosphamide ×4, G-CSF prime	High
06	F	61	DLBCL	Y	CHOP ×6	High
20	F	75	Thrombocythemia	Y	Hydroxyurea, Busulphan	High
26	F	51	AML	Y	ADE	High
80	M	66	Hairy cell leukemia	Y	Deoxycoformycin ×3	High
Mean		54 (SE=2)				
03	F	?	Low-grade NHL	Y	Oral chlorambucil	Low
12	F	70	Diabetic hypertensive	Y	Hydroxyurea, Glivec	Low
24	M	58	CML	Y	Interferon + Glivec	Low

Table 1 (continued)

ID number	M/F	age	Diagnosis	Chemotherapy (Y/N)	Regimen	Myelotoxicity classification
29	M	84	CML	Y	Glivec	Low
58.2	F	79	Myeloma	Y	Melphalan + prednisolone $\times 3$, cyclophosphamide $\times 3$	Low
66	M	57	Chronic lymphocytic leukemia	Y	Chlorambucil + pre, fludarabine oral ongoing	Low
Mean		70 (SE=5)				

MSC culture

Isolated MNCs were centrifuged and resuspended in MSC medium (consisting of DMEM with 10% FCS selected for the growth of MSCs (StemCell Technologies, London, UK) and 1% penicillin and streptomycin (Sigma-Aldrich, Gillingham, UK)). Vented flasks (25 cm²) containing 10 ml of MSC medium were seeded with 1×10^7 nucleated cells (seeding density=400,000 cells per square centimeter) for passage 0. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and nonadherent hematopoietic cells removed by a media exchange after 3–5 days. Cells were then cultured for 2 weeks at passage 0 and fed by half medium exchange.

To calculate MSC expansion, the adherent cells were resuspended using 0.25% trypsin (Sigma-Aldrich, Gillingham, UK) and reseeded at 7.5×10^4 cells per flask (seeding density=3,000 cells per square centimeter) into passage 1. Thereafter, the cells were replated at 7.5×10^4 cells per flask (seeding density=3,000 cells per square centimeter) every 14 days for up to five passages. During this time, cells were fed every week with MSC medium by half medium exchange.

Immunophenotyping MSC cultures

To ensure that a homogenous population of MSCs had been cultured, immunophenotyping of surface markers, using flow cytometric techniques, was carried out according to previous reports [1, 23, 24]. MSCs were examined using the fluorescently tagged monoclonal antibodies anti-CD105, anti-CD45, anti-CD166, anti-CD44, and anti-CD29 (BD Biosciences, Oxford, UK). For immunophenotypic analysis, MSCs were detached from culture flasks at second passage using 0.25% trypsin for 5 min, washed with phosphate-buffered saline (PBS) to remove trypsin, and resuspended in MSC medium at 10^6 cells per milliliter. The cell suspension was incubated in the dark at 4°C for 30 min with the specific monoclonal antibody. Cells were then washed with DMEM, centrifuged at 400 g for 5 min, and resuspended in MSC medium for analysis. At least 10,000 events were analyzed on a BD FACS vantage SE and

analyzed with CellQuest™ software (BD Biosciences, Oxford, UK). Gates were set on the analysis to remove cellular debris.

Purification and flow cytometric analysis of CD34⁺ cord blood cells

CD34⁺ cell isolation was undertaken after MNC separation as previously described in the “establishment of MSC culture” section. CD34⁺ cells were isolated from the MNC fraction obtained from cord blood harvests using the immunomagnetic MiniMACS (Magnetic-Activated Cell Sorter) CD34 isolation system according to the manufacturer’s instructions (Miltenyi Biotec, UK). The CD34⁺ cells obtained from the MACS-positive fraction were then assessed by cell counting and flow cytometry as described in the “Assessment of peripheral blood contamination of marrow MNC harvests” section above, with the exception that cells were assessed for CD34⁺ content by labeling with anti-CD34 clone HPCA-2 (BD Biosciences, Oxford, UK). Only CD34⁺ events with low side scatter were counted as CD34⁺ cells. CD34⁺ cells were cryopreserved in liquid nitrogen until use in long-term culture assay.

Long-term culture of CD34⁺ cells on MSC-derived stromal layer

Mesenchymal stem cell cultures, at second passage, were plated into 25-cm² vented flasks at 7.5×10^4 cells per flask (seeding density=3,000 cells per square centimeter) in 5 ml of long-term culture medium (LTCM; IMDM (Sigma-Aldrich, Gillingham, UK) containing 10% FCS (StemCell Technologies, London, UK), 10% horse serum (StemCell Technologies, London, UK), hydrocortisone (5×10^{-7} M), and 1% penicillin/streptomycin). Cells were fed every week with LTCM medium by half medium exchange. After 3 weeks of long-term culture, cryopreserved cord blood CD34⁺ cells were thawed and added to MSC long-term cultures at 2.5×10^4 cells per flask (seeding density=1,000 cells per square centimeter) in LTCM. All experiments were seeded with identical cryopreserved CD34⁺ cell populations derived from the same cord blood source. Flasks were

incubated at 37°C and fed weekly with LTCM by half media exchanges. All media removed each week when feeding was assessed for the numbers of supernatant cells present and colony-forming unit–granulocyte–macrophage (CFU-GM) content using the CFU-GM assay described below.

CD34⁺ cells are unable to produce stroma under the culture conditions used so it can be assumed that stromal elements grown in culture were MSC-derived [25]. As flow cytometric analysis indicated that there was no significant contamination of MSC cultures with cells of hematopoietic origin, it was decided not to irradiate MSC cultures prior to seeding as it was hypothesized that MSCs may have an altered sensitivity to radiation and DNA damage post-chemotherapy-exposure.

