

Isolation and identification of putative mesenchymal stem cells from bone marrow

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Abstract

Mesenchymal stem cells are pluripotent progenitor cells found in bone marrow that have the capacity of differentiating into bone, cartilage, tendon, fat, muscle, and early progenitors of neural cells under certain conditions. It has also been shown that they support haematopoiesis in culture. We have successfully isolated mesenchymal stem cells from a bone marrow sample of an anaemic patient by using DMEM supplemented with 10% of FBS. They could be distinguished from other cells by their tendency to adhere to tissue culture plastic. The adherent time was about three to seven days. Microscopically, the cultured cells showed morphology resembling fibroblast and they divided actively. Early passage cultures were heterogenous and contained four morphologically distinct cell types; long spindle-shaped cells, star-shaped cells, petal-shaped cells and large flattened granular cells with vacuoles. Identification of mesenchymal stem cells was carried out by cytochemical analysis and immunophenotyping by flow cytometry. Identification of these cells is vital as they have properties that appear to make them ideal candidates for studying differentiation and make them suitable for cellular and gene therapy.

Key words : Mesenchymal stem cells, bone marrow, DMEM, cytochemical analysis, immunophenotyping.

INTRODUCTION

Mesenchymal stem cells (MSC) are adult human pluripotent progenitor cells found in bone marrow^{1,2}, peripheral blood³, cord blood^{4,5} and liver⁶. They have self-renewal capability without differentiation in long term culture⁷. However, when stimulated under certain conditions, the cells could differentiate into adipocytes, chondrocytes, osteocytes, astrocytes, tenocytes, cardiomyocytes, hepatocytes, neurons, muscle cells, endothelial and endodermal cells^{5,8,9}. In baboon, autologous and allogenic MSC infused intravenously are capable of homing to the bone marrow and have the capacity to establish residence within the bone marrow¹⁰. It was also shown that MSC could incorporate into various types of tissues including thymus, liver, blood vessels, bone, cartilage, muscle, lung and spleen after systemic injection^{11,12}. It is plausible that the entire genome of the MSC has the potential to turn on any aspect of that genome and be any type of cell. MSC have low immunogenicity and suppress alloreactive T cell responses¹³. Thus, MSC have potential clinical utility as cellular vehicles for systemic or tissue-specific delivery

of therapeutic genes as well as in tissue engineering. Current application of MSC includes the use of MSC to treat osteogenesis imperfecta children, central nervous system disorders, diabetes melitus, Fabry's disease, myocardial infarction, hepatic failure and breast cancer patients¹⁴⁻¹⁷. MSC were also shown to express mRNA for IL-6, IL-11, GM-CSF, and M-CSF, thus they play a role in *in vivo* haematopoiesis¹⁸.

Despite the great interest in MSC, there is still no well-defined protocol for isolation and expansion of the cells in culture. Most experiments isolated MSC from bone marrow mononuclear cells based on their tight adherence to tissue culture plastic^{15,16,19}. The isolated cells were initially heterogenous, and were difficult to be distinguished from other adherent cells. Several methods have been developed to prepare more homogenous populations by sorting using flow cytometry or immunomagnetic beads selection based on their differences in size^{11,20}, or on some specific surface markers, such as Sca-1²¹, STRO-1²², CD 45, Glycophorin A and Nerve Growth Factor Receptor (NGFR)²³⁻²⁵, but none of these protocols has earned wide acceptance.

Here, we reported that we had successfully isolated MSC from the bone marrow of an anaemic patient by direct plating of mononuclear cells in culture medium, and characterized them by immunophenotyping and cytochemical staining.

MATERIALS AND METHODS:

Bone marrow preparation and cell culture

To isolate human MSC, bone marrow aspirates were taken from the iliac crest of a patient who came for routine check up in Hospital Universiti Kebangsaan Malaysia after informed consent and under a protocol approved by the UKM Research Committee and Ethics Committee. The patient suffered from megaloblastic anaemia.

