Multilineage Differentiation Capability Comparison between Mesenchymal Stem Cells and Multipotent Adult Progenitor Cells

Kai-Hong Ji, Jun Xiong, Li-Xing Fan, Kai-Meng Hu, Hou-Qi Liu

Research Center of Developmental Biology
Department of Histology and Embryology
College of Basic Medical Sciences
Second Military Medical University
Xiangyin Road 800, Shanghai 200433, P.R. China

Corresponding author: Prof Hou-Qi Liu, MD, PhD, Director, Developmental Biology Research Center; Department of Histology and Embryology, Second Military Medical University, Xiangyin Road 800, Shanghai 200433, P.R. China
E-mail: liuhouqismmu@yahoo.com.cn

Abstract

Mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPCs) are the 2 most studied adult stem cells isolated from bone marrows. However, to our knowledge there has been no report that systematically compared MSCs and MAPCs. The present study aimed to make a primary comparison of the two, in an effort to understand the similarities and dissimilarities in their properties and proliferation potentials. We successfully culture-isolated MSCs and MAPCs from bone marrows of adult rats with different culture conditions. The cell morphologies, surface markers, differentiation potentials, and the maximal passage generations at which the cell still retaining their properties as stem cells were compared between the 2 cell populations by RT-PCR, immunofluorescence, and cell cycle analysis. We found that MSCs gradually lost its multiple differentiation potential and phenotype with the increase of passages, whereas MAPCs well retained pluripotency. Therefore, MAPCs may hold a greater promise in the future clinical application than MSCs.
Keywords: Multipotent adult progenitor cell; Mesenchymal stem cell; Differentiation; Pluripotency; Rat

Introduction

Increasing evidences showed that isolation strategies of stem cells differ for different source tissues and different stem cell types; even from the same source tissues, different isolating protocols obtain different stem cell types [Ulloa-Montoya et al., 2005]. For example, the stromal stem cells screened out with different molecular markers demonstrated certain varieties in growth and plasticity, and were therefore given different names, including MSCs [Prockop, 1997; Pittenger et al., 1999; Tropela et al., 2004], MAPCs [Reyes et al., 2001; Jiang et al., 2002; Breyer et al., 2006], MPCs (mesodermal progenitor cells) [Reyes et al., 2001], MIAMI (marrow-isolated adult multi-lineage inducible cells) [Ippolito et al., 2004; Yoon et al., 2005], etc. Nevertheless, the relationships between these pluripotent stem cell populations remain unclear. In the present study, using different culture conditions, we undertook to assess the similarities and dissimilarities of the two most studied stem cells: MSCs and MAPCs.

Materials and methods

Isolation and passage of MAPCs

Bone marrow was obtained from four-week-old male Sprague Dawley (SD) rats (Laboratorial Animal Center of Second Military Medical University, Shanghai, China). Marrow stromal cells were flushed from the femurs and tibias by a 1ml-injector with Dulbecco’s phosphate-buffered saline (DPBS). The stromal cells were centrifuged at 1000 rpm for 5 min to remove the supernatant and fat cells and the cell pellets were resuspended in TrisNH4Cl solution at 4°C for 5 min for induction of hemolysis. Then the cells were centrifuged again and washed with DPBS to remove the red blood cells; the remaining cells were seeded at a density of 1×10^6 cells/cm^2 in 100 mm dishes coated with 10 ng/ml fibronectin (FN) (Sigma-Aldrich, St. Louis, Missouri, USA) at 37°C and 5% CO_2. The basal medium of MAPCs contained 60% low-glucose Dulbecco’s modified Eagle medium (DMEM-LG, Gibco-Invitrogen, Carlsbad, USA), 40% MCDB-201 (Sigma), supplemented with 2% fetal bovine serum (FBS, Gibco-Invitrogen), 100× insulin/ transferring/ selenium (ITS), 100× linoleic acid BSA (LA-BSA), 10^-9 M dexamethasone (Dex), 10^-4 M ascorbic acid 2-phosphate (all from Sigma-Aldrich), 100 U/ml penicillin, 1000 U/ml streptomycin (Gibco-Invitrogen), 10
ng/ml epidermal growth factor (EGF) (Sigma-Aldrich), PDGF-BB (R&D Systems Inc.), and 10 ng/ml leukemia inhibitory factor (LIF, Chemicon International, Temecula, California, USA). Cells were allowed to adhere to the substratum for 24 h and the flasks were rinsed twice with DPBS to eliminate unattached cells. The medium was changed every 3 days until 100% confluence was reached. Cells were passaged once with 0.25% trypsin and EDTA (Gibco-Invitrogen) and the passage 1 (P1) cells were replated at a 1:2 dilution under the same culture condition.