CFU-GM assay

Supernatant cells removed from long-term cultures each week were plated at a concentration of 10⁴ cells per well (seeding density=26,316 cells per square centimeter) into 0.25 ml of MethoCult® GF H4434 (StemCell Technologies, London, UK) in triplicate into 12-well tissue culture plates. The number of hematopoietic colonies, which are derived from the CB CD34⁺ cell population, present within each well after 2-week culture at 37°C in a humidified atmosphere containing 5% CO₂ was then assessed.

Differentiation

If sufficient numbers of MSCs were available at the end of second passage and after cells had been assigned for use in expansion, immunophenotyping, and long-term culture assays, they were used in differentiation studies. Mesenchymal stem cells were induced into adipogenic, osteoblastic, and chondrogenic differentiation by culturing identical numbers of MSCs, at second passage, in NH Adipodiff medium, NH Osteodiff medium, and NH Chondrodif medium (Miltenyi Biotec, Surrey, UK), respectively, according to the manufacturer's instructions. Adipogenic differentiation was visualized by the accumulation of lipid-containing vacuoles which stain red with oil red O and using immunofluorescent detection by labeling with anti-lipoprotein-lipase (Abcam, UK). Osteogenic differentiation was visualized morphologically and also by the presence of high levels of alkaline phosphatase stained with NBT substrate and using immunofluorescent detection by labeling with anti-alkaline-phosphatase (Abcam, UK). Finally, chondrogenic differentiation was characterized by Alcian blue staining and the production of the extracellular matrix proteoglycan Aggrecan, visualized using immunofluorescent detection by labeling of aggrecan using a mouse antihuman aggrecan (4F4) antibody (Santa Cruz Biotechnology, Heidelberg, Germany).

Immunofluorescence and confocal microscopy imaging of MSC and CD34⁺ cell cocultures

CD34⁺ cell fluorescent staining

CD34⁺ cells isolated from cord blood samples were fluorescently labeled using a general cell membrane labeling PKH26 red fluorescent cell linker kit according to manufacturer's instructions (Sigma-Aldrich, UK). Briefly, CD34⁺ cells were first washed twice in DMEM to remove FCS and centrifuged at 400×g for 5 min. All supernatant was removed and discarded, and cells were resuspended in 1 ml of diluent C/2×10⁷ cells. The cells were added to 4×10⁻⁶ M PKH26 dye, mixed gently, and incubated for 2–5 min at 25°C. The staining reaction was then stopped by adding an equal volume of FCS for 1 min, followed by an equal volume of MSC medium. Finally, the cell suspension was washed five times in DMEM to remove cells from the staining solution by centrifuging at 400×g for 10 min at 25°C and transferring to a new centrifugation tube. Cells were resuspended in MSC medium ready for seeding onto MSC cultures.

CD34⁺ cell seeding

Cord blood CD34⁺ cells were seeded onto MSC cultures at 70% confluence in 24-well plates containing one glass coverslip per well at a cellular density of 1×10⁴ cells per well (seeding density=5,263 cells per square centimeter). Cells were incubated in MSC medium at 37°C in a humidified atmosphere containing 5% CO₂ for 2 days at which point cultures were fixed for immunofluorescent staining.

CD44 immunofluorescent and confocal imaging

Cells on coverslips were washed with PBS and fixed in ice-cold acetone/methanol 1:1 for 10 min. Cells were then rehydrated in PBS and incubated with 2% bovine serum albumin (BSA)/7% glycerol/PBS to block nonspecific binding of antibody. After washing, mouse anti-human CD44 monoclonal antibody (1:100; BRIC235, kindly donated by the IBGRL, Southmead Hospital, Bristol) was applied in 2% BSA/PBS for 45 min at room temperature in a moist chamber. After washing in PBS, coverslips were incubated for 1 h with species-specific (1:200) Alexa-Fluor®-488-conjugated goat antimouse antibodies (Invitrogen). Finally, the coverslips were washed in PBS and mounted onto slides in DAPI Vectasheid™. Slides were then examined using both Nikon Eclipse TE300 and Nikon PCM2000 microscopes.

Statistics

All results within this study were expressed as the means ± one standard error. Statistical comparisons were made by

the unpaired *t* test, the Mann–Whitney *U* tests, or two-way analysis of variance (ANOVA) with Bonferroni corrections where appropriate. A value of less than $p < 0.05$ was considered as significant.

Results

Sample collection

The volume of each bone marrow aspirate sample was determined, the mean aspirate volume of the untreated patient group was 8.30 ml (SE=0.97), high-dose chemotherapy patients 8.04 ml (SE=1.19), and low-dose chemotherapy patients 8.75 ml (SE=1.62). There were no statistically significant differences in aspirate volume between any of the patient groups ($p=0.94$).

Peripheral blood contamination

Prior to seeding in primary culture, marrow samples were assessed for peripheral blood contamination using the

cellular expression of CD3. This method depends on the observation that the CD3⁺ T cell population is approximately 2% of nucleated cells in normal marrow uncontaminated with peripheral blood. In comparison, normal peripheral blood nucleated cells contain approximately 70% of CD3⁺ lymphocytes [26]. Thus, the level of CD3⁺ cells present in each aspirate can be used as an indicator of peripheral blood contamination of the bone marrow sample. The mean percentage of CD3⁺ cell contamination of the bone marrow aspirate within the untreated patient group was 22.09% (SE=4.60), high-dose chemotherapy patients 17.26% (SE=3.19), and low-dose chemotherapy patients 18.76% (SE=6.13). There were no statistically significant differences in CD3⁺ cell contamination of the bone marrow aspirates between any of the patient groups ($p=0.68$; Fig. 1a).

