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Research Article

Isolation and characterisation of Mesenchymal Stem Cells from Human Peripheral Blood

Abstract

Aim: We describe a comprehensible novel procedure for isolation and characterization of mesenchymal stem cells (MSCs) from human peripheral blood.

Background: MSCs are well known multipotent adult stem cells with a wide spectrum of divisibility including osteoblasts, chondrocytes, myocytes, adipocytes many isolations and characterization techniques have emerged to isolate MSCs from human peripheral blood, most which are expensive and time-consuming.

Methods: MSC isolation was carried out from human peripheral blood and cultured in a well-defined culture medium. For adherence of culture, cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ for 48h. Thereafter, medium was replaced with fresh one every 3-4 days for 21 days. Adherent cells were characterized by flow cytometry verified MSC specific markers.

Results: Isolated MSCs had a fibroblastic-like appearance with adherent property to the culture plate. After isolation of MSCs, purified MSCs from peripheral blood were seen to be positive for positive cell surface markers: CD73, CD90, and CD105 while being negative for CD14, CD34 and CD45 along with their respective conjugates.

Conclusion: These findings affirms that the above method described is capable of isolating MSCs from human peripheral blood with positive results of stem cell characterization through flow cytometry according to all minimum criteria defined by the International Society for Cellular Therapy (ISCT).

Introduction

Stem Cells (SCs) are defined as a “class of undifferentiated cells that has the ability to differentiate into specialized cell types each with their new characteristically specialized cell functions [1]. MSCs are multipotent adult SCs that are present in multiple tissues; including umbilical cord, bone marrow and fat tissue. MSCs can divide and differentiate into multiple tissues including bone, cartilage, muscle and fat cells, and connective tissue [2]. They have been derived from varied sources as bone marrow [3], human peripheral blood (hPBSCs) [4], adipose tissue [5], lungs [6], heart [7], placenta [8], cord blood [9], umbilical cord [10] and urine [11]. Despite of their easy culturing techniques, the process of isolation of MSCs from patients is complicated, painful [12], thus making it hard to find donors. Isolation of SCs from human peripheral blood can thus provide a less complicated and painful alternative for stem cell therapies involving autografts and allografts. In case of allografts involving SCs, hPBSCs allografts have been

seen to contain 3-4fold greater CD34+ cells as well as a 10-fold elevation in number of lymphoid subsets count when mobilized with recombinant human granulocyte colony-stimulating factor (rhG-CSF). Besides, it has been seen that peripheral blood stem cell transplant (which require hPBSCs) gives a better disease-free survival rate to patients suffering from advanced stage cancer than that of bone marrow derived MSCs [13]. Hence, for clinical applications; the hPBSCs seem to be more promising only if a solution to effectively isolation and culture of peripheral derived MSCs in vitro can be found. Thus, this research is focused on developing a novel and effective procedure or the isolation of hPBSCs from human peripheral blood.

Materials and Methods

Materials

For isolation of blood from human donors, sodium-heparin tubes were purchased from BD Bioscience (BD Vacutainer,

Reference no. 367671 G), BD Vacutainer push button blood collection set (Cat no.367344). For cell culture experiments, complete FBS culture media was prepared using MEM alpha modification (1X) medium (Cat. no. SH30265.01) was purchased from HyClone. Fetal bovine serum (FBS) (Cat. no. 12003C-500ML) from SAFC Biosciences, Penicillin/Streptomycin (Cat. no. 15140-122) and L-glutamine 200 mM (100X) (Cat. no. 25030-081) were obtained from Life Technologies. Dulbecco's Phosphate Buffer Saline (PBS) (Cat. no. D8537) purchased from Sigma-Aldrich, USA. Ficoll-Paque (Cat. no. 17-1440-02) from GE Healthcare, Trypsin-EDTA (0.25%) phenol red from Gibco (Cat. no. 25200072-500ML).

Isolation of human mesenchymal stem cell from human peripheral blood

The study was conducted after obtaining approval from the Institutional Review Board (IRB) of Christian Medical College; CMC, Vellore. Male participants (volunteers), between 18 and 30 years were screened, and medical history and examination, electrocardiograph, hematology, biochemistry, serology, urine analysis, and chest X-ray were done within 14 days before start of the study. Participants having a history of major illnesses, acute or chronic disease, habituated to tobacco or alcohol were excluded from the study. After being given detailed information about the study, the participants were requested to sign an informed consent form after affirming them that there was no harm was assigned to them. Human peripheral blood sample was thus obtained from donors as per procedure suggested by Jean M. Slockbower [14]. Venous blood was collected through venipuncture from volunteers into sodium-heparin tubes figure 1. Briefly, at first a suitable site of venipuncture was selected, tourniquet was placed 3 to 4 inches above the selected puncture site. Caution was maintained not put the tourniquet on too tightly or leave it on the donor for longer than a minute. Thereafter vein was selected, cleansed with 70% isopropyl alcohol in a circular motion. Subsequently, donor's arm was grasped firmly using thumb to draw the skin taut and anchor the vein. Needle was inserted swiftly through the skin into the lumen of the vein. Care was taken to see that the needle formed a 15-30-degree angle with the arm surface. Excess probing was avoided. After the tube got filled, needle from the donor's arm was withdrawn using a swift backward motion. Next, puncture site was gauzed. Thereafter, adequate pressure was applied to avoid formation of a hematoma. After holding pressure for 1-2 minutes, a fresh piece of gauze was taped to the puncture site. The blood so collected was uniformly mixed with heparin by a gentle tilt.