Isolation and passage of MSCs

The animals used for MSCs isolation were the same as those for MAPCs and the protocol for MSCs isolation was similar to that of MAPCs. Briefly, marrow stromal cells were flushed from femurs and tibias through a 1ml-injector with basal medium and were directly seeded in 100 mm dishes by direct adherence at 37°C and 5% CO2. The basal medium contained Dulbecco’s modified Eagle medium/Ham’s F-12 (DMEM/F12, Gibco-Invitrogen ), supplemented with 10% FBS(Gibco-Invitrogen ) and 1% penicillin/streptomycin (Gibco-Invitrogen). 24 h after plating, the non-adherent cells were removed by replacing medium. The medium was changed every 2 days until 80% confluence was reached. Cells were passaged once with 0.25% trypsin and EDTA (Gibco-Invitrogen) and the P1 cells were replated at a 1:3 dilution under the same culture condition.

Morphological observation of MAPCs and MSCs

Light phase-contrast microscopy

The morphologies of primary and passaged MAPCs and MSCs were observed under phase-contrast microscopy (Olympus IX70, Japan); the morphological changes of MAPCs and MSCs and the differences between the 2 cell populations were evaluated.

Electron microscopy

The ultrastructural characteristics of passaged MAPCs and MSCs were observed under the electron microscopes. Scanning electron microscope (SEM): cells cultured in the glass slide were immersed in 3% glutaraldehyde, transferred to Millonig buffer, pH 7.3 for 1 h, dehydrated in a graded acetone series, and dried at a critical point in a Hitachi HCP-2 (Japan) with CO2. The specimens were finally metalized with gold-palladium before observation with a Hitachi S520 SEM (Japan).

Immunocytofluorescence and cell cycle analysis

P4 MSCs and P4 MAPCs were fixed with 4% paraformaldehyde in phosphate buffer for 4 min at room temperature. After blocked with PBS containing 2% BSA, cells were permeabilized with 0.1% Triton-X 100 for 10 min. Slides were incubated sequentially overnight at 4°C with the following primary antibodies: CD71 (goat polyclonal, 1:50, Santa Cruz Biotechnology Inc.), Vimentin (mouse polyclonal, 1:200,
DAKO, CA, USA), and α-SMA (mouse polyclonal, 1:200, Boster Biotechnology, China), SSEA-1 (1:50, mouse polyclonal, Santa Cruz Biotechnology Inc.), and the major histocompatibility complex class I (MHC-I, mouse monoclonal, 1:400, abcam, Cambridge, UK). The slides were washed between each step with PBS ± 1% BSA. Finally, the cells were incubated for 40 min at room temperature with FITC or Cy3-coupled anti-mouse, anti-goat, or anti-rabbit IgG secondary antibody (1:500, Jackson), stained with diamidino-phenylindole (DAPI, Sigma–Aldrich), and observed under a fluorescence microscope (BX41TB, Olympus, Tokyo, Japan).

**Evaluation of DNA contents and cell growth patterns**

1 ×10^6 cells were fixed in 70% cold ethanol for 10 min at 4°C, treated with 100 μg/ml DNase-free RNase (Sigma, USA), and labeled with 50 μg/ml propidium iodine (PI, Sigma, USA) for 5 min at room temperature. Then the DNA contents of all samples were analyzed by a FACStar flow cytometer using Cellquest software (Becton Dickson, San Jose, Calif).

