Mesenchymal stem cells derived from bone marrow and leukapheresis show different putative subpopulations

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Abstract

Mesenchymal stem cells (MSCs), with their high proliferative and differentiation potential, in addition to their plasticity, are promising candidates for cell therapeutic applications. Bone marrow (BM) harvest is still the main source of MSCs in spite of being traumatic and painful. Clinical indications for peripheral blood-derived MSCs are rapidly increasing. This study was done to compare the biological properties of MSCs derived from BM and leukapheresis product regarding viability, fold expansion, cell cycle status and putative subpopulations. MSCs were isolated and cultured from BM and leukapheresis samples after stem cell mobilization. MSCs were characterized by morphology and immunophenotyping. Their viability, fold expansion and cell cycle status were compared. Estimation of putative cells among the mesenchymal population was done by dual expression of CD44 and Oct4. Leukapheresis derived MSCs were found to be comparable to BM-MSCs regarding their viability, fold expansion and cell cycle status, however they differ in their putative subpopulations. BM samples had significantly higher percentage of putative population than leukapheresis samples (18.38± 3.21% *vs* 5.43± 1.26%, P=0.009). These results indicated the possible isolation and expansion of MSCs from leukapheresis samples. The lower putative subpopulations among leukapheresis derived MSCs may be due to lack of BM microenvironment related factors needed to maintain pluripotency or due to the current methods of cell mobilization that have been optimized for hematopoietic stem cells rather than MSCs.

Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into a variety of cell types including; osteoblasts, chondrocytes, myocytes, adipocytes, endothelium and beta pancreatic islet cells.[1] MSCs are identified by typical morphology, adherence to plastic surface, trilineage differentiation potential in addition to their immunophenotypic pattern. They express variable levels of CD105, CD73, CD44, CD90 and CD271.[2] Oct4 (Octomer-binding Transcription Factor 4) is a master transcriptional regulator, which mediates pluripotency and regulates MSC cell cycle progression.[3]

Bone Marrow (BM) is the main source of MSCs although they can be isolated from adipose tissue, skeletal muscles, trabecular bones, liver, brain, placenta, cord blood, deciduous teeth and pancreas.[4] The procedure of BM harvest is traumatic and the amount of material extracted is limited. Therefore, exploring new sources and isolation techniques for obtaining such cells is of great interest. Major efforts have been made to examine techniques for isolation of MSCs from peripheral blood (PB); however, most of the studies suggested that MSCs which may be present in non-mobilized PB in healthy donors are too few to be detected and cultured for a long time.[5,6] MSCs were proved to be mobilized together with hemopoietic stem cells during G-CSF mobilization.[7] Mobilization involves disturbance of the normal cell cycle state and cell release mechanism that may compromise some of the biological properties of the stem cell population needed for their long term engraftment or regenerative capacity.

This study aimed at comparing BM and leukapheresis derived MSCs regarding their viability, proliferative capacity, cell cycle status and incidence of putative populations.

Materials and Methods

Samples

Institutional review board approval was taken before beginning of the work. Informed consent was taken from patients involved in the study. All patients were performing bone marrow aspiration or leukapheresis for other indications; none had to perform the procedure only for the study.

BM samples (n=15) were collected from individuals free from hematological diseases by posterior iliac instrumentation. 2-5 mL of BM was aspirated into heparinized vacutainers. Mononuclear cells (MNCs) were isolated by centrifugation over ficoll hypaque density gradient (density 1.077, Biochrom, Berlin) and suspended in Phosphate-Buffered Saline (PBS). Leukapheresis samples (n=15) were taken from stem cell donors who were under a G-CSF mobilization regimen consisted of injections of 10 μg/kg filgrastim once daily for 5days. Leukapheresis was performed on day 5 using an automated cell separator (COBE Spectra Apheresis System operated with version 7 LRS software, Gambro BCT, Lakewood, CO, USA). Samples were washed 3 times with PBS by centrifugation for 6 min at 1100 rpm. Cell debris was removed and the samples were re-suspended in PBS.

Culture and separation of mesenchymal stem cells

Culture and separation of MSCs were done as described before.[8] 5×10^6 MNC's was suspended in 10 mL Dulbecco's modified Eagle's medium low glucose (DMEM-LG) supplemented with 20% fetal calf serum (FCS), 100 μL penicillin (10,000 U/mL), 100μL streptomycin (10 mg/mL) and 20 μL amphotericin B (20 μg/mL) in T-75 flask and cultured for 7 days in humidified CO_2 incubator at 37°C. (All reagents are from Eurolone, Bedfordshire, UK). On day 7, the media was discarded and adherent cells were harvested by 5-10 min incubation in 0.05% trypsin with 0.02% EDTA in PBS to cover the bottom of the flask. One drop of FCS was added to stop the action of trypsin and left for 5 min. Two mL of Royal Park Memorial Institute medium (RPMI) was added and the contents were transferred to sterile tubes and centrifuged at 1500 rpm for 10 min. The supernatant was removed and the pellet was suspended in 2 mL RPMI. The harvested cells were examined for: i) Viability: using trypan blue dye exclusion test.[9] One part of 0.4% trypan blue was mixed with 1 part cell suspension (about 10^6cells/mL). Mixture was incubated for 1 -2 min at room temperature. A drop of the trypan blue/cell mixture was added to a hemacytometer and examined microscopically (X40). One hundred cells were counted and the percentage of viable cells was determined. The viable cells appear unstained and nonviable cells take up the dye and appear blue in color; ii) Immunephenotyping properties: the cells have been phe-

