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Original Article

Isolation and characterization of mesenchymal stem cells from human bone marrow

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Abstract: Objective: Human bone marrow mesenchymal stem cells (BMSCs) offer therapeutic potential for a broad range of diseases. Different approaches for the isolation of human BMSCs have been described, including the simple removal of non-adherent cells after 48 hours, density gradient centrifugation, and cell sorting, among others. However, many approaches have failed to effectively eliminate hematopoietic cells and harvest high-quality human BMSCs for subsequent research and regenerative medicine applications. A simple and efficient method for isolating human BMSCs was investigated in the present study. Methods: Human BMSCs from an adult patient were isolated by frequently changing the culture medium and were characterized according to their morphological features, pluripotent differentiation potential and cell surface marker expression at passage 3 and passage 9. Results: Human BMSCs of high purity (up to 99%) were harvested and characterized by their spindle-shape appearance, multipotent differentiation capability and cell surface marker expression at passage 3. The rate of expression of CD73, CD90, CD105, CD29, and CD44 was 98.67%-99.93% at passage 3 but decreased to 60.9%-98.34% at passage 9. Conclusion: Frequently changing the primary culture medium is essential for the isolation of highly purified BMSCs. The purity of human BMSCs gradually declined with subsequent passages.

Keywords: Isolation, characterization, human, bone marrow mesenchymal stem cells, cell surface markers, flow cytometry

Introduction

Stem cells are primitive cells capable of self-renewing and differentiating into multiple cell lineages [1]. Mesenchymal stem cells (MSCs) are one of the most extensively used types of stem cells. The unique and highly desirable properties of MSCs include high migratory capacities toward injured areas, immunomodulatory features, and the natural ability to differentiate into connective tissue phenotypes, which include bone and cartilage. These properties highlight the therapeutic potential of MSCs. In addition, MSCs elicit their tissue repair properties through paracrine mechanisms, in which the metabolism of target tissues is modulated [2]. Thus, MSCs offer therapeutic potential over a broad range of diseases such as acute graft-versus-host disease, Crohn's disease, multiple sclerosis, osteoarthritis, heart failure and myocardial infarction [2, 3]. Although MSCs from various organs have been isolated and characterized, the bone marrow is their most common source of MSCs for research and clinical applications [4].

For decades, bone marrow mesenchymal stem cells (BMSCs) have been isolated and purified based on their propensity to adhere to the plastic substrate of cell culture plates by removal of non-adherent cells after at least 48-72 hours [5, 6]. In addition, a range of approaches (e.g., density gradient centrifugation [7], cell sorting [8], negative and positive selection [4] not only decreased the proliferation and differentiation potential of MSCs but also failed to effectively eliminate hematopoietic cells from these cultures. The direct isolation and culture of murine BMSCs has been reported to yield a purified population of cells by frequently changing the culture medium and reducing the trypsinization time [9]. Additionally, BMSCs can be derived

from several animal species, including pigs [10], rabbits [11], horses [12], etc. It is known that human BMSCs are obtained from human bone marrow and can be safely transplanted back into patients after expansion and induction ex vivo [13].

With the goal of isolating highly purified human BMSCs reliably and reproducibly for research and regenerative medicine applications, we isolated high purified BMSCs from an adult patient by frequent change of culture medium and characterized their morphological features, pluripotent differentiation potential and expression of cell surface markers at passage 3 (P3) and passage 9 (P9).

