Immunophenotypic heterogeneity of bone marrow-derived mesenchymal stromal cells from patients with hematologic disorders: correlation with bone marrow microenvironment

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The immunophenotypic analysis of *ex vivo*-expanded mesenchymal stromal cells (MSC) has so far been confined to single or dual staining analysis in normal subjects. In this study, using a four-color cytofluorimetric protocol, we demonstrated that cultured MSC derived from the bone marrow of patients with hematologic malignancies showed alterations in the expression of CD105, CD90, CD184, and HLA-DR molecules. The decrease in the percentage of $CD105^+$ and $CD90^+$ MSC correlated with an increased bone marrow angiogenesis. This paper provides evidence that multiparametric flow cytometry is essential for the establishment of a standardized protocol to identify various MSCs subsets and aberrant phenotypes.

Key words: mesenchymal stromal cell subsets, flow cytometry, bone marrow microenvironment, hematologic disorders, angiogenesis.

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Mesenchymal stromal cells (MSC) can be isolated from various sources and their plasticity and multilineage potential to differentiate into other mesodermal cells have been widely reported.[1] The therapeutic application of MSC is based on their ability to enhance the engraftment of hematopoietic stem cells after transplantation,[2] on their use to correct disorders of mesenchymal origin,[3] and on their immunomodulatory properties.[4] Despite being apparently homogeneous, MSC heterogeneity has been previously reported.[5] The immunophenotype of *ex vivo* - expanded MSC is far from standardized and phenotypic characterization has been confined to single or dual staining analysis of culture-expanded specimens obtained from different tissues or commercially available preparations of mesenchymal cells.[6-7] Furthermore, despite extensive functional characterization of MSC from normal sources (NS), only a few studies have focused on primary cultured MSC from the bone marrow of patients affected by hematologic malignancies (HM).[8] Based on these considerations, we tried to develop guidelines for a standardized multicolor cytofluorimetric protocol which would allow accurate detection and phenotypic characterization of MSC grown in standard long-term culture conditions. We used a wide panel of monoclonal antibodies directed against adhesion molecules (CD29, CD106, CD105, CD166, CD36), extracellular matrix proteins (CD90, CD44), hematopoietic markers (CD31, CD34, CD11c, CD14, CD45-leukocyte common antigen), complement regulatory proteins (CD59), histocompatibility antigens (HLA-ABC class I, HLA-DR class II), and chemokine receptors (CD184-R, CXCR4-R), with the aims of characterizing different MSC subpopulations and determining whether there are any differences in the antigen expression profile between NS-MSC and HM-MSC. Fibroblasts from skin were also expanded and analyzed for comparison. Furthermore, changes in the immunophenotypic profile of MSC were tentatively correlated with the angiogenic potential of patients' bone marrow.

Patients

Forty-three patients (mean age: 48 years, range 25-70 years; 22 males: 21 females) with newly diagnosed hematologic malignancies were investigated: 7 had acute myeloid leukemia (AML), 7 had acute lymphoblastic leukemia (ALL) (5 B-ALL and 2 T-ALL), 7 had non-Hodgkin's lymphoma (NHL; 2 out of 7 with bone marrow involvement), 7 had multiple myeloma (MM), and 15 had myelodysplastic syndromes (MDS). Human bone marrow samples obtained from 8 healthy subjects (mean age 39 years; range 27-49; 5 males: 3 females) were used as normal controls (NS). Primary cultured skin fibroblasts used for comparison were obtained from skin biopsies from five healthy donors who underwent different surgical interventions (mean age 51 years; range 32-66).

Cell cultures

Cell suspensions from healthy donors and patients were obtained as previously described.[9] Colony forming units-fibroblasts (CFU-F) were scored. Primary stromal layers were evaluated at the first passage (P1) of culture (mean age of culture 18-25 days) and, when possible, MSC were further expanded over 60 days (P2-P4) and re-analyzed by flow cytometry.

