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.

Haematologica 2006; 91:364-368
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Cell cultures

Cell suspensions from healthy donors and patients were obtained as previously described. 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 reanalyzed 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 FACS Calibur 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 monoclonal antibodies was used: anti-CD45 (clone 2D1, APC), CD11c (S-HCL-3, PE), CD14 (M°P9, PE), CD31 (WM-59, PE), CD36 (NL07, FITC), CD90 (5E10, FITC), CD59 (P282-H19, PE), CD184 (12G5, PE), CD166 (3A6, PE) (Becton-Dickinson-Phar-Mingen, CA, USA); anti-CD106, (1G1b1- PE) (Southern Biotechnologies Associates-USA); HLA-DR (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 CD105⁺/CD166⁺ 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. 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.

Results and Discussion

Bone marrow-derived stromal culture

CFU-F assessment. The frequency of CFU-F/1x10⁶ 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
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 7-AAD+ 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 (8.3%±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 cytometry 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.10 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...
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 (4th 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 MSC12 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 microenvironmental 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 CD457AAD cells (Table 1). However, no reactivity for CD106 monoclonal antibody was found in skin fibroblasts 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%

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

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 tube length (MTL) and the total tube 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 treated 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.
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 cytofluorimetric 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 interest.

The authors would like to thank Dr. Massimo Dominici (University of Modena-I) for the critical reading of the manuscript. We thank Bob and Helga Johnson (Caltag Laboratories) for providing some MoAbs.

Manuscript received September 7, 2005. Accepted January 30, 2006.

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