**Results**

**Morphological observation of MAPCs**

On the 3rd day of primary culture, very few triangle-like or thin spindle-shaped cells adhered to the culture surface under phase contrast microscope, co-existing with plenty of hematopoietic cells (round in shape and small in size, shining under microscope). During the 4th to 7th day of culture, the triangle-like or thin spindle-shaped cells gradually increased and had a typical colony growth, with obvious three-dimensional appearance and growth circle, demonstrating the classical characteristics of MAPCs: polygonal-shaped, with scant cytoplasm, and with granules around the nuclei (Fig. 1A P0). After treated with trypsin, the cultured cells no longer grew in clusters, but in a homogeneous manner (Fig. 1A P4). It should be noted that neither the primary confluent MAPCs nor the passaged MAPCs had the helix growth pattern. Scanning electron microscope revealed villus-like structures and processes on the surface of P1 MAPCs (Fig. 1A P1).

**Morphological observation of MSCs**

MSCs were also isolated by direct adherence of whole bone marrow and were cultured with DMEM/F12 and 10% FBS. The cell cycle and proliferation rate of MSCs and MAPCs were similar in primary culture. Under phase contrast microscope, MSCs were larger than MAPCs and were fibroblast-shaped. Cell proliferation was in a more classical colony manner (Fig. 1B P0). With the prolongation of culture, fibroblast-shaped cells were seen growing out from the cell colonies and reached confluence after about 7 days, in an obvious helix growth manner (Fig. 1B P0). The cells grew fast after passaged; Fig. 1B P4 showed that P4 cells were homogeneous in size and grew in a classical helix manner.
**Identification of molecular markers MAPCs and MSCs**

Both MSCs and MAPCs lack specific markers, so their identification depends on multiple markers. We had chosen the P4 MSCs and P4 MAPCs for identification and found that P3 MSCs were positive of CD71 (cell surface antigen of stem cells), Vimentin (marker of stromal cell skeleton protein), and α-SMA (marker of early smooth muscle) (Fig. 2). P3 MAPCs were also found positive of the above 3 markers (data not given). As for the expression of SSEA-1, a marker for more primitive stem cells, MAPCs were positive but MSC were negative, implying that MAPCs is more primitive than MSCs (Fig. 2). Moreover, we noticed that P4 MSCs and P4 MAPCs were both positive of MHC I molecules, which indicates that the 2 cell populations both possess certain immunogenicity (Fig. 2).
Figure 2. Immunofluorescence detection in MSCs and MAPCs. P4 MSCs were positive for CD71 (original magnification 200×), Vimentin (original magnification 400×) and α-SMA (original magnification 200×). P4 MSCs and P4 MAPCs were both positive for MHC I antigen (original magnification 200×). P4 MSCs were negative for SSEA-1 but P4 MAPCs were positive for it.

Cell cycle analysis of P1, P4 MAPCs and MSCs

P1, P4 of MAPCs and MSCs were subjected to cell cycle analysis. We found that for both P1 and P4 MAPCs, 80% of them were in the resting phase of cell cycle (G0/G1) and 17% were in the S + G2 + M phase, which indicates that, under the present culture condition, only a few MAPCs were at the active proliferation phase, consistent with the cell cycle property of stem cells (Fig. 3). We also found that 80% P1 MSCs were at the G0/G1 phase, but only 51% P4 MSC were at G0/G1 phase and some of them were at S + G2 + M phase, i.e., having entered the differentiation phase. This finding indicates that early passages of MSCs are preferred for cell transplantation therapy.
Figure 3. FACS analysis of cell cycles of MAPCs and MSCs at various passages. 80% of P1 and P4 MAPCs were at the G0/G1 stage (resting stage), and 17% were at the S + G2 + M stage; but for MSCs, 80% of P1 MSCs were at the G0/G1 stage and only 51% P4 MSCs were at G0/G1 stage, with some entering the S + G2 + M stage.

