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ISOLATION AND CHARACTERIZATION OF ADULT STEM CELLS FROM BONE MARROW

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ABSRACT

Bone marrow is considered one of the most widely used sources for obtaining stem cells. The ease of techniques, absence of ethical dilemmas makes it one favorable source for regenerative medicine trials. In the present study we isolated MSCs from the bone marrow of Sprague Dawley rats. Characterization of the isolated cells revealed their positive CD29, 90 (Mesenchymal stem cells) and negative CD 45 denoting their mesenchymal lineage and the supporting the possibility of isolating MSCs from bone marrow. As such, this would invite us to use BMSCs for further preclinical and clinical trials in the area of regenerative medicine.

INTRODUCTION

There has been continuing interest in both the biology and potential therapeutic applications of the adult stem like cells from bone marrow, referred to as either mesenchymal stem cells or marrow stromal cells (MSCs). MSCs from human and rat bone marrow have been the most extensively characterized, in part because they are relatively easy to isolate by their adherence to plastic and can be extensively expanded in the culture. Also, the human and rat MSCs can differentiate into multiple cell phenotypes, including bone, fat, cartilage, muscle,

epithelium, and early neural progenitors [1,2]. In experiments performed here, we have developed a protocol of the isolation , expansion and immunophenotyping of MSCs. The isolated rat MSCs were expanded extensively and showed their markers and colonogenic potentials.

MATERIALS & METHODS

***Isolation of Mesenchymal stem cells from bone marrow:**

Eight-week-old male Sprague-Dawley rat was sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from skin by pulling toward the foot, which is cut at the anklebone . Remove the muscle and the connective tissue from both the tibia and the femur by scraping the diaphysis of the bone clean then pulling the tissue toward the ends of the bone. Cut the ends of the tibia and femur by sharp Scissors. Insert a 27-gauge needle and flushing with Dulbecco's Modified Eagle's Medium DMEM low glucose (Thermo scientific, USA) and collect in a 15-ml tube. Filter the cell suspension through a 70-mm filter mesh. Culture BM cells in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (Thermo scientific, USA)in 25 cm2 flask . Incubate flask at 370 C with 5% CO2 .

***Cell culture:**

After 1 days, non adherent cells were removed by two to three washes with phosphate-buffered saline PBS (Thermo scientific, USA) and adherent cells further cultured in complete medium. The medium was changed every 3 days until the monolayer of adherent cells reach 70-80% confluence . Then, we made trypsinization for cell splitting by trypsin-EDTA solution(0. 25%,sigma Aldrich, USA) for passage 1. Evaluate the number of cells by hemocytometer and cellular viability by the Trypan Blue exclusion test. Inoculate each 250-300 × 103 cells in 75cm2 culture flask that were incubated at 370 C and 5 % CO2. Cell cultivation was maintained up to the 3th passage.

***Flow cytometry analysis:**

Cells were characterised using cell surface markers by fluorescence-activated cell sorting (FACS) analyses. The cells were stained with different fluorescently labeled monoclonal antibodies (mAb) (eBioscience). In brief, 5×105 cells (in100 µl PBS/0.5% BSA/2mmol/L EDTA) were mixed with 10 µl of the fluorescently labeled mAb and incubated in the dark at 2-8 C0 for 30 min.Washing with PBS containing 2% BSA was done twice and the bellet was resuspended in PBS and analyzed immediately on flow cytometry. Using the mouse anti-rat CD45-FITC mAb, CD29-PE mAb and CD 90-PCY5. The fluorescence intensity of the cells was evaluated by EPICS-XL flowcytometry (Coulter, Miami, Fl,USA).

***CFU-F assay:**

For colony forming unit-fibroblast (CFU-F) assays, about 100 cells were plated per 100-mm tissue culture dish (Falcon) in complete culture medium. Cells were incubated for 10–14 d at 37° C in 5% humidified CO2, and wash with PBS and fixed in 95% ethanol for 5 minutes, and then the cells were incubated for 20- 30 minutes at room temperature in 0.5% crystal violet in 95% ethanol. Then plate was washed twice with distilled H2O. The plates were dried and the CFU-F units counted.

