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ISOLATION AND IDENTIFICATION OFADIPOSETISSUE-DERIVED STEM CELLS

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ABSRACT

Stem cells are considered new promising tool for treating many disorders. However, the difficulties in isolation of stem cells, the immunorejection problems and the ethical debates about their sources could slow down the progress in their application in clinical trials. In the present study we were able to isolate mesenchymal stem cells from adipose tissue. This would overcome the difficult isolation from elsewhere, the immunorejection problems (as they can be isolated from the same patient) and of course the ethical debate as they will be isolated form a known source after taking conste of the patient. The characterization of the isolated adipose tissue derived stem cells (ADSCs) revealed mesenchymal markers (CD29,90) and absence of Hematopoietic markers (CD45). So, these results support the capacity of adipose tissue to be an easy source for obtaining functional stem cells

INTRODUCTION

Stem cell transplantation has become a promising therapy for many disorders. However, the ethical debate, teratoma formation and the technical inconsistency regarding embryonic stem cells (ESCs) favored the use of adult stem cells. Moreover, immunorejection seems to be a limiting obstacle infront of stem cells trials in humans. Clearly,

to overcome this, autologous stem cells are practically of value [(1)]. One putative source of stem cell has been identified within the stromal compartment of adipose tissue [(2)]. The main benefit of this ADSCs is that they can be easily harvested from the patients by a simple, minimally invasive method, and they can be easily cultured. Moreover, ADSCs can be propagated more rapidly, and they retain their mesenchymal pleuripotency after some passages [(3)]. ADSCs characterization is based on the expression of cell-specific markers. Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy put a minimum of four points to identify human mesenchymal stem cells [(4)]: (1) MSCs have to be plastic-adherent when maintained under standard culture conditions, (2) MSCs must have the ability for osteogenic, adipogenic, and chondrogenic differentiation, (3) Human MSCs must express CD73, CD90, and CD105, and (4) MSCs must lack the expression of hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79 and human leukocyte antigen (HLA)-DR. However, the phenotypic characterization of ADSCs is still controversial especially regarding the expression of hematopoietic and embryonic stem cell-related genes.

In the present study, rat ADSCs were isolated from the inguinal fat pad of adult rats. Isolated ADSCs identified positive for CD29 and CD90 markers, and they showed the lack of CD45 expression. These results showed that white adipose tissue contains a stem cell population that seems to be a good multipotential cell candidate for the future cell replacement therapy.

MATERIALS & METHODS

***Adipose tissue harvest:**

The inguinal fat pad was collected under sterile conditions from eight-week-old male Sprague-Dawley rats and wash with phosphate-buffered saline (PBS) containing 1% antibiotic–antimycotic solution (Thermo scientific, USA). The washing step is repeated until all blood vessels and connective tissues appear to have been liberated (usually 3 washes).

***Isolation of stromal vascular fraction cells:**

Tissue sample was minced into small pieces. Then adipose tissue was digested in trypsine (Sigma-Aldrich , USA) 0.125 % at 37 ºC with shaking 100 rpm.(3ml for each 1 g. Adipose tissue) for 60 min. After digestion, trypsine activity was neutralized by adding equal volume of DMEM containing 10% fetal bovine serum (Thermo scientific, USA) to the tissue sample. And for further disintegration, aggregates of the tissue sample were Pipetted up and down several times. Then the cell suspension was filtered through 100 µm filters (BD Falcon, USA) for avoiding the solid aggregates.