Oct4 expression in MAPCs and MSCs of P1, P3 and P4

To further understand the pluripotency changes of MAPCs and MSCs during culture, we examined the expression of Oct4, the self-renewal gene of stem cells, in MAPCs and MSCs of P1, P3, and P4. As one of the major determining factors for the pluripotency of stem cells, the abundance of Oct4 can reflect the difference in pluripotency of MAPCs and MSCs. Fig. 4 showed that P1-4 MAPCs all had high expression of Oct4, but for MSCs the expression of Oct4 decreased gradually as passaging continued, and almost disappeared in P6 MSCs (result not shown), suggesting that under the present condition, MSCs were gradually losing or almost lost their pluripotency when passaged for more than 4 times. On the contrary, MAPCs well retained their pluripotency under culture condition different from that of MSCs.
Figure 4. RT-PCR analysis of Oct4 expression in P1-4 MAPCs and MSCs. P1-4 MAPCs all had a high expression of Oct4; but for MSCs, the expression of Oct4 gradually decreased with the increase of passage. 18S was taken as an internal control (n = 3).

Discussion

MSCs and MAPCs are both derived from the bone marrow of adult animals. MSC, firstly isolated from bone marrow adhesions by Friedenstein in 1976 [Friedenstein, 1961], is fibroblast-like, non-hematopoietic and can adhere to a plastic culture surface. Presently the general protocol for isolating MSCs from bone marrow involves the isolation of mononuclear cells (by direct adherence or gradient centrifugation) and seeding these cells on tissue culture plates in medium containing FBS. After attachment of the adherent cell fraction, the medium was replaced to eliminate the non-adherent cells and the adherent cells could be expanded for several passages. MAPCs were accidentally isolated from human bone marrow in an attempt to isolate MSCs, and were believed to be mesodermal progenitor cells. Subsequently, MAPCs were proven capable of differentiating into neurons, hepatocytes and endothelial cells [Jiang et al., 2003; Schwartz et al., 2002; Snykers et al., 2007; Liu et al., 2007], and could contribute to 3 somatic germ layers. Isolation of MAPCs was achieved by plating bone marrow mononuclear cells depleted of CD45$^+$ and glycoporphin-A-positive (GlyA$^+$) in fibronectin coated surfaces. When cultured at 2000-8000 cells/cm$^2$, MAPCs maintained their telomere length and pluripotent potentials even when expanded for more than 50 population doublings [Ji et al., 2008].
Whether MAPCs and MSCs belong to the same cell population at different stages or two different cell populations remains a controversy. We found that, MSCs and MAPCs, both derived from the bone marrow of adult rats but under different culture conditions, were different in the morphology, cell surface markers, differentiation potential, and the maximal passage generations at which the cell still retaining their properties as stem cells. Under our experimental condition, MSCs could be passaged for more than 30 times and these over-passaged MSCs no longer had differentiation capability (data not shown), becoming almost immortalized and incapable of differentiation, which is different from the earlier belief that MSCs could be extensively expanded \textit{in vitro} while maintaining differentiation potential. The above findings further indicate that MAPCs represent a group of earlier precursor cells and are more homogeneous than MSCs. Therefore, MAPCs hold more promise than MSCs in the future clinical application.

In conclusion, the relationships between the above-mentioned multi-lineage stem cells need to be further clarified, and the present study, however, only aims to compare the characteristics between MSCs and MAPCs. The statement that “MAPCs as a subpopulation of pluripotent stem cells are stored in some tissues/organs during the process of human development” [Verfaillie \textit{et al.}, 2003] urges us to reexamine the relationship between the primitive cells of early embryogenesis stage and their descendant cells, especially those rare ones with extensive proliferation ability and existing in other tissues or nurtured by other tissues. How to isolate this cell population with suitable molecule marker and how to maintain their self-renewal and differentiation capability are both very important, for they remain to be the largest challenge of the clinical application of adult stem cells. Presently, our limited knowledge about the conditions needed for retaining the pluripotency of adult stem cells and the lack of suitable markers for the pluripotency hinder the wider application of stem cells. Nevertheless, due to the easy availability of bone marrow-derived stem cells and their specific advantages in treatment of some human diseases (There are no ethical problems and immune rejection in autografting), more related studies are expected to be carried out in the future.

\textbf{Acknowledgments.}

This work was supported by grants from the National Natural Science Foundation of China (No. 30600651) and the Cooperation Foundation for Overseas Young Scientists (No.30428001).

\textbf{References}


Received: November, 2008