Mononuclear cells (MNCs) were isolated from peripheral blood by procedure as described hereafter. Briefly, 5ml of blood was taken in a 15 ml centrifuge tube. To it 5ml of phosphate buffered saline (PBS) was added. To ficoll-paque, PBS-blood suspension was added in a ratio of 1:2. Thereafter, first round of centrifugation (Figure 2), was done at 400Xg at room temperature for 30 minutes. Subsequently plasma layer was discarded and MNC layer collected. Caution was maintained in collection of MNC layer to avoid mixing of MNC layer with ficoll or plasma layer. Following collection of MNC layer a second round of centrifugation with MNC layer alone was performed at

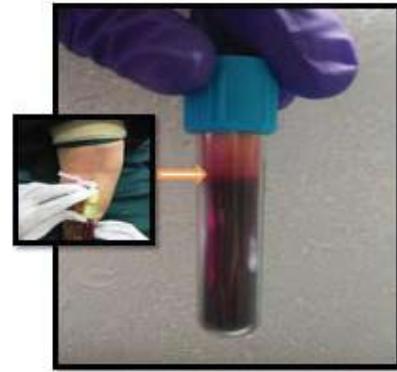


Figure 1: Collection of Human Peripheral Blood via venipuncture in Heparin Tubes.

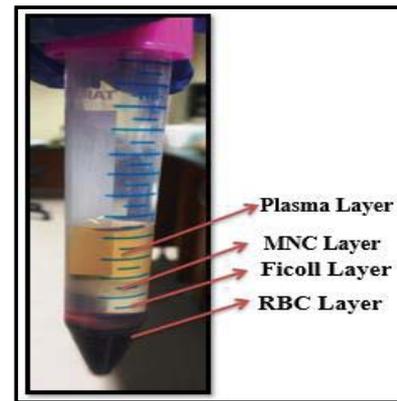


Figure 2: Layers of plasma, MNC, Ficoll and RBS after 1st round of centrifuge.

700Xg for 10 minutes. After 2nd round of centrifugation cell pellet was collected and to it 10% complete FBS media was added. The pelleted MNC's were cultured for 21 days. It is to be noted here that cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Suspended cells were discarded after 5-7 days of culture. Adherent cells were left to grow on the flask surface. Culture medium was changed every 3 days till 21 days.

Microscopic analysis of human mesenchymal stem cell

Microscopic analysis of peripheral blood derived MNCs revealed the morphology of MNCs at Day 0 and MSCs after Day 21. For morphological analysis inverted microscope (Leica Inverted Microscope Imaging System, USA) was used in phase contrast mode.

Characterization of human mesenchymal stem cell through flowcytometry

For characterization (phenotype analysis) of hPBSCs flow cytometry (FACS Calibur, Becton Dickinson, USA) was used. Adhered cells were trypsinized for their harvestation. Trypsinization was followed by centrifugation to pellet down harvested cells. The cell pellet obtained was then resuspended in 300µl PBS. The procedure following resuspension of pellet is depicted in Figure 3. The anti-human antibody markers used were as follows CD45-FITC, CD34-PE, CD14-PERCP as negative markers; and CD73-PE, CD90-PERCP and CD105-FITC

as positive (hematopoietic lineage) markers (Becton Dickinson, USA). All the anti-human antibodies were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin-chlorophyll proteins (PERCP): IgG FITC/PE/PERCP. Approximately 1×10^5 cells were analysed using a flow cytometer and data interpreted. Replicate experiments were performed in triplicate.

Results

Microscopic analysis of human mesenchymal stem cell

Morphology of cultured hPBSCs in complete FBS media was analysed under inverted microscope figure 4. Phase contrast micrographs of MNCs & hPBSCs cultured in a tissue culture flask in complete FBS culture medium. The confluent cells also showed spindle-shaped morphology as that of earlier reported MSCs, which further confirmed that the isolated cells were MSCs [15].

Characterization of human mesenchymal stem cell through flowcytometry

MSCs express CD105, CD73, and CD90 and are void of CD45, CD34, CD14 expression [16]. Flow cytometry was used to characterize hPBSCs using stem cell surface marker antibodies figures 5,6. Figure 5 shows the grouping of around 6×10^3 cells from around a cell population of 1×10^4 cells for flow cytometry analysis. Figure 6 shows results of flow cytometric analysis for the typical surface markers expressed in hPBSCs. Events P3, P5 and P7 signals from hBMSCs surface marker antibodies and events P2, P4 and P6 represent the isotype controls for fluorescence from that negative markers and background. Had the cell not been SCs one would not find signals from hBMSCs surface marker antibodies, thus confirming that the cells isolated from human bone marrow belonged to human mesenchymal stem cell lineage [17].