Five ml of bone marrow aspirates was layered on top of 3 ml Ficoll-Paque (Amersham Biosciences; Uppsala, Sweden) and centrifuged at 127.05 g for 30 minutes. The mononuclear cells in the interface (density gradient 1.073 g/l) were extracted and washed twice with culture medium by centrifuging at 31.15 g for 10 minutes. The pellet cells were then suspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco; Grand Island, New York, USA) and the viability of cells was counted by haematocytometer and Trypan Blue staining. Our result showed that the percentage of viable cells was 99.7%. The cells were then seeded at a density of 1×10^7 cells in a 25 cm² plastic flask containing DMEM supplemented with 10% Fetal Bovine Serum (FBS) (Gibco; Grand Island, New York, USA). The flask was then incubated in 5% CO₂ in air and monitored daily. Once the cells reached confluency, they were detached by 1 ml of 0.25% Trypsin-EDTA (Gibco; Grand Island, New York, USA) and replated again into new flasks at the similar cell density. Characterization of DMEM-derived adherent cells was performed by using cells from the third and fourth passages after four to five weeks from the initial culture.

Cytochemical staining of DMEM-derived adherent cells

The DMEM-derived adherent cells *in situ* were analysed for the following cytochemical markers: periodic acid-Schiff (PAS), α -naphthyl acetate esterase (NSE), sudan black B (SBB), naphthol AS-D chloroacetate esterase (NASDA), and alkaline phosphatase (ALP). In all cases, analyses as well as the selection of positive and negative controls were performed according to the manufacturer's guidelines (Sigma Diagnostics Kits; St. Louis, Missouri, USA).

Immunophenotyping of DMEM-derived adherent cells

To detect surface antigens, aliquots of DMEM-derived adherent cells were washed twice with phosphate-buffered saline (PBS) (Gibco; Grand Island, New York, USA) pH 7.2 after detachment with 0.25% trypsin-EDTA. The pellet cells were then diluted again with PBS according to pellet size. The diluted cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) -conjugated monoclonal antibodies for 20 minutes. The antibodies used are CD3, CD 11c, CD13, CD14, CD 34, CD 45, CD 54, CD 56, and HLA-DR. The antibodies were purchased from Becton-Dickinson (Ontario, Canada, USA). After incubation, the cells were washed again with PBS to remove the untagged antibody. The cells were rediluted with PBS and were subjected to flow cytometry analysis. The preparation of sample for immunophenotyping was performed according to the procedure recommended by Becton Dickinson. The CellQuest software and FacsCalibur (Becton Dickinson; Ontario, Canada, USA) were used for the flow cytometry analysis.

RESULT

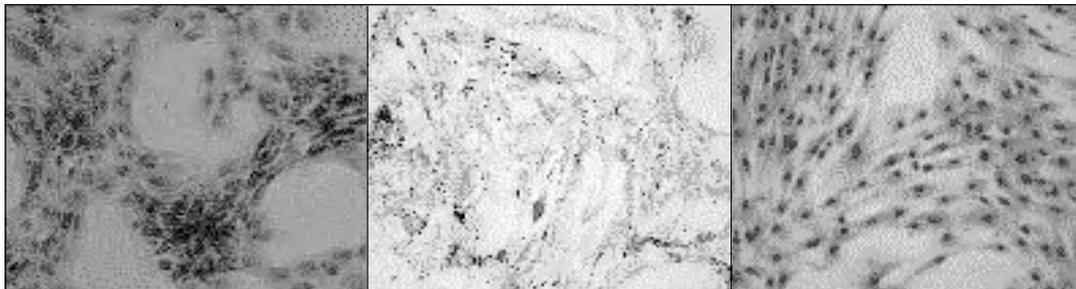
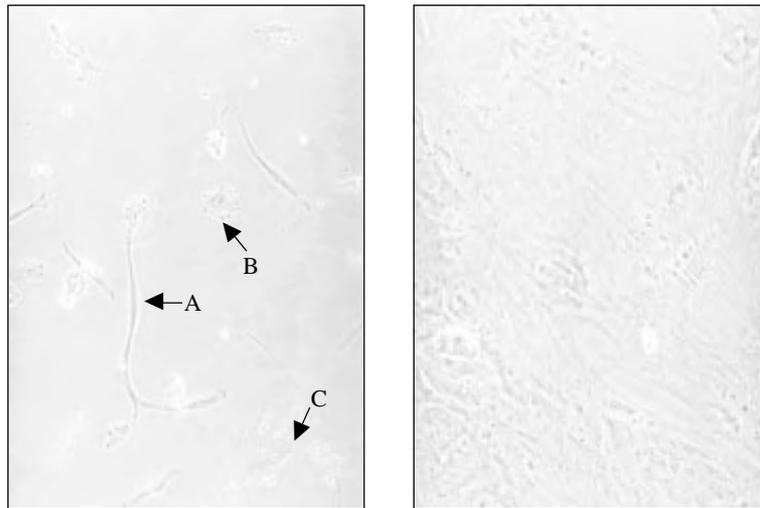
Microscopic observation