Materials and methods

Isolation and expansion of human BMSCs

Human bone marrow cells were obtained from an iliac bone fragment of an adult patient who underwent bone graft following a car accident. This study was approved by the Ethics Committee of the Shanghai No. 6 People's Hospital, and informed consent was provided by the patient. A 1.5 × 1 × 1 cm^3 bone fragment was immediately immersed in Dulbecco's modified Eagle's medium (DMEM) in a 50 ml tube on ice for temporary storage. And the bone fragment was put into the clean bench as soon as possible. A 27-gauge needle attached to a 10 ml syringe containing DMEM was inserted into the exposed spongy bone, and the marrow was repeatedly flushed out of the iliac fragment with 5-10 ml of complete media until the bone fragment turned white. Then the complete media were pipetted and collected in a 10 ml tube on ice. The cell suspension was filtered through a 70 μm mesh filter to remove bone spicules and cell clumps. The cell numbers were counted using a hemocytometer. Bone marrow cells were carefully plated in 5 ml of complete medium in a 25 cm^2 culture flask incubated at 37°C with 5% CO_2 in a humidified chamber without disturbing them. After 3 h of culture, the nonadherent cells were gently removed by changing the medium and replacing them with fresh complete medium. After an additional 8 h of culture, the medium was replaced with 5 ml of fresh complete medium. From then on, the medium was replaced every 8 h for up to 72 h after the initial culture. Upon reaching 80-90% confluence, the cells were washed with phosphate-buffered saline (PBS) and detached by incubation in 0.5 ml of 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid for 2 min at room temperature. Trypsin was neutralized by adding 1.5 ml of complete medium, and all detached cells were replated in a 25 cm^2 flask. Thereafter, the medium was changed every 3 days. Typically, cell confluence is achieved in 10 d (**Figure 1**).

Multipotent differentiation assays in vitro

To determine the multi-lineage differentiation potential of the isolated human BMSCs, human BMSCs at P3 were induced into trilineage differentiation (osteogenic, adipogenic and chondrogenic lineages) using the standard induction media. Osteogenic and adipogenic differentiation of human BMSCs was accomplished using OriCell™ osteogenesis differentiation kit and OriCell™ adipogenesis differentiation kit (Cyagen, China) in accordance with the manufacturer's instructions. Pelleted cells in conical tubes (**Figure 2C**) were also conditioned towards chondrogenic differentiation using OriCell™ chondrogenesis differentiation kit. The resulting cells were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek, USA) at -20°C, cut into 6 μm sections in Leica CM1950 (Leica Biosystems, Germany) and stained with alcian blue for evaluation of the formation of cartilage matrix. The differentiation potential was assessed by histological staining with alizarin red for osteogenic differentiation, oil red for adipogenic differentiation and alcian blue for chondrogenic differentiation at day 28 of the induction of differentiation.

Flow cytometry analysis

Confluent cell layers at P3 and P9 were detached and collected after incubation with 0.25% trypsin-EDTA for 5 min. The single cell suspensions were washed with PBS prior to staining. For direct staining, the cells were centrifuged (250 ×g, 5 min) and re-suspended in cold PBS. A total of 1 × 10^7 cells were incubated for 30 min on ice in the dark in cold PBS with known concentrations of phycoerythrin (PE)-conjugated mouse monoclonal anti-human CD9, CD14, CD29, CD31, CD34, CD38, CD44, CD59, CD80, CD86, CD90, CD105 and CD106 antibodies (Becton-Dickinson Bioscience, Franklin Lakes, NJ, USA). The cells were then washed twice by centrifugation (250