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notypically characterized by using a flowcytometer (Coulter Epics Elite, Miami, FL, USA). Cells were incubated with fluorescence-conjugated antibodies (marked with FITC-fluorescein isothiocyanate and PE-phycoerythrin fluorochromes, R&D Systems Inc., Minneapolis, MN, USA) for 15 min at room temperature in the dark. After two washing steps with PBS, cells were acquired in flowcytometer and analyzed. Cells were analyzed for expression of CD44, CD 90, CD 73, CD 105, and CD271. CD44 was used to evaluate proliferative capacity. Positive cells were counted out of the CD45 negative population; iii) Proliferative properties: i) Fold expansion of $CD44^+$ cells after 7 days: After detection of percentage of $CD44^+$ cells before and after culture, data was expressed as the fold expansion that was calculated using this formula: (CD44% after culture - CD44% before culture) / CD44% before culture; ii) Cell cycle status: Harvested cells after culture were examined for cellular DNA content using Coulter DNA Prep reagents kit (Beckman Coulter, Miami, FL, USA). Cell cycle phase distribution of nuclear DNA [*i.e.* quiescent (G0/G1) versus cycling cells (S)] was determined using Cell Quest software (Becton Dickinson, San Jose, CA, USA); iv) Incidence of putative cells: Oct4 expression in $CD44^+$ cells: percentage of the cells expressing both CD44 and Oct4 was determined after culture by flow cytometric analysis using (Coulter Epics Elite, Miami, FL, USA).

Statistical methods

Statistical analysis was done using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Data were statistically described in terms of range, mean ± standard deviation (± SD) and percentages when appropriate. Comparison of quantitative variables was done using Mann Whitney *U* test for independent samples when not normally distributed. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlation between various variables was done using Spearman rank correlation equation for non-normal variables. A probability value (P) less than 0.05 was considered statistically significant.

Results

Morphology and viability

MSCs were identified morphologically as colonies of fibroblast like cells adherent to plastic surface (Figures 1 and 2). The viability of leukapheresis derived MSCs was comparable to that of BM-MSCs (89.30±8.06% *vs.* 90.45±7.41%, P=0.914).

Immunophenotyping results

The results of Immunophenotyping are shown in Table 1 and Figure 3.

Proliferative status

i) Fold expansion

The mean fold expansion of leukapheresis derived MSCs was comparable to that of BM-MSCs (20.69±37.78 *vs* 25.09±33.36, P=0.624).

ii) Cell cycle analysis

The cell cycle phase distribution of leukapheresis derived MSCs was; 94.51%±3.21% quiescent cells and 4.78%±2.87% cycling cells.

The cell cycle phase distribution of BM-MSCs was; 96.24%±3.02% quiescent cells and 3.33%±2.71% cycling cells.

No statistically significant difference could be detected between Leukapheresis derived MSCs and BM-MSCs regarding the mean percentage of quiescent cells (P=0.231) and cycling cells (P=0.274) after culture (Figure 4).

Figure 1. Mesenchymal stem cells colonies (Light inverted microscope X40).

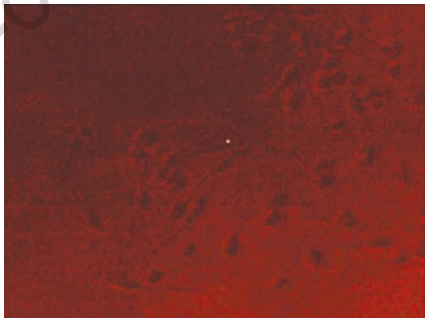

Figure 2. Mesenchymal stem cells appear as fibroblast like cells (Light inverted microscope X200).

Putative populations

Oct4 expression

The mean percentage of putative clonogenic population expressing both CD44 and Oct4 among BM samples after culture was significantly higher than that of leukapheresis samples (18.38± 3.21% *vs* 5.43± 1.26%, P=0.009).

Discussion

MSCs comprise a heterogeneous population of cells. Whether the source of MSC is the cause of this heterogeneity or it is the property of this lineage is yet to be determined. The growing interest in the use of MSCs in regenerative medicine raises the needs for easy and

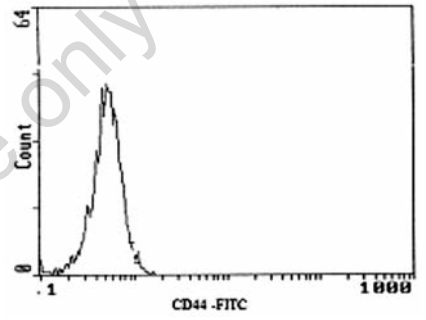

Figure 3. FACS analysis of immunephenotype profile for mesenchymal antigen CD44.