Initial culture of mononuclear cells extracted from bone marrow yielded a heterogenous population of cells adhered on the plastic flask. The initial adherent time was three to seven days. There were four types of morphologically distinct cells, i.e., the long spindle-shaped cells, star-shaped cells, petal-shaped cells, and large-flattened granular cells with vacuoles (Fig. 1). The long spindle-shaped cells were the cells that divided actively. The divided new cells were lifted before they were separated from the parent cells and readhered again as single cells. As the cells were passed twice, the long spindle-shaped cells decreased in number, whereas the large-flattened granular cells with vacuoles became more abundant when they reached confluency (Fig. 2 and 3). When these large cells were detached and replated, we noticed that the large cells attached as star-shaped cells before turning into large-flattened granular cells with vacuoles again. The long spindle-shaped cells were observed to appear in small amount, dispersed, and divide very slowly at this time. The large cells were passed six times and were noticed to experience apoptosis. The petal-shaped cells were never seen again after the first passage.

Characterization of DMEM-derived adherent cells

The large-flattened granular cells from passage three and four were then cytochemically stained and immunophenotyped to characterize their biological properties. The result showed that the cells were positive to PAS (strongly) (Fig. 4)

and NSE stains (Fig. 5), but negative to NASDA, SBB and ALP stains. As detected by flow cytometry, these cells expressed CD 13 strongly, CD 14 and CD 56. However, they did not express CD3, CD 11c, CD 34, CD 45, CD 54 and HLA-DR (Fig. 6).



(Clockwise from above: FIG. 1, FIG. 2, FIG. 3, FIG. 4, FIG. 5)

FIG. 1: The morphological characteristics of the DMEM-derived adherent cells in early culture under X100 magnification. There were four distinct types of cells, i.e., the long spindle-shaped cell (A), petal-shaped cell (B), large flattened granular cell with vacuoles (C) and star-shaped cell (not shown).

FIG. 2: The confluent state of the large flattened granular cells with vacuoles (X200) in the 3rd passage. The long spindle-shaped cells and petal-shaped cells were not seen.

FIG. 3: This picture depicts the enhanced feature of MSC after being stained with May Grunwald Giemsa. Note that the cells showed fibroblastoid feature and they spreaded their body broadly.

FIG. 4: This picture clearly shows that the MSC were positive to Periodic Acid Schiff stain. The positive cells had red granulations.

FIG. 5: α -Naphthyl Acetate Esterase stain. Black granulations were noticed in the cytoplasm of the cells indicating that MSC have this cellular enzyme.