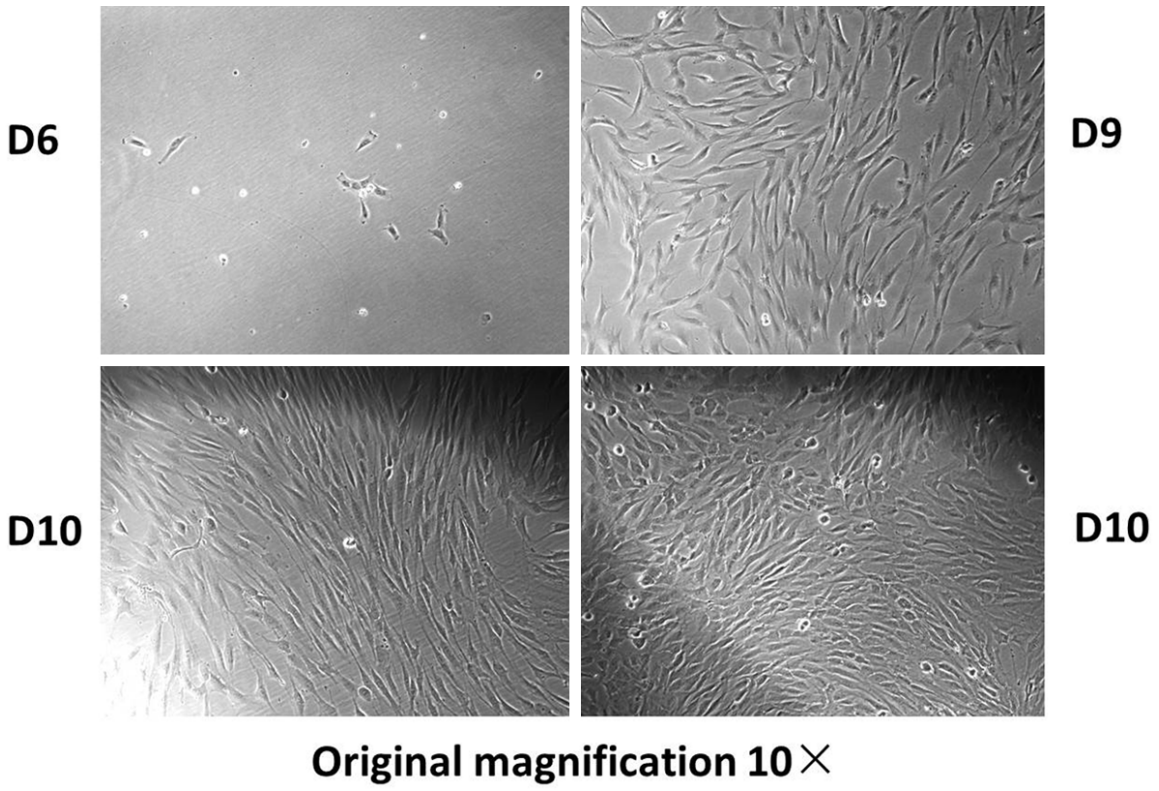

Figure 1. Morphological characteristic of human BMSCs. Adherent spindle-shape human BMSCs were rare day 6 and rapidly appeared confluent at day 10.