Establishment of MSC primary cultures

When observing cultures at passage 0, it was noted that several primary cultures established from patient marrows after in vivo HDC displayed a mixed morphology with

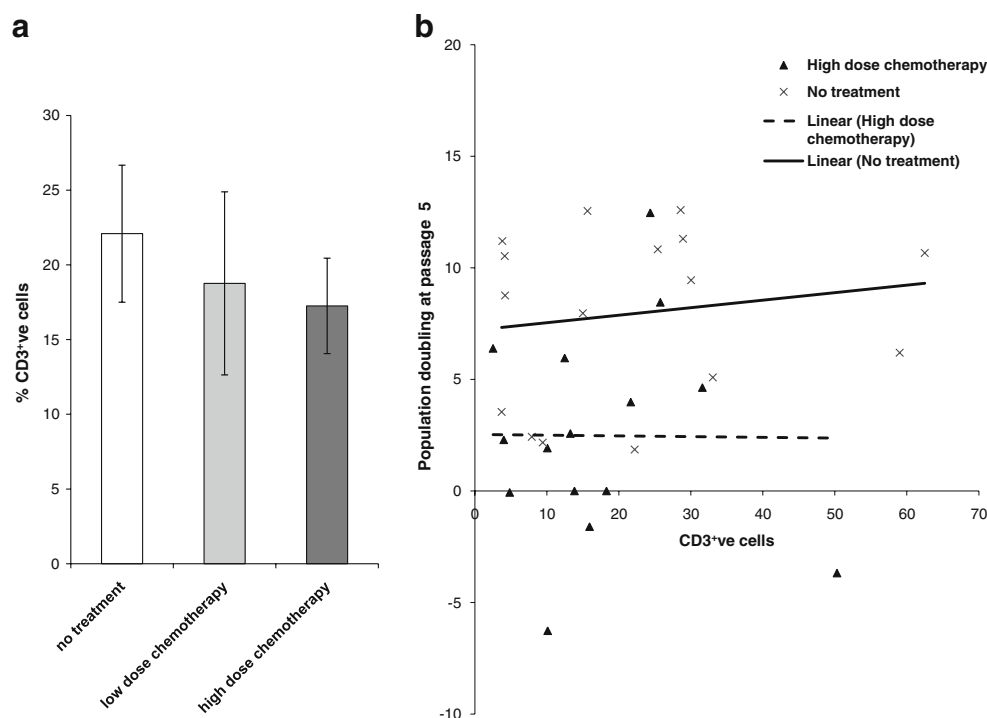


Fig. 1 **a** There were no differences in the level of peripheral blood contamination of bone marrow aspirates between any of the patient groups. The graph shows the mean (\pm SEM) percentage of CD3-positive cell contamination of original marrow aspirates from patients who have received no prior treatment ($n=16$) and high-dose ($n=15$) and low-dose ($n=6$) chemotherapy regimens. There were no statistically significant differences in CD3⁺ cell contamination of the bone marrow aspirate between any of the patient groups ($p=0.68$; ANOVA). **b** Peripheral blood contamination of the bone marrow

aspirate did not affect MSC expansion. The graphs shows the relationship between the percentage of CD3-positive cell contamination of marrow aspirates and the MSC expansion of fifth-passage MSCs from patients who have received no prior treatment ($n=16$) or high-dose ($n=15$) chemotherapy regimens. Broken line = regression line for high-dose chemotherapy group. Continuous line regression line for no treatment group. (No prior treatment vs high-dose chemotherapy $p > 0.05$; ANOVA)

large proportions of cells presenting a more rounded membrane structure, compared to those from untreated marrows that grew as typical densely packed fibroblast-like monolayers, characteristic of MSC cultures, when visualized under phase-contrast microscopy (Fig. 2). However, by first passage, the morphology of treated patient MSCs were similar to MSCs from untreated patients, with cells harvested from both patients groups displaying all the typical characteristics of MSCs in culture, producing adherent layers of elongated fibroblast-like cells in mesenchymal culture conditions.

Expansion capacity of patient MSC cultures

To examine the *in vitro* expansion capacity of MSCs derived from patients receiving no treatment, low-dose chemotherapy, and high-dose chemotherapy, the cells were replated every 14 days at 7.5×10^4 cells per flask, and the total number of cells harvested at the end of each passage was found to calculate the cell population doubling rate (Fig. 3). The expansion of marrow MSCs from patients with untreated hematological malignancy, $n=20$, was 8.79 population doublings (SE=1.04) after fifth passage. The expansion of treated patient MSCs was lower at fifth passage, with a population doubling of 4.67 population doublings (SE=2.44; low-dose chemotherapy, $n=6$) and 3.13 population doublings (SE=0.97; high-dose chemotherapy, $n=21$). Differences in the expansion of MSCs derived from patients who had received no treatment and patients receiving high-dose chemotherapy regimens were statistically significant ($p<0.01$). No significant differences in expansion were evident when comparing MSCs derived from patients receiving no treatment and low-dose chemotherapy regimens ($p>0.05$).

Peripheral blood contamination vs MSC proliferation

A regression plot and analysis were used to investigate the relationship between the CD3⁺ cell contamination of the

marrow aspirate and the cellular population doubling for both patients who had received no prior treatment or high-dose chemotherapy regimens. The regression analysis showed no correlation between CD3⁺ cell contamination of the bone marrow aspirate and MSC expansion at any passage. The correlation between CD3⁺ cell contamination of the bone marrow aspirate and MSC expansion at fifth passage in culture, expressed as population doubling, is shown in Fig. 1b ($p>0.05$).