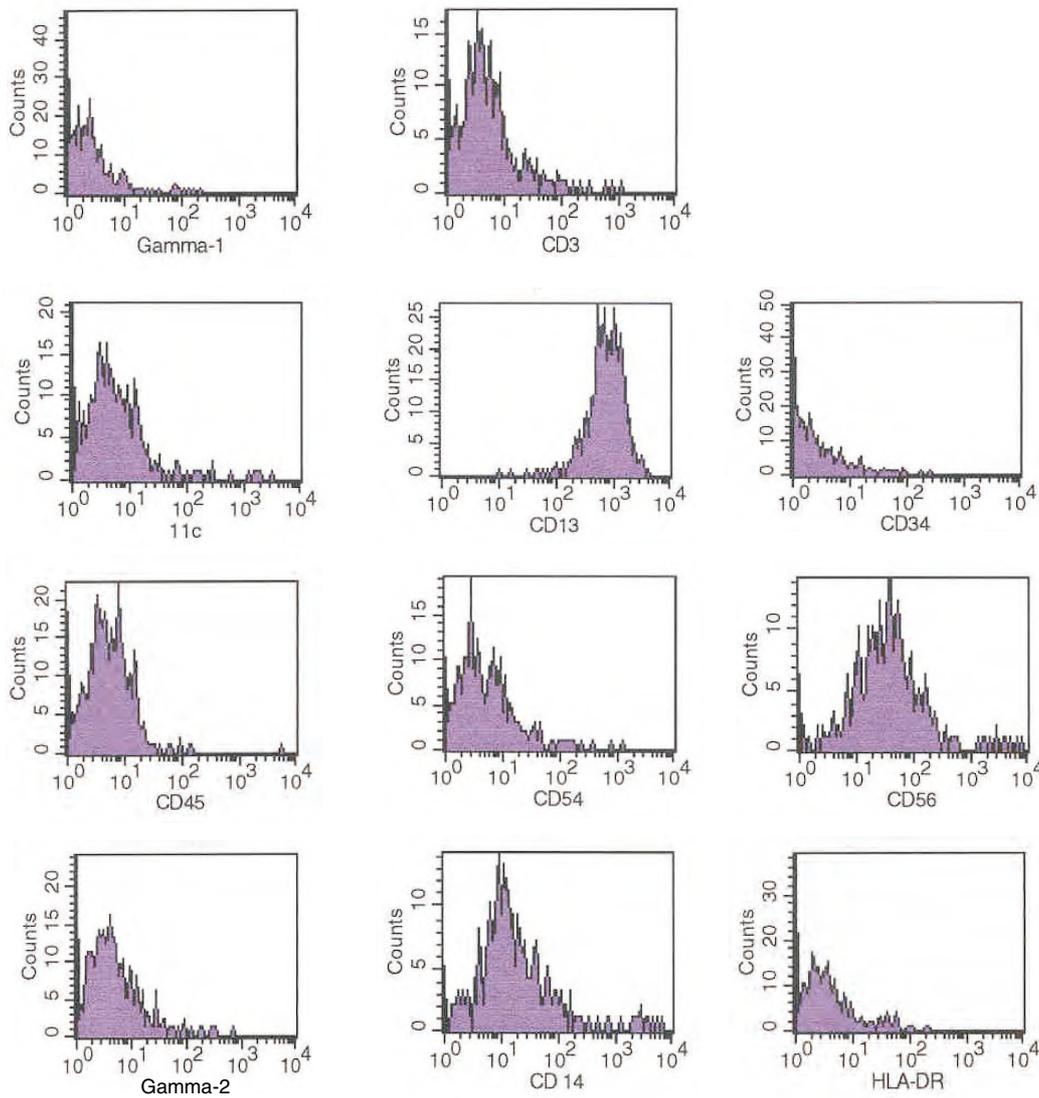


FIG. 6: Immunochemical surface characteristics of the large-flattened granular cells derived from culture of bone marrow mononuclear cells in DMEM. Gamma-1 acts as a control for CD3, CD11c, CD13, CD34, CD45, CD54 and CD56, whereas Gamma-2 acts as a control for CD14 and HLA-DR.

DISCUSSION

Our results showed that marrow-derived mononuclear cells were able to generate heterogenous adherent cells when cultured in DMEM containing FBS. The initial adherent time was three to seven days. Adherent time indicates the length of time for the cells in suspension to attach to the culture surface. We observed that there were four types of morphologically distinct cells, i.e., the long spindle-shaped cells, star-shaped cells, petal-shaped cells, and the large flattened granular cells with vacuoles. The long spindle-shaped

cells formed a larger colony compared with the other three types of cell that were more dispersed.

Heterogeneity in the culture of bone marrow cells has been reported since the first discovery of MSC by Friedenstein in the 1970s and other investigators^{1,7,26-28}. Friedenstein plated the whole bone marrow sample in plastic culture dishes, and after 4 hours, poured off the cells that were nonadherent. Although the adherent cells varied in size and phenotype, the most abundant cell noticeable were the long spindle-shaped cells. These fibroblastoid cells were quiescent for 2 to 5 days before beginning to replicate rapidly

form colonies. After several passages, the adherent cells became more uniformly spindle-shaped in appearance. His most impressive experiments were the successful differentiation of the cells into osteoblasts, adipocytes, and chondrocytes *in vitro* and after implantation *in vivo*^{16,27}. Since then, many investigators have repeated and even extended his experiments because MSC may be a source for cell replacement in the body.

