In Search of Mesenchymal Stem Cells: Bone Marrow, Cord Blood, or Peripheral Blood

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Objective: Mesenchymal stem cells (MSC) are capable of self-renewal and differentiation into various connective tissue lineages. Therefore, they have attracted a lot of attention from investigators in the context of stem cell therapies. In our study, we have evaluated the frequency, phenotype and differentiation potential of MSC in bone marrow (BM) cord blood (CB) and mobilized peripheral blood (mPB).
methods: Sixteen CB, 11 BM and 19 mPB were obtained from normal donors. Mononuclear cells suspended in culture medium and seeded in culture flasks. Flasks were incubated in a CO2 incubator with a change of culture medium every 4 days and passaged when fibroblast like cells reached confluence. For every other passage, MSC were examined for CD13, CD44,CD34 by flow cytometry and induced to differentiate into adipocytes and osteocytes.
Results: All BM samples produced MSC that survived multiple passages in mesenchymal culture medium over 4 months. CB and mPB samples produced a non-confluent adherent layer of heterogeneous cells, and did not proliferate beyond the first passage. Immunophenotype of BM-derived MSC in every other passage were $CD34^-$, $CD13^+$ and $CD44^+$, the adipogenic and osteogenic differentiation were confirmed by Oil-red O and Von Kossas staining, respectively.
The mentioned evaluation for mPB and CB were not attempted because these were not confluent even in the first passage.
Conclusion: In our study, only human BM cells produced MSC. These cells are positive for MSC surface proteins and differentiate into MSC lineages.

Introduction:

Many years of work on the origins of blood cells led to the concept of the hematopoietic stem cell that could serve as a progenitor for all blood cell types. The concept of a similar multipotential bone marrow stem cell for connective tissues was first presented by Owen [(1)] and suggested that differentiated cell types found in bone marrow stroma might derive from a common progenitor, or stem cell. This concept was further developed by Caplan to include all of the mesoderm-derived lineages [(2)] . The presence of Mesenchymal stem cell (MSCs) in bone marrow is consistent with accumulated data from many labs using multiple species[(3-5)].
MSCs comprise a rare population of multipotent progenitors capable of supporting hematopoiesis and differentiation into multiple lineages including: osteogenic[(6)], adipogenic[(7)], chondrogenic[(8)], neural[(9)] and cardiomyogenic[(10)].
Due to this ability, the cells are currently being tested for their potential use in cell and gene therapy for a number of human diseases.

Because the MSCs coexist with hematopoietic stem cells (HSCs), in bone marrow and both of these stem cells originate from the mesodermal germ layer in embryogenesis, we intended to isolate MSCs from sites which HSCs normally exist and circulate thought, meaning bone marrow (BM), cord blood (CB) and mobilized peripheral blood (mPB).

Materials and Methods

In this study we collected 11 BM, 16 CB and 19 mPB samples. All samples were cultured to obtain MSC. Each culture where MSCs proliferated, was passaged sequentially, for about 130 days. For every even passage, some of the expanded MSC was used to examine their ability to form differentiaed adipocyte and osteocyte, and immunophenotyping. For every odd passage, the cells were examined for telomere length.

Samples Collection

Ten ml of each BM and 2 ml of each mPB was obtained from healthy hematopoietic stem cell transplant donors at the time of collections. The mean age of BM donors was 25 (range 2.5 to 63

years) and for mPB donors it was 26.7 (range 14 to 55 years).
Sixteen Umbilical Cord Blood (UCB) were obtained from full-term deliveries after receiving informed consent according to a protocol approved by the cord blood Bank's ethics committee. The volume of CB collected ranged from 20-55 ml. The majority of RBCs were depleted by hydroxyethylstarch (HES 6% feresenius) sedimentation method.

Ex Vivo MSC Culture

Collected samples were mixed with two volumes of RPMI 1640 (Sigma-Aldrich) and then loaded on ficoll (density = 1.077 g/ml) (R&D). Cells were centrifuged at 2000 rpm (540 × 9) for 20 minutes at 25° C. The mononuclear cells (MNCs) at the interface were washed twice in RPMI 1640 supplemented with penicillin (20U/ml), and streptomycin (20 μg/ml) (Gibco). Pelletes were resuspended at 1×10^6 cells/ml (BM) and 2×10^6 /ml (CB and mPB) in Dulbecco's modified Eagle medium, low glucose (DMEM-LG) (Gibco) with 10% felal bovine serum (FBS) (Gibco) for BM and 20% for CB and mPB cells. Twenty-one ml of Cell suspension supplemented with penicillin/streptomycin was plated in a 75 cm^2 flask (Greiner bio-one).
MSC cultures were incubated in humidified incubators with 5% CO2 and initially allowed to adhere for 48 hours, followed by media change every 4 days. When fibroblast-like cells at the base of the flask reached more than 90% confluence, adherent cells were detached with 0.25% trypsin EDTA and replated (passaged) at a density of 1×10^4 cells/ml in two 75 cm^2 flasks.