A, B and C are hPBSCs stained with phycoerythrin (PE), peridinin chlorophyll (PERCP) or fluorescein isothiocyanate (FITC) conjugated antibodies against the indicated negative surface markers: CD45-FITC; CD34-PE and CD14-PERCP. **D, E and F** are hPBSCs stained with phycoerythrin (PE), peridinin chlorophyll (PERCP) or fluorescein isothiocyanate (FITC) conjugated antibodies against the indicated positive surface markers: CD105-FITC; CD73-PE and CD90-PERCP. *Events P3, P5 and P7* signals from hBMSCs surface marker antibodies

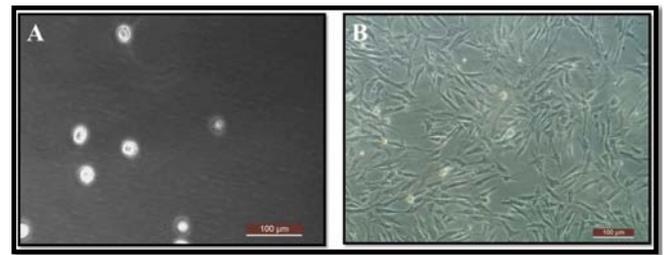


Figure 4: Phase contrast micrographs of MNCs & hPBSCs cultured in a tissue culture flask in complete FBS culture medium. A). Phase contrast micrographs of MNCs obtained from peripheral blood on Day 0; B). Cultured hPBSCs after Day 21 at around 60% confluency.

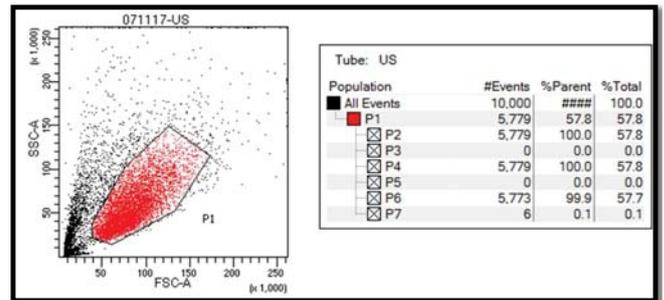


Figure 5: Grouping of cells for Flow Cytometry Analysis.

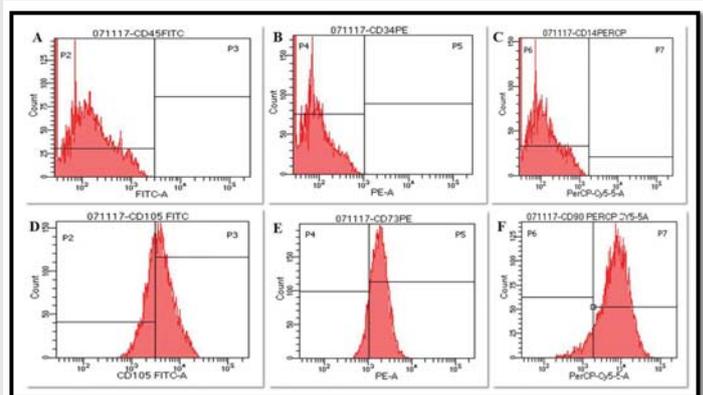


Figure 6: Flow cytometric analysis for the typical surface markers expressed in hPBSCs.

and *events P2, P4 and P6* represent the isotype controls for fluorescence from that negative markers and background.

Conclusion

Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells which give rise to marrow adipose tissue). To the best of our knowledge the novel procedure being investigated, is the first of its kind for the isolation of MSCs from human peripheral blood. This endeavour was based in the backdrop of current scenario whereby it was suggested that there are few clinically relevant techniques for hPBSCs isolation and that hPBSCs is better than bone marrow derived MSCs in many ways. hPBSCs were isolated from blood of donors, characterized morphology analysis using inverted microscope and the results of the same

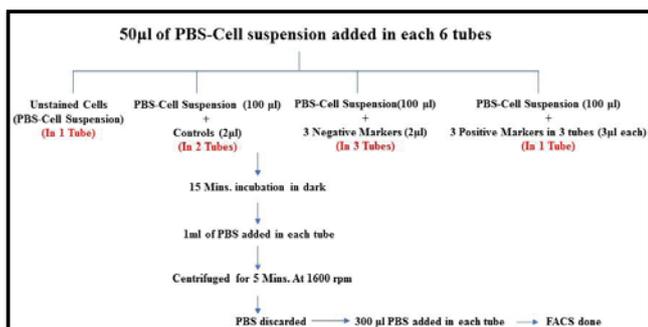


Figure 3: Procedural flowchart for Stem Cell Characterization.

confirmed through flow cytometry. Thus, with the results of the present study, clinical grade hPBSCs can be isolated by the established method.

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