Multicolor flow cytometric analysis

Primary culture-expanded MSC from patients and controls were detached with trypsin /EDTA (GIBCO), then suspended in 0.5 mL phosphate-buffered saline supplemented with 0.2% bovine serum albumin (BSA, Sigma) and trypsin-inhibitor (Becton-Dickinson). The four-color cytometric analysis of bone marrow-derived cultured MSC was performed on a FACSCalibur equipped with the four-color option (Becton Dickinson). In order to optimize the FACS analysis non-viable cultured MSC were identified by uptake of 7-amino-actinomycin D (7-AAD) (Molecular Probes, Leiden, The Netherlands). The following panel of labeled monoclonal antibodies was used: anti-CD45 (clone 2D1, APC), CD11c (S-HCL-3, PE), CD14 (MºP9, PE), CD31 (WM-59, PE), CD36 (NL07, FITC), CD90 (5E10, FITC), CD59 (P282-H19, PE), CD184 (12G5, PE), CD166 (3A6, PE) (Becton-Dickinson-Pharmingen, CA, USA); anti-CD106, (1G1b1- PE) (Southern Biotechnology Associates-USA); HLA-DR (Tu36, FITC), CD105 (SN6, PE), CD44 (MEM 85, FITC), HLA-ABC (Tu149, FITC), CD80 (MEM-233, PE), CD29 (MEM101A, PE) (Caltag Laboratories, CA, USA), CD31 (CBL468F, FITC, Cymbus Biotechnology Ltd.); CD34 (Q-Bend/10, PercP, Serotec Ltd., Oxford, UK). The combinations of monoclonal antibodies used in this study are listed in Table 1.

In vitro angiogenesis assay

Conditioned media obtained from the bone marrow of 24 patients (CM1) with a low percentage of $CD90^+/CD105^+$ MSC (mean 28%±19SD), and from four normal controls (CM2) were compared for their capacity to induce angiogenesis *in vitro*. The assessment of the *in vitro* angiogenic pattern of bone marrow samples, from which the conditioned media were taken, was performed by evaluating the formation of *in vitro* microvessel structures using the Angiokit assay (TCS Cell Works Ltd, Buckingham, UK/TEMA Ricerca, Italy) as previously reported.[10] The angiogenic pattern was further correlated with the MSCs immunophenotypic profile. CM1 and CM2 were cryopreserved before use.

Effects of conditioned medium on MSC immunophenotype

After thawing the CM1 and CM2 with 10% fresh fetal bovine serum (StemCell Technologies Inc., Vancouver, B.C., Canada). The conditioned media obtained from patients with hematologic malignancies (MDS: 8 cases; ALL: 5 cases, CM1) and controls (CM2) were used to determine whether there were any changes in the immunophenotypic profile of cultured MSC obtained from four normal samples. Experiments were done in duplicate.

Table 1. The mean percentages of cultured MSCs for the various immunological markers are shown. Data were analysed by 4-color cytofluorimetric analysis.

Antigen expression on CD45-/7AAD-gated MSC	*BM HM-MSC*	*p value*	*BM NS-MSC*	*Skin fibroblast*
$CD90^+$	52±25 s.d. (6-93)	$p<0.05$	85±5 s.d. (80-90)	88±1 s.d. (85-89)
$CD105^+$	48±26 s.d. (5-97)	$p<0.05$	80±11 s.d. (71-92)	89±2 s.d. (88-90)
$CD90^+/CD105^+$	48±24 s.d. (8-90)	$p<0.05$	74±16 s.d. (68-89)	88±9 s.d. (87-90)
$CD59^+$	71±21 s.d. (65-93)	ns	93±6 s.d. (89-95)	94±3 s.d. (91-98)
$CD90^+/CD59^+$	50±19 s.d. (10-81)	ns	87±7 s.d. (83-91)	86±4 s.d. (76-89)
$CD29^+$	98±2 s.d. (95-100)	ns	98±2 s.d. (96-100)	99±1 s.d. (96-100)
$CD44^+$	84v12 s.d. (63-96)	ns	90±8 s.d. (86-98)	85±2 s.d. (84-89)
$CD44^+/CD29^+$	81±15 s.d. (74-97)	ns	90±7 s.d. (88-97)	84±5 s.d. (84-90)
$CD106^+$	25±17 s.d. (4-58)	ns	35±8 s.d. (23-60)	<1
$CD90^+/CD106^+$	24±19 s.d. (4-55)	ns	34±19 s.d. (19-57)	<1
$CD166^+$	78±4 s.d. (75-93)	ns	63±13 s.d. (48-73)	65±18 s.d. (39-76)
$CD90^+/CD166^+$	51±31 s.d. (39-91)	ns	61±11 s.d. (38-70)	63±10 s.d. (40-74)
$CD105^+/CD166^+$	47±15 s.d. (10-95)	$p<0.01$	68±28 s.d. (36-90)	64±3 s.d. (40-48)
$CD184^+$	4±8 s.d. (0-12)	ns	3+/-6 (0-8)	< 1
$CD90^+/CD184^+$	3±2 s.d. (0-3)	ns	2±16 s.d. (0-5)	<1
$CD36^+$	6±7 s.d. (0-21)	ns	<1	<1
$CD36^+/CD105^+$	5±13 s.d. (0-32)	ns	<1	<1
$CD31^+$	5±10 s.d. (2-21)	ns	3±5 s.d. (1-9)	1±2 s.d. (0-3)
$CD31^+/CD59^+$	4±6 s.d. (0-24)	ns	3+/-3 s.d. (0-6)	0
$HLA\text{-}DR^+$	15±10 s.d. (0-25)	$p<0.01$	0	0
$HLA\text{-}DR^+/CD80^+$	4±2 s.d. (0-8)	ns	0	0
HLA-ABC cl.I$^+$	98±2 s.d. (85-100)	ns	99±2 s.d. (98-100)	99±1 s.d. (99-100)

Cell positivity was assessed on CD45-/ 7AAD- events +/- standard deviation, s.d.