Flow Cytometry

Surface expression of CD44, CD13 and CD34 was determined at every even passage on culture-expanded MSCs. The monoclonal antibodies used were anti-CD44 fluorescein isothiocyanate (FITC), anti-CD13 phycoerythrin (PE), and anti-CD34 FITC (all from Dako). Relevant isotope control antibodies were also used. Flow cytometry was performed on a FACScan (Becton Dickinson), and data were analyzed with Cellquest software.

Differentiation of MSC

The differentiation ability of MSCs was assessed in every even passage culture. Adipogenic differentiation was assessed by incubating the cells with DMEM-LG and 10% FBS supplemented with 0.5 μM hydrocortisone, 0.5 μM isoboutyl methylxanthine, 60μM indomethacin (Sigma-Aldrich), and 10μg/ml insulin (R&D) for 2 to 4 weeks. Adipocyte was recognized by the accumulation of lipid-containing vacuoles which stained red with Oil red O. Osteogenic differentiation was assessed by incubating the cells with DMEM-LG and 10% FBS supplemented with 0.1 μM dexamethasone, 10 μM β-glycero-phosphate, and 50 μM ascorbate (all from Sigma-Aldrich) for 2 to 4 weeks. To assess mineralization, cultures were stained with silver nitrate (Von Kossa's staining).

Determination of telomere length

Telomere length of human cell samples may range over one order of magnitude. Even within a population of and on single cell level, considerable heterogeneity of telomere length was shown. Therefore, analyzing a population of cells provides the average telomere length of the telomeres in the sample, indicated by a smear at a specific length compared to the molecular weight standard.
The Telemere Restiriction Fragments (TRFs) comprise not only the variable terminal array but also the subtelomeric region, which has been estimated to be about 3-4 kbp in length.
After exposure of the blot to an X-ray film, an estimate of the mean TRF length can be obtained by visually comparing the mean size of the smear to the molecular weight marker.
However, for quantitative measurements of mean TRF length, the chemilumines-cence signals were scanned and calculated by the multianalizor (Bio-rad) software.
The positive control DNA's (Control-DNA, low and Control-DNA, high) supplied with the *T*elo *TAGGG* Telomere Length Assay are purified genomic DNA from immortal cell lines. The mean TRF length of these cell lines has been determined to be 3.9 kbp and 10.2 kbp, respectively.

Statistical analysis

All data are presented as mean ±SD. The data collected in ten passages was compared by Repeated Measurement analysis of variance test.

P. values of less than P‹ 0.05 were considered significant.

RESULTS

The results of BM-culture

Three days after plating the mononuclear cells of BM in culture flasks, the mean number of fibroblast like cells were about 6800 (90 cell/cm^2) of total heterogeneous cells. Gradually, the fibroblast like cells (MSC) increased and the heterogeneous cells decreased in the culture.

The predominant cell after 2 weeks of culture was fibroblast like (figure 1) and dislodged readily upon trypsinization in 3 minutes.

Primary culture took between 11 and 25 days (mean 15.6 day). The total count of harvested cells from the primary culture was between 5.4 $\times$ 10^5 and 39.0 $\times$ 10^5 (mean: 17.0 $\times$ 10^5 cells). The mean number of BM harvested cells and the mean time of confluency from the second culture on was about 23.9 $\times$10^5 cells and 14 days respectively. The average number of cells that proliferate during each passage and the average confluency time in the various passages were different. Therefore, to analyze these differences we performed the Repeated Measurement Analysis of Variance statistical test, but we did not find any significant difference between the different passages. Keeping in mined that the count of plated cells were 4.2 x 10^5 cells (except primary culture) in two 75 cm^2 flasks for each passage, the mean cell dose in every passage was increased 5.5 times (range: 2.6-9.5 times).

The goal of this study was keeping the MSC in the culture by multiple passages up to 120 days. Eight of the 11 BM samples survived between 112 and 164 days. Three samples survived up to 63, 95, and 100 days, respectively, and didn't finish the procedure because of contamination.

From 11 BM samples 5 were passaged 10 times, 2 were passaged between 5 and 6 times (because of contamination) and the 4 remaining samples were passaged between 8 to 9 times during the procedure.