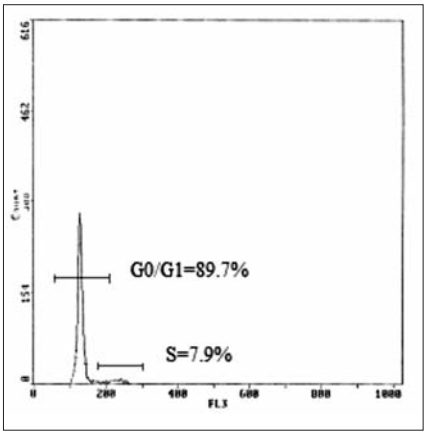

Figure 4. Histogram plot of mesenchymal stem cell cycle status showing quiescent cells (89.7%) and cycling cells (7.9%).

Table 1. Immunophenotyping of bone marrow and leukapheresis samples.

Variable	BM	Leukapheresis	*P*
CD44+ cells B (%)	0.16±0.14	0.95±1.27	0.214
CD44+ cells A (%)	4.19±5.39	6.94±9.71	0.359
Fold expansion	25.09±33.36	20.69±37.78	0.624
Putative population (CD44/Oct4+) A (%)	18.38 ± 3.21	5.43 ± 1.26	0.009

Data is expressed as mean ±SD. B, before culture; A, after culture; BM, bone marrow.

safe sources for large numbers of MSCs. The introduction of hematopoietic stem cell (HSC) mobilization was a milestone in the practice of HSC transplantation. Likewise, if a constant, safe source of MSCs harvest can be achieved; it will be a breakthrough in the use of MSCs in regenerative medicine.

In this study, we have isolated, expanded and compared colonies of MSCs from BM and leukapheresis regarding their number, viability, progenitor status or *stemness*, and cycling status. MSCs were identified according to the minimal criteria to define human MSCs proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy.[10] MSC colonies were plastic-adherent when maintained in standard culture conditions and expressed CD44, CD 90, CD73, CD105 and CD 271 that are markers of MSCs.[11] These cells retained the capacity to differentiate into adipogenic, osteogenic and chondrogenic lineages (data not shown). These cells expanded in culture with a mean fold expansion of 25.09 for BM-MSCs and 20.69 for leukapheresis derived MSCs. The fold expansion, viability and cell cycle status of leukapheresis derived MSCs were comparable to that of BM-MSCs.

However, the BM samples have significantly higher percentage of putative progenitor population expressing both CD44 and Oct4 compared to leukapheresis samples after culture. In a previous study, MSCs isolated from BM, mobilized PB and umbilical cord blood were found to express Oct4; however, the three sources were not compared regarding the level of expression.[12] Oct4 is a transcriptional binding factor present in undifferentiated cells. It is considered as pluripotency marker as it is responsible for self-renewal of embryonic stem cells. Down regulation of Oct4 coincident with stem cell differentiation and loss of its expression leads to differentiation.[13] The lower percentage of putative progenitor population among leukapheresis derived MSCs may limit the potential of future use of leukapheresis as a source of MSCs for clinical applications. The number of putative progenitors should be enough to sustain a steady supply of cells that upon proliferation and commitment may serve as precursors for a number of nonhematopoietic tissues after stem cell transplantation.

The lower putative subpopulation among MSCs derived from leukapheresis product in this study may be due to loss of BM microenvironment related factors needed to maintain pleuripotency. It is more likely that tissue-specific stem cells exist in a multipotent state in vivo, and that this potency is governed not only by Oct4 but rather by intrinsic and extrinsic factors from the microenvironment in which they exist.[14] Also the protocol used for mobilization of stem cells may be another factor as it has not been optimized for MSCs yet. Other investigators reported that the number of MSCs isolated from mobilized and non-mobilized PB by conventional plastic adherence that could be further expanded was negligible in all the samples tested.[6,15]

Although G-CSF is relatively effective in mobilizing $CD34^+$ cells in the circulation,[16] this strategy has not been optimized for non-hematopoietic progenitors, which show an inverse relationship with the number of mobilized $CD34^+$cells.[17]

It is possible that other cytokines such as IL-8 may be more effective for their mobilization.[18] Also, in a recent study, urokinase receptor (uPAR) was required to mobilize MSCs from BM of mice stimulated with G-CSF in vivo. An insignificant amount of MSCs was mobilized in $uPAR^{-/-}$ mice, whereas in wild-type animals G-CSF induced an eight-fold increase of mobilized MSCs.[19]

We concluded that, MSCs isolated from leukapheresis could be expanded in vitro in a potential similar to BM-MSCs, however, they differ in their putative subpopulations. MSC mobilization, isolation and culture need to be optimized before their use for clinical application being easier for collection and less invasive and painful than BM harvest. Further studies including analysis of clonogenic and differentiation potential of MSCs derived from both BM and leukapheresis are recommended for determination of the best source for MSCs isolation.

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