Results and Discussion

Bone marrow-derived stromal culture

CFU-F assessment. The frequency of CFU-F/1x10^6 BMMNC in samples from patients with hematologic malignancies was usually lower (8±4 SD; range 2-15) than that of normal samples (mean value 12±3 SD ; range 8-12). The lowest number of CFU-F was found in patients with acute leukemia (ALL: 3±1 SD; AML: 6±4) and MDS (6±3 SD), while patients with MM had higher amounts (7±6 SD). The CFU-F value of NHL patients (10±3 SD) was similar to that of normal samples (12±1). After 30 days of extended long-term *in vitro* culture, the

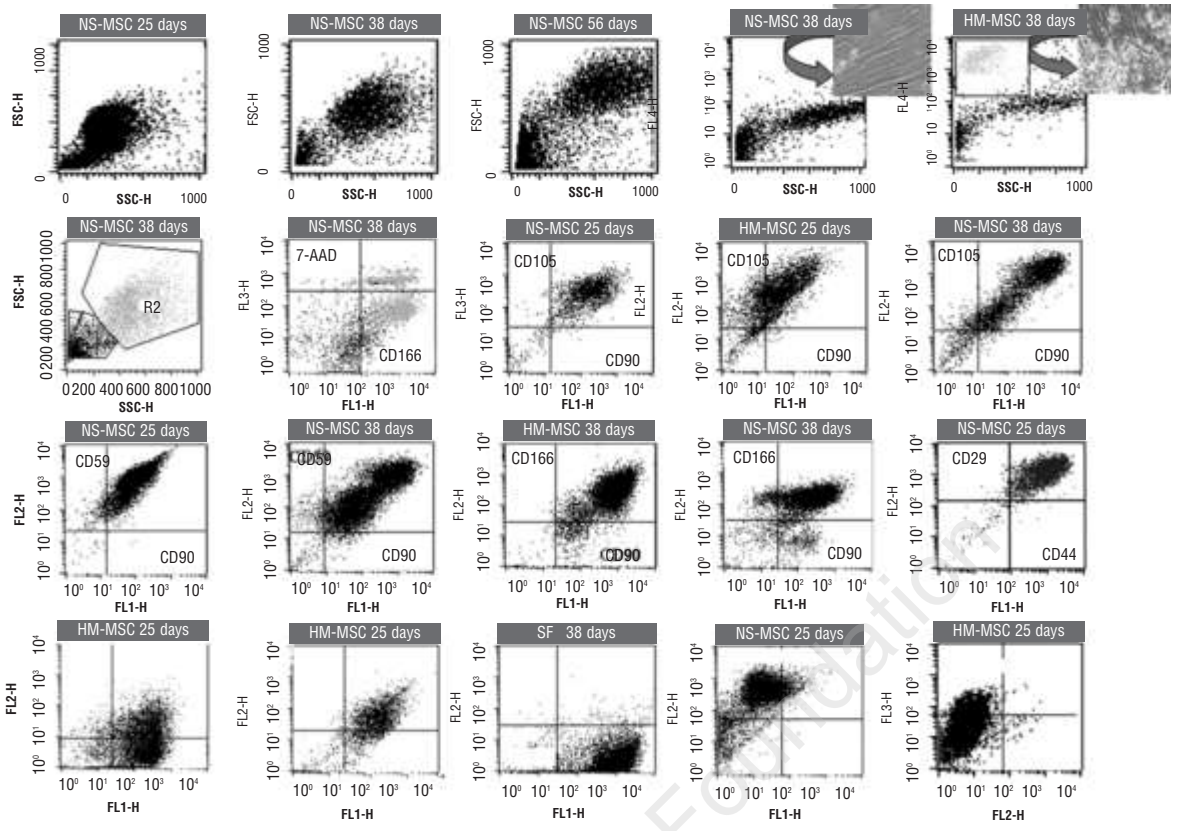

Figure 1. Flow cytometry analysis of *in vitro* cultured bone marrow-derived HM and NS-MSCs. The morphological characteristics (SSC and FSC light scattering parameters) exhibited by NS-MSCs at different time points of the culture (25 and 56 day) are shown (upper row). CD45 antigen expression in a MM sample is illustrated in the right part of the upper row (gate R1 identifies contaminant CD45$^+$ leukocytes; the inset shows the corresponding adherent stromal layer (little square). An example of 7-AAD staining is illustrated in the second row. Some representative flow cytometry dot plots obtained from HM-MSC and NS-MSC analyzed at P1-P2 passages are shown. MSC from all samples were gated on CD45$^-$/7-AAD$^-$ events.