RESULTS

***Cell culture:**

Attachment of spindle-shaped cells to tissue culture plastic flask was observed after 1 days of culture. After 6 days, spindle-shaped cells reached 80% confluency .

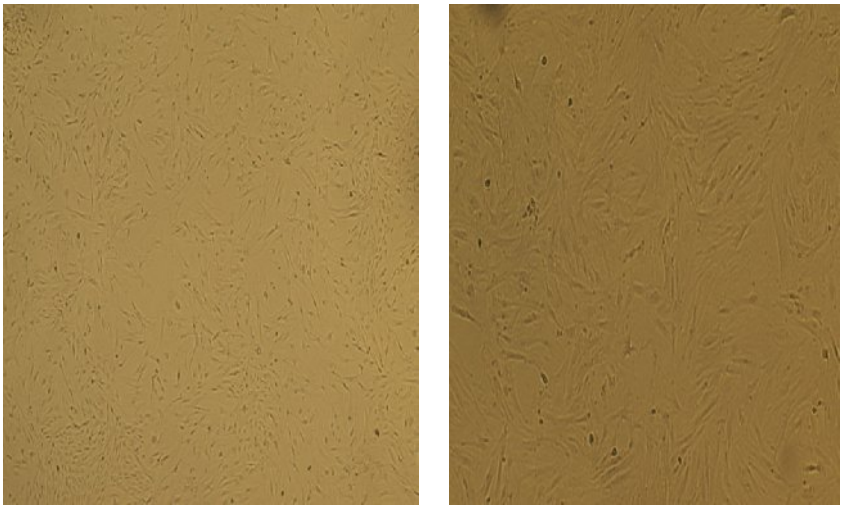

Figure 1.(a)Image of BMSCs in passage 3 with 80% confluence Scale bar=100 µm..(b) Image of BMSCs in in passage 3 Scale bar=200µm

***Immunophenotypic characterization:**

Cultures of BMSCs were analyzed for the expression of cell-surface markers. BMSCs were negative for the hematopoietic lineage marker CD45 with percentage 1.72.BMSCs were positive for CD29 and CD90 with percentage 98.4 and 93.7 respectively.