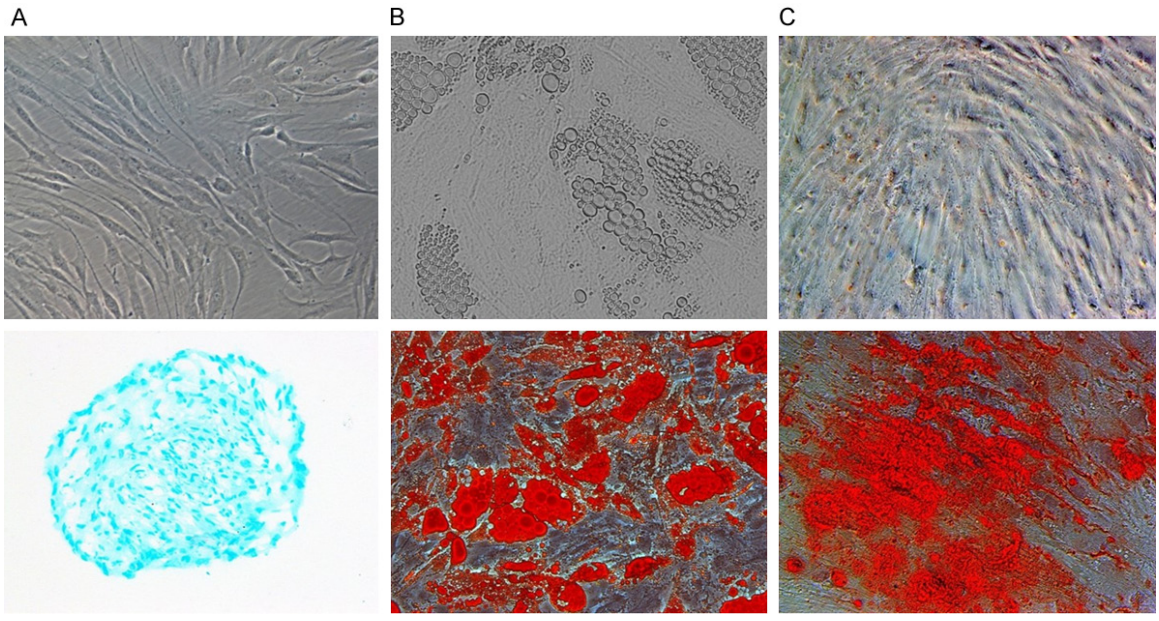

Figure 2. Differentiation capability of human BMSCs in vitro. A. Chondrocytes are stained with alcian blue. B. Adipocytes are stained with oil red. C. Osteocytes are stained with alizarin red. Original magnification 20 ×.

×g, 5 min) and re-suspended with cold PBS prior to flow cytometry. Samples were analyzed on a FACS Calibur flow cytometer (Becton-Dickinson Bioscience, Franklin Lakes, NJ, USA).