mean percentage of *in vitro* stromal confluence capacity was lower for samples from patients with hematologic malignancies (65%±24 SD) than for normal samples (97%±12 SD). These data support previous data showing a defective bone marrow microenvironment in some patients with hemalogic malignancies.[9-10]

Flow cytometry analysis of MSCs

In this study, a multicolor flow cytometric protocol was used to characterize cultured MSC.This protocol was based on the exclusion of cells expressing CD45$^+$ (known to be a hematopoietic marker) and 7-AAD (a nucleic acid staining compound, used for the recognition of dead and apoptotic cells). The mean percentage of 7AAD$^+$ cells was comparable in patients and controls (9%±3 SD and 8%±3 SD, respectively). As far as concerns the detection of contaminant hematopoietic cells within the stromal layers, the mean number of CD45$^+$ cells observed in HM-MSC at the first passage of culture (P1) was higher (25%±23 SD) than that in NS-MSC (13%±15 SD). The contaminant CD45$^+$ cells in normal donors coexpressed CD14 and CD11c antigens, thus indicating their monocytic nature, and tended to decrease with the age of the stroma. Interestingly, the flow cytometry analysis of acute leukemia samples showed that both fresh and cultured CD45$^+$ cells expressed several immunological markers, such as CD105 (8.8%±19.6 SD in ALL; 43.5%±39 in AML), CD44 (99.6%±8 in ALL; 94.6%±9 in AML); CD184 (93.5%±2.5 in ALL; 65.2%±26.6 in AML), CD106 (62.4%±54 in ALL; 39.7%±39 in AML), which were commonly used to characterize the MSC CD45-negative counterpart. Based on these results, it can be stated that the multiparametric flow cytometery approach used in this study proved to be a valid tool for identifying cultured viable CD45$^-$ MSC and discriminating contaminant malignant and normal hematopoietic CD45$^+$ cells. Based on forward and side scatter characteristics, two distinct MSC cell subpopulations were identified: (i) small-medium sized agranular cells, (ii) large hypergranular cells (Figure 1) partially supporting Colter's data.[11] Extensively cultured MSC (>30 days) showed a progressive increase in cell volume, and granularity. However, none of the immunological markers used in this study was capable of specifically distinguishing the agranular from the hypergranular cell components in either normal or pathological samples.

Cultured MSC showed significant differences in antigen expression between normal and malignant samples. In particular, we identified a group of antigens such as

Conditioned medium CM	% of BM CD90/CD105+ MSCs from which CM was obtained (Mean±SD)	N° junctions (Mean±SD)	N° tubules (Mean±SD)	TTL (Pixels) (Mean±SD)	MTL (Pixels) (Mean±SD)
CM1 (obtained from the BM of HM)	28±19	48±16	108±74	3737±2095	39±17
CM2 (obtained from the BM of NS)	88±2	33±31	63±60	2744±2566	39±25
VEGF	–	104±64	213±136	5169±1848	34±18
CTR	–	8±6	22±12	512±316	22±6