The results of mPB and CB culture

mPB and CB were cultured the same as BM, but produced a scanty heterogeneous adherent population of cells (Fig 2& 3). The mean frequency of fibroblast like cells in primary culture was 51% (range 20-90%), and 10% (range 2-20%) respectively. The mean count of harvested cells from the first passage was 2.4 $\times$ 10^5 (range 0.2-3.8), and 2.67 $\times$ 10^5 (range 0.03-3.9) respectively, which indicated a reduction with regard to reseeded cell does (4.2 $\times$ 10^5 cell).

Duration time of mPB cell culture was 30.3 days, with an average range of 12-47 days and the mean time of CB culture was 29.75 days (range 4-73 days).

The cultures, which had more adherent cells in their flasks, were passaged, but did not proliferate again. Within the first passage, these fibroblast like cells were reduced or disappeared completely.

Immunophenotype of BM MSCs

The immunophenotype of BM MSCs was determined by flow cytometry using the antibodies against CD44, CD13 and CD34. The mean percent of CD44$^+$ cells in different passages was 50 to 70% and the mean percent of CD13$^+$ cells was around 50%. A small population (mean ~ 0.2%) of MSCs express the CD34 antigen in different passages. The mean percent of expression of the three CD antigens in 5 different passages was compared using Repeated Measurement Analysis of Variance test, however there was no significant difference in their expression .

For the heterogeneous population of mPB and CB cultures which were relatively resistant to trypsinization, immunophenotyping was not performed because there was no proliferative or morphological evidence that these were a kind of stem cell like mesenchyme in origin.

Differentiation of BM MSCs

BM-derived MSC were differentiated along adipogenic and osteogenic lineages. Oil red O staining for adipocyte and Van Kossas and Alkaline phosphatase staining for osteocyte was positive. Adipogenic differentiation of MSC after 2 weeks incubation in adipocyte culture media demonstrated scattered aggregates of Oil red O positive staining cells. But within the same time limit, the osteocyte culture media exhibited a homogeneous and relatively abundant number of Van Kossas positive cells.

The differentiation ability of MSC varied in the 5 different passages. Every one of the samples cultured in osteogenic and adipogenic mediums

differentiated to osteocyte and adipocytes up to the 4th passage. In the 6th passage, 10%, in the 8th passage 25% (6 of 8), and in the 10th passage 20% (4 of 5) of the samples lost their osteogenic differentiation potential. While in the same passages (6, 8, 10), the adipogenic differentiation potential declined to 90%, 50% and 40% respectively.

Telomere length of BM MSC

Telomere signals appeared as a broad smear of densities. The range of the Mean telomere restriction fragment length (mTRFL) varied between 10.2 kbp to 7.8 kbp in different BM samples and in different passages. mTRFL was decreased from an average of 9.19 kbp in the first passage to 8.75 kbp in the 9th passage, giving a telomere shortening of about 0.44 kbp during 130 days of expansion.

DISCUSSION

The stem cell nature, that is, the ability of hMSCs to differentiate along multiple lineages of mesenchymal tissues, and the ease with which these cells can be cultured and expanded in vitro, are an attractive area of tissue engineering and cell-based therapies in several human diseases.

In our morphological study, the adherent cells in the MSC culture of BM produced a homogeneous confluent monolayer (Fig 1), whereas CB and mPB cultures produced a heterogeneous mixture of non-confluent cells, which could not be passaged (Fig 2 & 3).

It was reported that MSCs could be purified on the basis of their ability to adhere to plastic [11]. In our BM cultures, the adherent MSCs were detached easily using 0.25% trypsin in less than 3 minutes, but the adherent cells in CB and mPB cultures were partially resistant to trypsinization even after 30 minutes of incubation at 37ºC. We passaged the partially detached cells from the CB and PB flasks and refed the remaining attached cells at the base of the flasks. In spite of changing the culture medium regularly and keeping them alive in a CO2 incubator, they didn't proliferate even in two months. The characteristics and behavior CB and PB cultures were not similar to BM cultures. The fibroblast-like cells, which appeared in the first few days of primary CB and PB cultures, disappeared with time or sub-culturing.

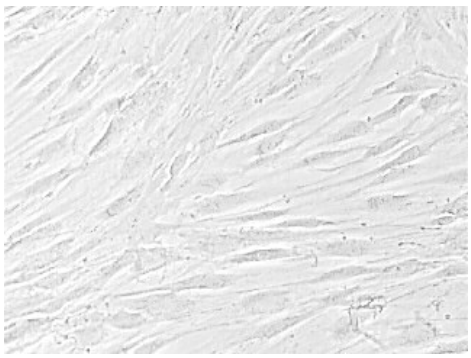

Fig 1.Homogenous monolayer of BM MSC culture.