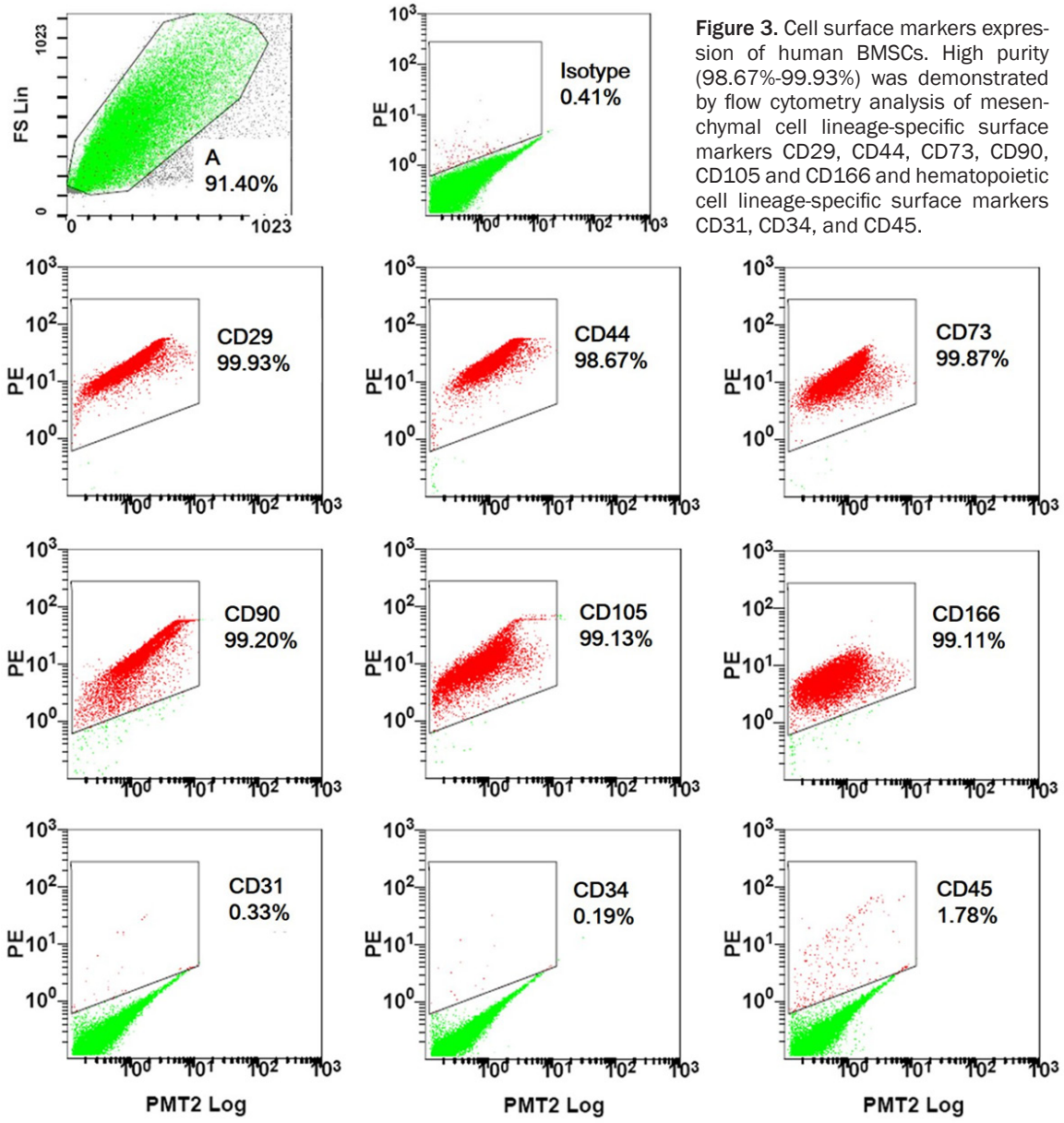

Figure 3. Cell surface markers expression of human BMSCs. High purity (98.67%-99.93%) was demonstrated by flow cytometry analysis of mesenchymal cell lineage-specific surface markers CD29, CD44, CD73, CD90, CD105 and CD166 and hematopoietic cell lineage-specific surface markers CD31, CD34, and CD45.

At least 10 000 events were captured by the system. Data analysis was conducted using FCS Express V2 software (De Novo Software, Los Angeles, CA, USA) following gating of the cell population to be analyzed. A non-specific isotype control (IgG1) was used to determine the background fluorescence emission.

Statistical analysis

All experiments were performed in triplicate. Means and Standard deviations were calculated. The paired *t*-test was used to analyze the differences between P3 and P9. A P value of < 0.05 was considered statistically significant.

Results

Morphology and expansion characteristics of human BMSCs

On the first day after harvesting marrow cells, rare adherent spindle-shape cells and a few round, bright hematopoietic cells were observed under low power microscopy. The mesenchymal/hematopoietic ratio is far below 1. With the frequent change of culture medium, more adherent and fewer hematopoietic cells were observed. On day 6, the mesenchymal/hematopoietic ratio gradually rose close to 1. Subsequently, the mesenchymal stem cells

Table 1. The positive rate of cells expressing cell surface markers at passage 3 and passage 9 were demonstrated and compared as below

Cell surface markers	P3	P9	P3 *vs* P9	
	Mean ± SD	Mean ± SD	*P* value	
CD29	99.93 ± 0.12	92.40 ± 3.26	0.0538	NS
CD44	98.67 ± 1.97	90.07 ± 2.76	0.0160	*
CD73	99.87 ± 0.15	93.90 ± 1.49	0.0170	*
CD90	99.20 ± 0.88	98.34 ± 0.51	0.1745	*
CD105	99.13 ± 1.17	70.03 ± 1.85	0.0012	*
CD166	99.11 ± 0.79	60.90 ± 3.84	0.0043	*
CD31	0.33 ± 0.13	1.56 ± 0.16	0.0024	*
CD34	0.19 ± 0.05	2.82 ± 0.67	0.0221	*
CD45	1.78 ± 0.17	2.11 ± 0.18	0.1919	*

became in excess of the hematopoietic cells. On day 10, this ratio became far beyond 1. The morphology of human BMSCs resembled that of fibroblasts, and these cells grew in parallel or whorls (**Figure 1**).