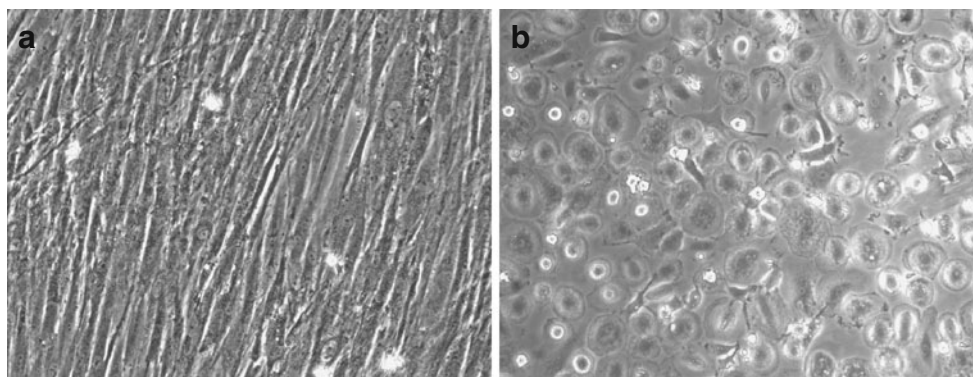
MSC phenotype

Cells were examined at second passage for the expression of CD105, CD29, CD44, CD166, and CD45 using antibody labeling and flow cytometry. At second passage, MSCs from all patient groups were uniformly positive for the mesenchymal markers CD105, CD166, CD44, and CD29 but negative for CD45 which is consistent with the known MSC phenotype and excludes contamination of cultures with hematopoietic cells [24]. When comparing the expression of these cellular markers on MSCs derived from untreated patients with MSCs derived from patients who have received HDC, it was shown that MSCs from treated and untreated patients were phenotypically similar with regards to the percentage of cells CD105⁺, CD166⁺, CD29⁺, CD44⁺, and CD45^{neg} ($p>0.84$; Fig. 4a). However, the level of MSC CD44 expression, calculated using mean fluorescence intensity (MFI) values, after second passage from patients receiving HDC was significantly lower than in untreated patients ($p<0.05$; Fig. 4b). No significant differences in expression levels of CD105, CD166, and CD29 were evident.

Hematopoietic colony-forming assay

The ability of MSC-derived stromal cells to support hematopoiesis was compared using MSCs derived from patients receiving HDC or no treatment in long-term hematopoietic cell cultures. MSCs from all groups in

Fig. 2 Cells from HDC patients display a mixed morphology in primary culture. Representative phase-contrast images of primary cultures derived from **a** patients who have received no prior treatment and **b** patients who had received prior high-dose chemotherapy



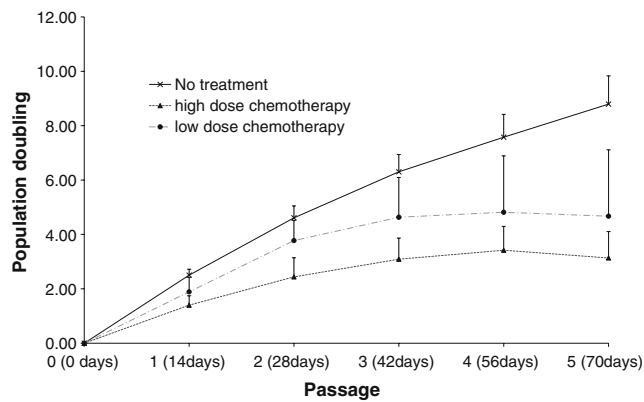


Fig. 3 High-dose chemotherapy damages the in vitro long-term expansion capacity of MSCs. The graph depicts the expansion of MSCs derived from patients who have received no prior treatment ($n=20$) and low-dose ($n=6$) or high-dose ($n=21$) chemotherapy regimens. Results are expressed as the mean \pm (SEM). (No prior treatment vs low-dose chemotherapy $p>0.05$; no prior treatment vs high-dose chemotherapy $p<0.01$; low-dose chemotherapy vs high-dose chemotherapy $p>0.05$; ANOVA)

long-term culture conditions developed the same microscopic appearance as stroma derived from fresh marrow buffy coat samples as reported by Wexler et al. [23]. All cultures were primarily seeded with hematopoietic CD34⁺ cells derived from the same cord blood source. Assessing hematopoietic activity, using a two-way ANOVA with Bonferroni corrections, tests indicated that over the 10-week culture period there were no significant differences in the numbers of CFU-GM present in cultures seeded onto MSC-derived stromal cells from patients who received HDC, when compared to the numbers of CFU-GM supported by MSC cultures derived from patients who had received no treatment ($p>0.05$; Fig. 5a, b).

Differentiation of MSCs

Differentiation of MSCs was investigated at second passage of culture. Mesenchymal stems cells, from patients who had received no prior treatment, were successfully induced to