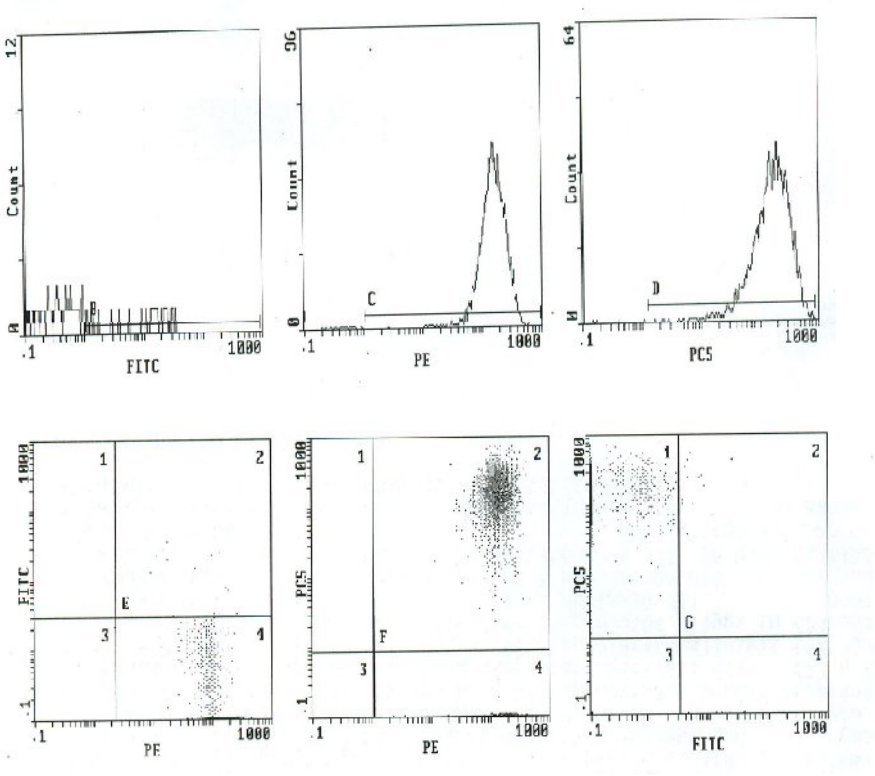

Figure 2. Immunophenotypic analysis of BMSCs. (a,b,c)Histogram representing the flow cytometry performed on the BMSCs .The BMSCs were stained with monoclonal antibodies conjugated to FITC, PE and PCY5 against the following markers: CD45, CD 29 and CD90 respectively . (d) 97.1 % of cells positive for CD 29 and negative for CD45 (e) 93.7 % of cells positive for both CD29 and CD 90. (f) 93.1 % positive for CD 90 and negative for CD45 .

Table (1) Immunophenotypic characterization of BMSCs

Marker	%
CD90	93.7
CD29	98.4
CD45	1.72

CFU

CFU-F assay is a suitable tool for evaluating the proliferation and colonogenic capacity of the cells (3th passage) expanded in the culture. The colony number of 100 ADSCs per 100-mm tissue culture dish is 5 ± 2.

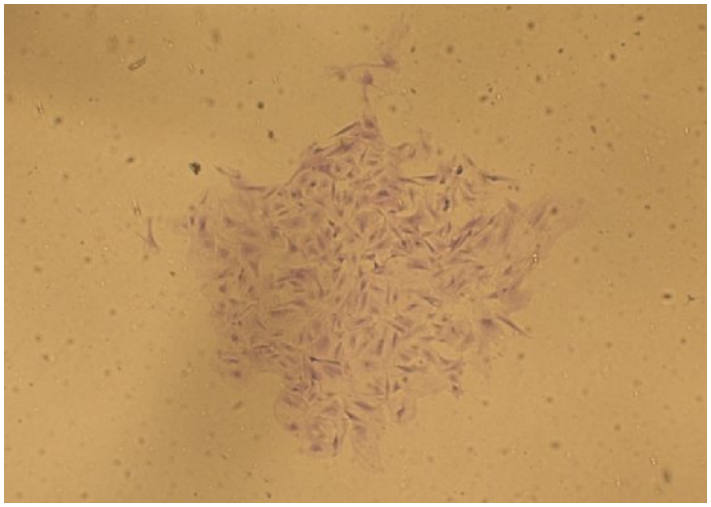

Fig 3 . (A) Crystal violet stained plate of CFU-F assays (B) one colony magnified under microscope

DISCUSSION

In the present study, rat BMSCs were isolated from the femurs and tibiae of adult rat and were cultured in DMEM containing 10% FBS. In this condition, the cells adhered to the plastic tissue culture plates and constituted a rapidly expanding population of polygonal or fibroblast-like cells. Previous studies have demonstrated a strong phenotypic resemblance between ADSCs and BM-MSCs (3,4). Moreover, Greco et al. (2007) (5) showed similarities between mesenchymal stem cells and embryonic stem cells.

In the present study, flow cytometric analysis showed that 93.7% of BMSCs expressed CD90 and 98.4% of BMSCs expressed CD29, after three passages. CD29 (beta-1 integrin) is a mesenchymal cell marker that is expressed in both BM-MSCs and ADSCs (6,7). Nevertheless, the presence of hematopoietic cell markers on the human (8,9) MSCs has been reported. In the present study, the expression of CD45 was detected in the freshly isolated rat BMSCs (1.72%).

In summary, the results of the present study showed that bone marrow is containing a population of stem cells that express the mesenchymal cell-specific markers and most of them lack the expression of hematopoietic markers. Phenotypical characteristics of BMSCs combined with their the proliferation and colony forming capacity suggest that BMSCs is a good multipotential cell candidate for the future cell replacement therapy.

CONCULSION

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