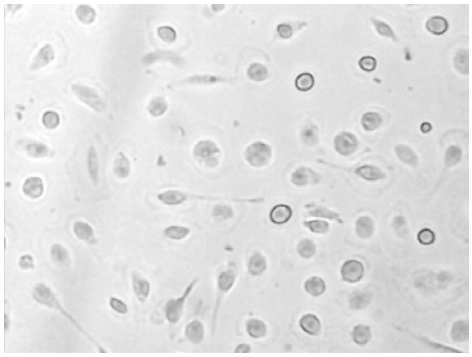

Fig 2. Heterogeneous cells of CB culture.

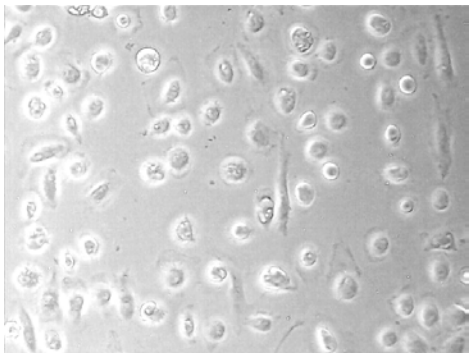

Fig 3.Heterogeneous cells of mPB culture.

The harvested cell counts were less than seeded cell count and heterogeneous cells remained non-confluent until discarded.

All these criteria indicated that MSC did not proliferate in our CB and mPB cultures.

In contrast to our study, some investigators were able to isolate mesenchymal progenitor cells from umbilical cord blood (UCB). Erices et al [12] have shown that 24% of their UCB harvests from preterm and term deliveries produced a homogeneous adherent population layer, with the expression of the mesenchymal-related antigens. However, they used different culture conditions (α-MEM containing 20% FBS) and most of their samples were from preterm deliveries.

Romanov et al[13] have suggested that MSC-like cells are present in the sub-endothelial layer of

the human umbilical cord vein and can be successfully isolated, cultured, and expanded using routine technical approaches. In contrast, Waxler et al [14] (like our current study) claimed MSC could not be isolated or successfully cultured from UCB.

The controversial data concerning the presence of MSCs in UCB, maybe related to UCB harvest methods. Keeping in mind the presence of MSC in the subendothelial layer of cord vein in Romanov's reports, milking or pushing the umbelical cord at the time of CB harvest can cause the release of a few MSCs from the damaged subendothelial layer. All our CB samples were collected after full-term normal deliveries and our method allowed blood flow to the collection bag by gravity without any pressure. So, the presence of MSCs in CB samples in other studies might be related to gestational age or the collection method.

Ralf Huss et al[15] reported the presence of the precursor of $CD34^{-/low}$ fibroblast-like cells in the peripheral blood. Fernandez and colleagues detected MSC from the PB collections of patients with breast cancer[16] whereas Wexler et al[14] reported no evidence of MSC in mPB from normal donors.

In contrast to other studies[12, 14,] which reported $CD34^{-}$ for MSC, we found that our BM-derived MSC were low level positive (mean 0.2%) for the CD34 antigen. To examine whether this low positive result is a consequence of inaccuracy and non-specific reactions or not, we had a control for every sample tested. First we set up the flowcytometer with the negative control of the sample in mind (which was completely matched) and constructed the graph, and then we superimposed the result over the sample graph. Eventully, we analyzed the obtained comparative graph. Therefore, if the result is positive we can be satisfied that we have a reliable answer.

Some studies reported that $CD34^{+}$ cells originate from $CD34^{-}$ adherent cell. For many years, it was thought that stromal cells are $CD34^{-}$, while even quiescent stem cells express at least low levels of CD34 antigen.[17]

Singer et al [18] described adherent common precursors for stromal and hematopoietic cells. Ralf Huss[15] believed that there is a common progenitor $CD34^{-}$ cell for both hematopoietic and mesenchymal stem cells. This type of cell can give rise to various specified tissues, depending on growth factor mediated signals and internal signal control. Nevertheless, marrow stroma-derived fibroblast-like $CD34^{-}$ cells can give rise to $CD34^{+}$ cells with hematopoetic characteristics with regard to colony formation and long term culture initiation.[19] These fibroblast-like cells can spontaneously differentiate into $CD34^{+}$ nonadherent cells, because of the outocrine and paracrine production of SCF [20]. Therefore, maybe the presence of a few $CD34^{+}$ cells was due to spontaneous differentiation of the $CD34^{-}$ adherent MSC.

We conclude that MSCs from CB and mPB do not proliferate in our culture condition. In contrast, MSC from 100% of our BM samples expanded easily in our culture medium. Therefore, BM is the best source of human-mesenchymal stem cell for both research purposes and cell or gene therapy. How useful will they be in therapy? Some studies show hopeful signs ([21], Mohyeddin et al unpublished data), but only the future can tell.

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