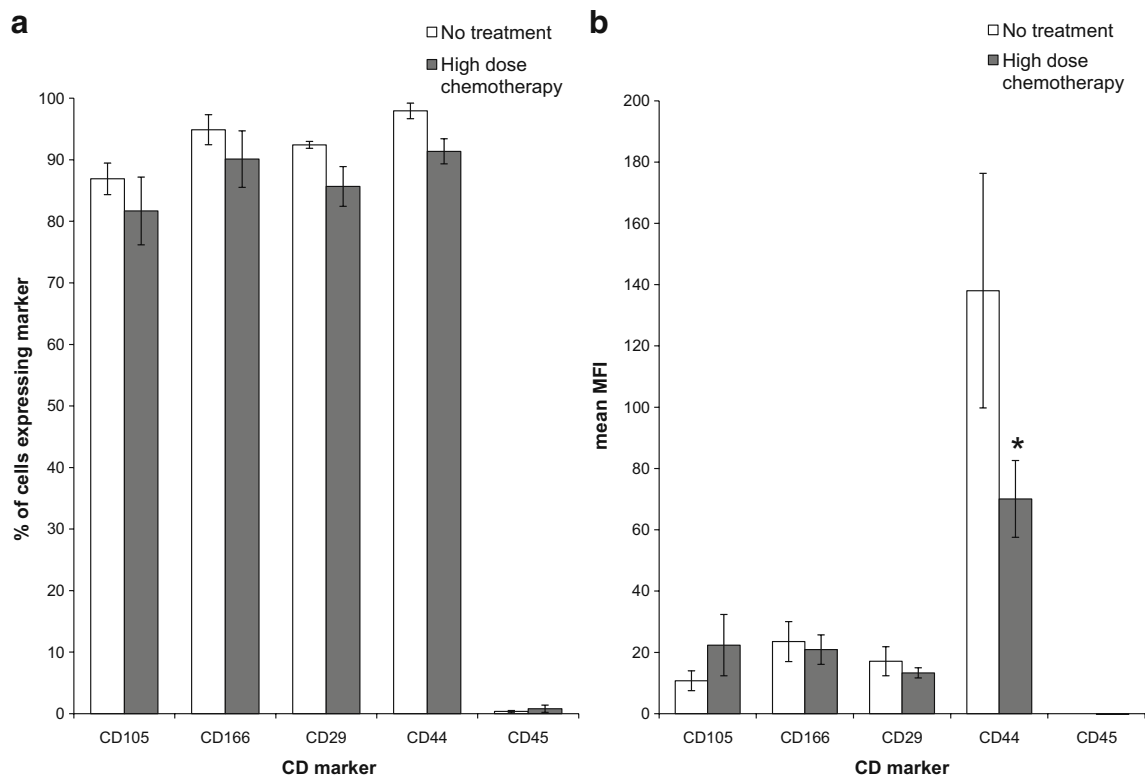
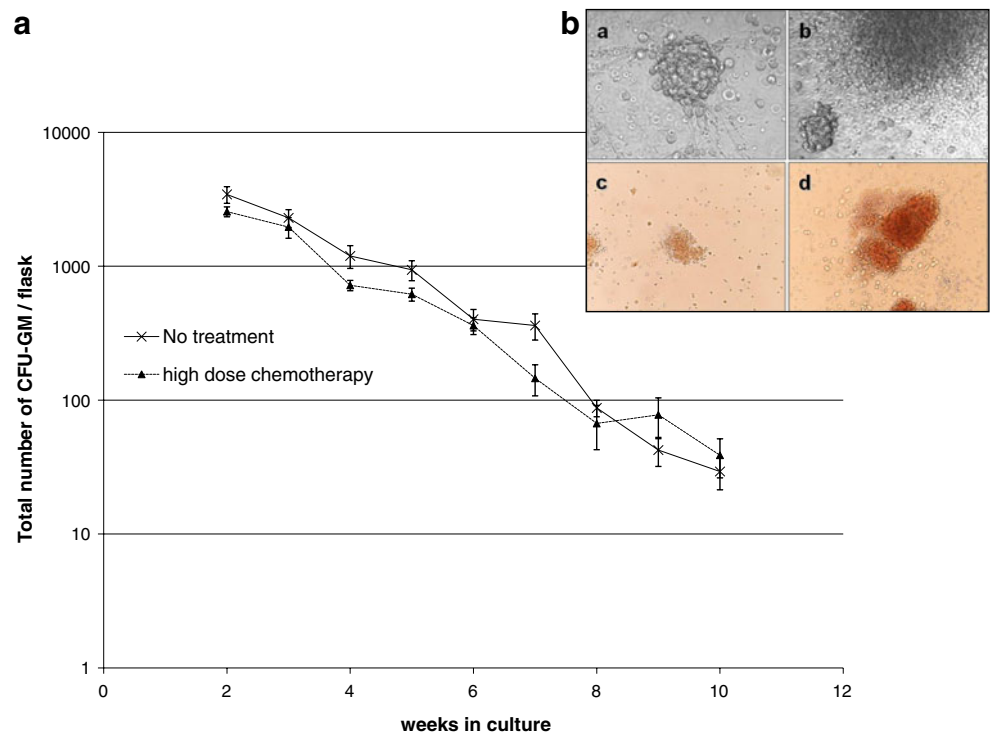


Fig. 4 a The phenotype of cells within MSC culture at second passage, derived from patients who have received no treatment or high-dose chemotherapy regimens. No treatment $n=14$, high-dose chemotherapy $n=9$. Results are expressed as the percentage of cells expressing the phenotypic cellular markers CD105, CD166, CD29, CD44, and CD45 in MSC culture (\pm SEM). MSCs from treated patients were phenotypically similar to MSCs from untreated patients with regards to the percentage of cells CD105⁺, CD166⁺, CD29⁺,

CD44⁺, CD45^{neg} ($p>0.84$). **b** High-dose chemotherapy causes a significant reduction in MSC-CD44 expression. The graph shows the comparison in the levels of CD105, CD166, CD29, CD44, and CD45 expression, using MFI values, on MSCs derived from patients who have received no treatment or high-dose chemotherapy regimens at second passage. No treatment $n=14$, high-dose chemotherapy $n=9$. Results are expressed as the mean MFI level (\pm SEM; no prior treatment vs high-dose chemotherapy; * $p<0.05$)

Fig. 5 **a** MSC-derived stroma from patients who have received HDC display a normal stromal function. The *graph* depicts the number of CFU-GM present in culture after seeding cord blood CD34⁺ cells into coculture with MSC-derived stromal long-term cultures from both patients who have received no prior treatment ($n=6$) or high-dose chemotherapy ($n=6$). Results are expressed as the mean (\pm SEM). (*No prior treatment vs high-dose chemotherapy* $p>0.05$; ANOVA). **b** Insert depicting images of hemopoietic colonies derived from cord blood CD34⁺ cells grown in the CFU-GM assay, *a* (CFU-GM), *b* (CFU-GEMM), *c* (CFU-E), *d* (BFU-E)



differentiate towards osteogenic, adipogenic, and chondrogenic lineages using methods described by others [1, 27] (Fig. 6). Unfortunately, comparison of the osteogenic, adipogenic, and chondrogenic differentiation potential of

MSC cultures from the different patient groups could not be quantified due to the low cell numbers obtained from high-dose chemotherapy patient group after passing caused by low expansion rates.