Multipotent differentiation capability of human BMSCs in vitro

Human mesenchymal stem cells at passage 3 and passage 9 both successfully differentiated into the three cell line ages using the standard induction media. Adipocytes were positively stained with oil red on day 21 post induction (**Figure 2B**). Osteocytes were positively stained with alizarin red on day 23 post induction (**Figure 2C**). Finally, frozen pellets of induced human MSCs were cut into 6 μm thick sections, and chondrocytes were positively stained with alcian blue on day 28 post induction (**Figure 2A**).

Cell surface marker expression on human BMSCs according to passage number

At passage 3, the positive rate of cells expressing the mesenchymal cell lineage-specific surface markers CD29, CD44, CD73, CD90, CD105 and CD166 was 99.93%, 98.67%, 99.87%, 99.20%, 99.13% and 99.11%, respectively (**Figure 3**). The positive rate of cells expressing the hematopoietic cell lineage-specific surface markers CD31, CD34 and CD45 was 0.33%, 0.19%, and 1.78%, respectively, compared with an isotype control positive rate of 0.41% (**Figure 3**). Thus, human BMSCs differentiated into mesenchymal lineages and were not contaminated with hematopoietic cells. At passage 9, the positive rate of cells expressing mesenchymal cell lineage-specific surface markers CD29, CD44, CD73, CD90, CD105 and CD166 was 92.4%, 90.07%, 93.90%, 98.34%, 70.03% and 60.90%, respectively (**Table 1**). The positive rate of cells expressing the hematopoietic cell lineage-specific surface markers CD31, CD34 and CD45 was 1.56%, 2.82%, and 2.11%, respectively, compared with an isotype control positive rate of 0.62% (**Table 1**). Although human BMSCs at passage 9 still expressed high levels of mesenchymal cell lineage-specific surface markers and rarely expressed hematopoietic cell lineage-specific surface markers, comparison of our surface marker results between passage 3 and passage 9 demonstrated that the purity of human BMSCs decreased to some extent with subsequent passages.

Discussion

Human BMSCs have been widely used in many studies in regenerative medicine in vitro and in vivo. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human MSC in culture: adherence to plastic, specific surface antigen expression and multipotent differentiation potential. The level of MSC purity (≥ 95% expression of CD105, CD73, CD90; ≤ 2% expression of hematopoietic cell surface markers) should be considered as minimal guidelines [14]. To date, nonadherent cells are usually removed after 48 h or longer in primary culture [15, 16]. However, changing the culture medium after 48 h or longer tend to increase the adherence of hematopoietic lineage cells to plastics, with the positive rate of hematopoietic lineage surface markers being approximately 20%-30% in the first 3 or 4 passages [6, 17]. Our experimentations suggest that highly purified human BMSCs can be harvested simply by frequent change of the primary culture medium. That is, it is essential to remove nonadherent cells after 3 h to eliminate most hematopoietic cells. By doing so, human BMSCs of high purity (up to 99%) could be harvested and characterized based upon the expression of cell surface markers, which is consistent with the result first obtained in murine BMSCs [18]. Hence, frequent change of the primary culture medium is essential for the isolation of highly purified BMSCs.

In addition, the proliferation rate and the positive rates of human BMSCs expressing mesenchymal surface markers gradually decreased as the number of passages increased [16]. In their study, P3 and P7 human BMSCs were compared and showed positive expression of CD73, CD90, CD105, CD29, and CD44 in 63.0%-99.5% of cells at P3, but that number decreased to 47.6%-99.7% at P7. In our study, we further compared the P3 and P9 human BMSCs and showed positive expression of CD73, CD90, CD105, CD29, and CD44 in 98.67%-99.93% of cells at P3 but that number decreased to 60.9%-98.34% at P9. In conclusion, highly purified and viable human BMSCs collected before P4 may be more suitable for regenerative medicine applications in vitro and in vivo than those obtained at higher passages.

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Disclosure of conflict of interest

None.

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