In 1981, two researchers have isolated stromal cells in bone marrow and found two types of cells named as Type I and Type II cell. Type I cells were small and spindle-shaped while Type II cells were large and flat. During subcultivation, type I cells gradually disappeared, whereas the type II cells formed an increasing fraction of the population²⁹. Colter and his colleagues, in another similar study, identified large cells referred as mature MSC, and small and agranular cells as rapidly self-renewing cells (RS-1). Subcultivation has seen appearance of small and granular (RS-2 cells). During the log phase growth, the population of mature MSC rapidly expanded, the RS-2 cells declined in number, and the RS-1 cells increased. They explained that RS-1 cells might generate RS-2 cells which in turn would give rise to mature MSC. RS-2 cells might also have role in regenerating RS-1 cells during the late log phase²⁸. Both reports suggested that mature MSC might arise from a MSC progenitor that could be distinguished morphologically in culture.

In the current study, the rapid expansion of the MSC in culture was found to depend on a minor population of long spindle-shaped cells. These cells doubled actively in initial culture. When the cells reached confluency and were subcultivated, the cells adhered as star-shaped cells, and these star-shaped cells turned into large flattened granular cells with vacuoles. The long spindle-shaped cells were seen in small quantities and were able to maintain their capability to replicate. We believe that the spindle-shaped cells are the progenitor cells and they give rise to large flattened granular cells with vacuoles referred as mature MSC. The mature MSC divided slowly and became the more predominant cell as the cultures approached senescence and/or apoptosis in the 7th passage. Therefore, the number of long spindle-shaped cells obtained from early passage cultures probably reflects the number of MSCs in a sample of bone marrow. The star-shaped cells and petal-shaped cells might be the intermediate

cells of a MSC progenitor cell changing into a mature MSC.

While some preparations of MSC can be expanded through over 50 or more cell doublings^{23,30}, others ceased replicating after four cell doublings¹⁵. These observations may arise from several determinants, among them the procedure used to harvest the marrow⁷ and conditions of storage of MSC³¹, the age or condition of the donor (steady state *vs* post chemo-or radio-therapy) from which MSC were prepared⁷, and the low frequency of MSC in marrow harvests³². There are also variations in the quality and number of MSC obtained from different bone marrow aspirates, even when obtained from the same donor at the same time³³.

MSC could be identified by carrying out a series of assays that include adherence to plastic and glass, and morphological appearance as have been discussed above. Next, we tried to characterize the MSC by immunophenotyping and cytochemical staining.

Immunophenotyping of the large flattened granular cells showed that they expressed CD 13, CD 14 and CD 56. They did not express CD 3, CD 11c, CD 34, CD 45, CD 54 and HLA-DR. Our results are comparable to observations reported previously^{4,6,14}. However, we found that these cells showed positive expression of CD 14 and negative expression of CD 54. We are not sure if there is any underlying pathology from where the bone marrow sample was obtained might have influenced the outcome of the immunophenotyping. It is difficult to compare the results of different groups of investigators obtained because the variability of markers expression not only exist between cells strains, but also within a cell strain, as a function of time in culture³⁴. Since MSC did not express typical haematopoietic lineage markers, the bone marrow may host at least two main different stem or progenitor cells which give rise to mature haematopoietic cells and stromal cells respectively.

When these cells were stained cytochemically, they displayed positive reaction to PAS and NSE, but negative reaction to SBB, NASDA, and ALP. Our results was similar to the work of Erices and his colleagues where they had successfully turned the MSC derived from cord blood into adipocytes and osteocytes. The MSC were demonstrated to have cellular glycogen and α -naphthyl acetate esterase, but no lipid, naphthol AS-D chloroacetate esterase, and alkaline phosphatase.

CONCLUSIONS

Marrow-derived mononuclear cells were able to generate adherent cells when cultured in DMEM containing FBS. Initially, the cells were heterogenous but as they proceeded to the third passage, only the large-flattened granular cells with vacuoles could be seen. The large flattened granular cells with vacuoles were positive to PAS and NSE, but negative to SBB, SE, and alkaline phosphatase. These cells expressed CD 13, CD 14, CD 56, but did not express CD 3, CD 11c, CD 34, CD 45, CD 54 and HLA-DR. We believe that these cells show morphological, cytochemical and immunochemical characteristics of mesenchymal stem cells. To further confirm the authenticity of the isolated mesenchymal stem cells, we plan to carry out *in vitro* and *in vivo* differentiation into osteoblasts, chondrocytes, adipocytes, and perhaps neural cells in the future. Isolation of these would enable them to be used as a vehicle for genetic manipulation and expanded *in vitro* for cellular therapy.

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