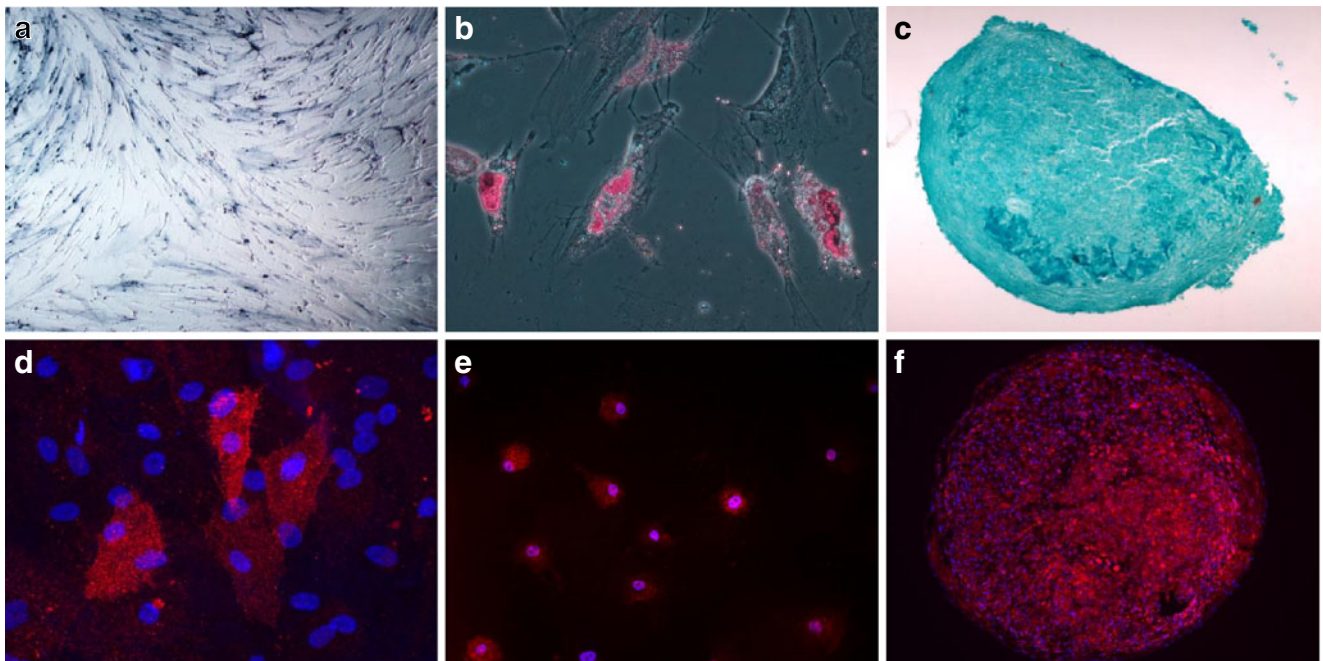


Fig. 6 Images depicting MSC cultures, at second passage, differentiated down osteogenic, adipogenic, and chondrogenic lineages. Osteogenic differentiation was visualized by the presence of high levels of alkaline phosphatase (**a**) and using immunofluorescent detection of alkaline phosphatase (red)/nuclei (blue; **d**). Adipogenic

differentiation was visualized by the accumulation of lipid-containing vacuoles which stain red with oil red O (**b**) and using immunofluorescent detection of lipoprotein lipase (red)/nuclei (blue; **e**). Chondrogenic differentiation was characterized by Alcian blue staining (**c**) and the immunofluorescent detection of aggrecan (red)/nuclei (blue; **f**)

Immunofluorescence and confocal microscopy imaging of MSC and CD34⁺ cell cocultures

To examine the interaction of CD44 on the MSC membrane with hematopoietic stem cells, cocultured bone marrow MSCs and cord blood CD34⁺ cells were fluorescently labeled and visualized using both fluorescence and confocal microscopy. Human mesenchymal stem cells displayed a homogeneous fibroblast-like morphology and an even labeling of the CD44 protein present on the cell membrane. CD44 staining revealed a complex and dynamic membrane structure consisting of many membrane extrusions interlinking with adjacent cells (Fig. 7a, b). Using confocal microscopy, the interaction of CD44, on the MSC surface membrane, and HSC in coculture systems was visualized (Fig. 7a, b). It was shown that CD44 was highly localized at the point at which there was contact between HSC/MSC membranes with the MSC membrane extrusions holding the HSC within its niche.

Discussion

Patients who have undergone HDC commonly display disruption of the marrow architecture with hemorrhaging, loss of fat, and loss of stromal compartments [28]. Studies have also demonstrated that a recipient's stromal cells are damaged after bone marrow transplantation [10, 11, 13] or even from chemotherapeutic drugs alone [6, 8].

Within this study, examining the effects of HDC given clinically on marrow MSCs, bone marrow samples from patients who have received chemotherapeutic treatment for various hematological diseases, and from patients who had received no prior treatment were obtained from diagnostic or staging posterior iliac crest marrow aspirates. Firstly, it was demonstrated that there was no significant differences in aspirate volume between any of the patient groups, thus ruling out any bias for further experiments. Secondly, marrow samples were assessed for peripheral blood contamination using the cellular expression of CD3 to