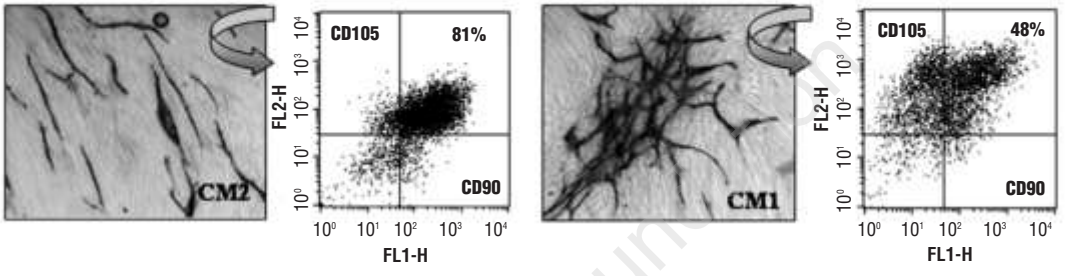

Figure 2. The angiogenic pattern of normal and pathologic bone marrow samples from which the conditioned medium were obtained are reported. Results from studies on angiogenesis are expressed as the number of junctions, number of tubules, the mean tubule length (MTL) and the total tubule length (TTL) (expressed in pixels). Vascular endothelial growth factor (VEGF, 50 ng/mL) was added to the wells as a positive control. The activity of TCS fresh medium without addition of endothelial factors was also evaluated as negative control to verify the adequacy of the system. The flow cytometry dot plots show the pattern of expression for CD90/CD105 antigens on NS-MSC after treatment with patients' CM (CM1) and normal CM (CM2, used as control); the correspondent angiogenic pattern generated by BM samples from which the CM were taken are showed in the pictures. A down regulation of $CD105^+/CD90^+$ MSC was detected in MSC cultured with angiogenic CM1.

CD59, HLA-ABC (class I), CD44 and CD29 that were homogeneously expressed in almost all cultured $CD45^-/CD34^-/7AAD^-$ MSCs, regardless of the cell source and time of the culture (Table 1). Interestingly, the mean fluorescence intensity for CD59 and CD29 antigens expressed by MSC and skin fibroblasts was very high (4^{th} logarithmic decade, Figure 2), thus supporting the diagnostic role of these molecules for the phenotypic characterization of cultured MSC. The large majority of cultured MSC were also positive for CD90, and CD105 molecules (Table 1 and Figure 1), but the frequency of $CD90^+/CD105^+$ MSC was significantly lower ($p< 0.05$) in HM-MSC (48%) than in skin fibroblasts (88%) or NS-MSC (74%). These changes did not correlate with the age of the culture and were statistically significant in ALL (31±8 SD, $p<0,041$), and MDS (58±20 SD, $p<0.048$) as compared with normal samples (Table 1 and Figure 1). This phenomenon could be explained by microenvironmental perturbations, or by the occurrence of aberrations in MSC[12] involving chromosomes 9 and 11, where CD105 and CD90 genes are located Interestingly, in MDS and ALL patients, a reduction in this MSC cell subset correlated with an increased angiogenic potential of bone marrow cells (Figure 2). These results are in agreement with data previously published by our group,[10] showing enhanced angiogenesis in MDS patients, thus suggesting that relatively undifferentiated MSCs can adopt a particular phenotype according to physiological requirements and to the micronenvironmental factors to which they are exposed. Our data further showed that the use of bone marrow-derived conditioned medium from HM with a reduced percentage of $CD90^+/CD105^+$ MSCs is capable of inducing a significant decrease in the number of $CD90^+/CD105^+$ MSCs (baseline mean value : 79%; after CM1 treatment: 48 %; after CM2 treatment, used as control, 81%, $p<0.002$) (Figure 2), thus suggesting an abnormal release of angiogenic cytokines by patients' bone marrow microenvironmental niches, which could negatively affect the expression of CD105 and CD90 immunological markers on MSC. These changes could be responsible for the defective hematopoiesis in the bone marrow of MDS.

The MSC subset co-expressing CD90 and CD106 antigens (Figure 1) comprised, respectively, less than 24% and 34% of the total HM-MSC and NS-MSC $CD45^-7AAD^-$ cells (Table 1). However, no reactivity for CD106 monoclonal antibody was found in skin fibroblastic suggesting the use of CD106 marker for the discrimination of bone marrow-derived MSC from fibroblasts residing in the skin. Our data further showed that cultured MSC from two out of 15 MDS patients expressed HLA-DR on the surface membrane of > 15%

MSC (range 16-25%); the biological significance of this anomaly is under investigation. With regard to the assessment of chemokine receptors on MSC, we showed that the percentage of $CD184^+$ (CXCR4-R, receptor for the stromal derived factor 1 -SDF-1) MSCs could be increased in some HM-MSC (mostly MDS), possibly supporting a more pronounced migration potential of MSC taken from pathological samples. In conclusion, the effects of the *in vivo* and *in vitro* microenvironment on MSC phenotype should be considered. In conclusion, we have described for the first time a 4-color cytofluorimetric protocol for the phenotypic characterization of HM- MSC, NS- MSC and skin fibroblast cell subsets as an important step in establishing the cell composition of *ex vivo* expanded MSC before their use in transplantation settings.

DC performed the experiments, isolated and cultured the cells, collected the cytofluorymetric data and wrote the paper. SM, LF, and MP conducted statistical tests and performed the cytofluorimetric analysis. GLC contributed to the conception of the study. FL was responsible for the interpretation of the flow cytometric data, designed the study and reviewed the manuscript draft. The authors declare that they have no potential conflicts of interst.

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