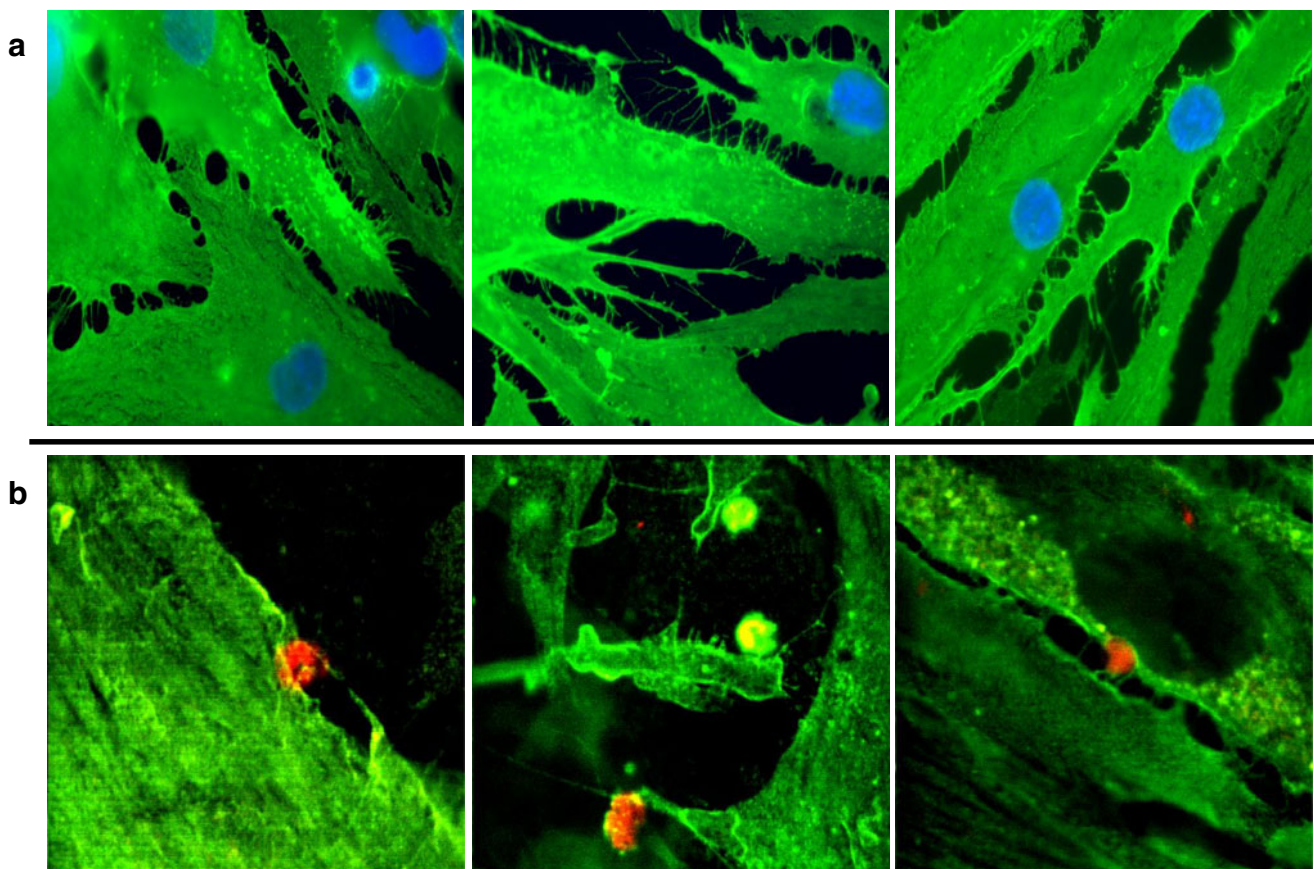


Fig. 7 **a** Immunofluorescence images of cultured MSCs at second passage. Images depict a uniform CD44 expression demonstrating cell–cell interactions between adjacent MSC. CD44 (green) and nuclei (blue). **b** Images depict the important interaction between CD44 and

CD34⁺ umbilical cord blood cells. Confocal microscopy images of cultured MSCs and cord blood CD34⁺ cells. CD44 (green), CD34⁺ cells (red)

ensure that any effects on MSCs caused by HDC were not due to peripheral blood contamination of the original bone marrow aspirates. Again, it was demonstrated that there were no statistically significant differences in CD3⁺ cell contamination of the bone marrow aspirate between any of the patient groups.

In primary culture, it was noted that several cultures established from HDC-treated patients displayed an abnormal morphology with large proportions of cells presenting a more rounded membrane structure. However, by first passage, HDC-treated patient MSCs were morphologically similar to MSCs from untreated patients, with cells harvested from both patients groups displaying all the typical characteristics of MSCs in culture, producing adherent elongated fibroblast-like cells under normal mesenchymal culture conditions. When investigating the expansion potential of MSCs, the current study shows that the differences in expansion capacity of MSCs isolated from untreated patients and patients receiving HDC were significantly different ($p < 0.01$). The mean expansion of untreated MSCs at the fifth passage was more than double that seen of MSCs derived from patients receiving HDC. These data suggest that HDC given to treat hematological malignancy damages the *in vitro* expansion capacity of MSCs. A dose–response effect also seems to be evident as a significant reduction in the MSC expansion capacity was not seen in the low-dose chemotherapy patient group. This may however be associated with the relatively low sample numbers in this group as the proliferation of MSCs was highly variable, giving large standard errors across the mean values. These findings are consistent with published reports that MSC and stromal elements are susceptible to damage by both radiation and chemotherapy administered to cells cultured *in vitro* [5–14, 29, 30]. In contrast, there are little data on stromal cell toxicity of HDC given *in vivo* as demonstrated in the current study. Whether HDC resulted in a decrease in MSC expansion due to a reduction in CFU-forming frequency, through the reduction in MSC proliferation, or from the enhancement in MSC apoptosis is not known. Although investigating the mechanisms by which HDC promotes a decreased MSC expansion would be desirable, these results do show disadvantages in the use of autologous MSCs in chemotherapeutically pre-treated patients for future therapeutic strategies, especially in any therapy where MSC harvest and expansion is desirable prior to transplantation.

Regression analysis of MSC expansion and the percentage of CD3⁺ cell contamination of the iliac crest marrow aspirate was also undertaken to show that any apparent alteration in MSC expansion was not due to peripheral blood contamination of the bone marrow aspirates. The analysis indicated that there was no significant correlation between the number of peripheral blood-derived CD3⁺ cells and the expansion capacity of MSCs at all passages for both

patient groups. This therefore excludes that any differences in MSC expansion between groups were due to differences in the frequency of MSCs present within the initial primary mononuclear cell preparation seeded into passage 0.

To ensure that a homogenous population of MSCs had been cultured, the phenotype of the MSC cultures were studied. At second passage, MSCs from each group were uniformly positive for the mesenchymal markers CD105, CD166, CD29, and CD44 but negative for CD45 which is consistent with the known MSC phenotype [1, 24]. It was shown that MSCs from treated and untreated patients were phenotypically similar with regards to the percentage of cells CD105⁺, CD166⁺, CD29⁺, CD44⁺, and CD45^{neg} ($p > 0.84$). Our findings using MFI values did however indicate significant reductions (49% decrease) in the cell surface expression of CD44 on MSCs from HDC-treated patients. The MFI is a measure of surface antigen density determined by the amount of binding of fluorescently labeled antibody. It was therefore used as a quantitative method of determining the amount of antigen expression on the MSC surface membrane.

By using both immunofluorescent and confocal imaging techniques, it was demonstrated that CD44 is an integral part of the MSC plasma membrane and is seemingly important for interactions between MSCs and HSCs *in vitro*. CD44 is an adhesion molecule that plays a critical role in normal hematopoiesis [31, 32], and it is the stromal microenvironment consisting of stromal cells and extracellular matrix that is thought to regulate and support the future fate of stem cells and committed progenitors along specific lineages [33–35]. The role and function of CD44 have been extensively studied on the surface of hemopoietic cells, however, much less so when concerning its presence on MSCs. It is known that CD44 influences the production of progenitor cells and bears and influences myeloid differentiation [31, 36]. Anti-CD44 mAbs significantly affect both the formation of cobblestone areas within the adherent layer of long-term bone marrow cultures and the production of mature cells [37, 38] and also completely prevented emergence of myeloid cells, and they also blocked lymphocyte growth in Dexter-type long-term bone marrow cultures [38]. In addition, it has shown that CD44 present on stromal cells is critical for the adhesion and development of NK cells in culture [39]. Thus, alterations in CD44 expression on MSCs are likely to contribute to functional changes in a variety of the major activities ascribed to the CD44 molecule. As CD44 is required for vital interactions between stroma and hematopoietic cells, the decrease in CD44 expression, induced by chemotherapy and bone marrow conditioning regimens shown in our study, could lead to a reduced hematopoietic activity and delayed engraftment after stem cell transplantation.

In long-term hematopoietic cell cultures, the CFU-GM assay is used as an indirect measure of primitive hemato-

poietic cell numbers engrafting on MSC-derived stromal cells. Thus, the CFU-GM readout may be used to assess the ability of MSC-derived stromal cells to support hematopoiesis [3, 40]. We have shown that MSC-derived stroma from patients who have received HDC display a normal stromal function in terms of supporting the growth of early hemopoietic progenitor cells when compared to MSC-derived stroma from untreated patients over 10 weeks in long-term cultures. This finding was in concurrence with previous studies reporting that MSCs, derived from patients who had undertaken chemotherapeutic treatments for various different malignancies, produce normal levels of hemopoietic support in vitro [6, 8, 41]. The role of MSC damage in engraftment kinetics in vivo is unknown; however, it is known that MSCs can produce a number of early-acting cytokines that maintain HSC in quiescence or promote their self-renewal and also a variety of interleukins and cytokines which act on more mature hemopoietic progenitors [33, 34].

The in vitro expansion and characterization of MSCs from patients receiving HDC for hematological malignancies have important implications for basic research and future therapeutic strategies. The results of this study demonstrate changes in MSC expansion capacity and MSC phenotype in patients with hematological malignancy after HDC. Patients who received HDC had been treated for on average 501 days before inclusion in this study; thus, their MSCs must have sustained prolonged injury. We have not been able to demonstrate a functional defect in mature marrow stroma derived from patient MSCs in vitro using long-term hemopoietic cell cultures (LTC). An explanation to why these differences were not observed may be due to the most severely affected patient samples within the HDC group, with very low proliferative capacities, not yielding sufficient numbers of cells for differentiation or hemopoietic support cultures. Thus, only the higher proliferating, less-affected MSC-derived stromal cultures were used in the comparative analysis between the high-dose and no-treatment patient groups in these experiments. Two previous studies have suggested that MSCs are resistant to chemotherapeutic agents at clinically relevant doses [30, 42]. In contrast to our study, one focused on the effects of several chemotherapeutic agents used alone in vitro [30]; the other study did however present the effects of chemotherapy on MSCs in vivo but it did not focus on hemopoietic cell support. In addition, while their findings suggest that chemotherapy has no effect on MSC expansion and phenotype, there were several differences between our studies, for example differences in population sizes, experimental methods, times between last chemotherapy administration and bone marrow harvest, and most importantly chemotherapy dose and/or regimens used in the patient cohorts [42].

Together, MSCs derived from untreated patients with hematological malignancies display all defining characteristics of multipotent mesenchymal stem cells in culture; however, the results of this study do implicate potential disadvantages in the use of autologous MSCs in chemotherapeutically pretreated patients for future therapeutic strategies. The clinical importance of the high-dose chemotherapy-induced defects we have observed in vitro could be investigated in prospective randomized trials of the effects of MSC cotransplantation on hemopoietic recovery in the setting of HDC with and without hemopoietic stem cell rescue.

Acknowledgements We wish to express our gratitude to the doctors and nurses at the Royal United Hospital, Bath, for bone marrow collections and helpful discussions and to all patients who donated marrow samples. We would also like to thank midwives at the Central Delivery Suite, Southmead Hospital, Bristol, for cord blood collections and mothers who donated cord blood. This work was supported by a Ph.D. bursary from the University of the West of England and also in part by the funding provided by the Transplant Trust.

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