The sample was centrifuged at 2000 rpm for 5 min at room temperature. The samples was taken out of the centrifuge and shaked vigorously to completes the separation of the stromal cells from the primary adipocytes. We removed supernatant without disturbing the cells then, re-suspended the pellet in 1 ml of lysis buffer(Promega, Germany) to lyse RBCs, incubated for 10 min and washed with 10 ml of PBS containing 1% antibiotic–antimycotic and centrifuge at 2000 rpm for5 min. The supernatant was removed and re-suspended the cell pellet in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution in 25 cm2 culture flask and maintained in an incubator supplied with humidified atmosphere of 5% CO2 at 37◦C

***Cell culture:**

After 1 days, non adherent cells were removed by two to three washes with PBS 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 . Trypsinization was made for cell splitting by trypsin-EDTA solution (0. 25%,sigma Aldrich, USA) for passage 1 .Number of cells was evaluated by hemocytometer and cellular viability by the Trypan Blue exclusion

test. Each 250-300 × 103 cells was inoculated in 75cm2 culture flask that were incubated at 370 C and 5 % CO2. Cell cultivation was maintained up to the 3rd 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/LEDTA) 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 flowcytometry 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 for20- 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 ADSC. After 5 days, spindle-shaped cells reached 80% confluency . Morphology of cells changed gradually with passage number. Cells become more flat-shape with increasing in passage number.

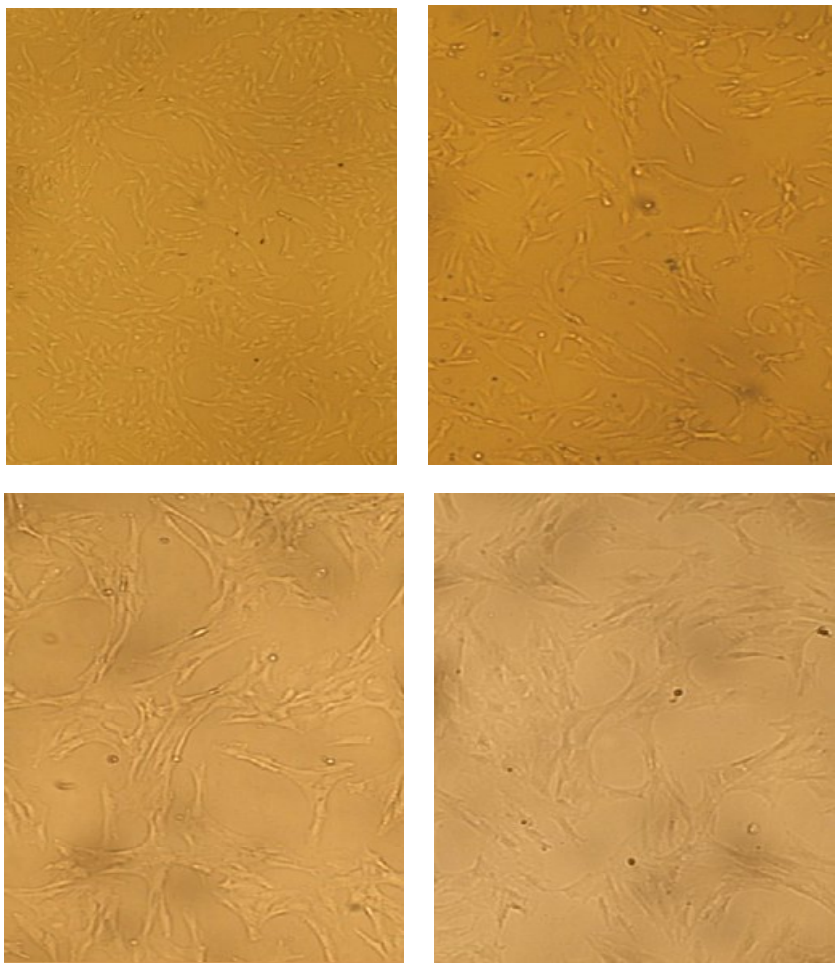

Figure 1.(a)Image of ADSC in passage 2 with 80% confluence Scale bar=100 µm.(b) Image of ADSC in passage 1 Scale bar=200 µm.(c) Image of ADSC in passage 2 Scale bar=200 µm. (d)Image of ADSC in passage 3 Scale bar=200 µm

***Immunophenotypic characterization:**

Cultures of ADSCs were analyzed for expression of cell-surface markers. ADSCs were negative for the hematopoietic lineage marker CD45 with percentage 1.55%. ADSCs were positive for CD29 and CD90 with percentage 99.9% and 91% respectively.

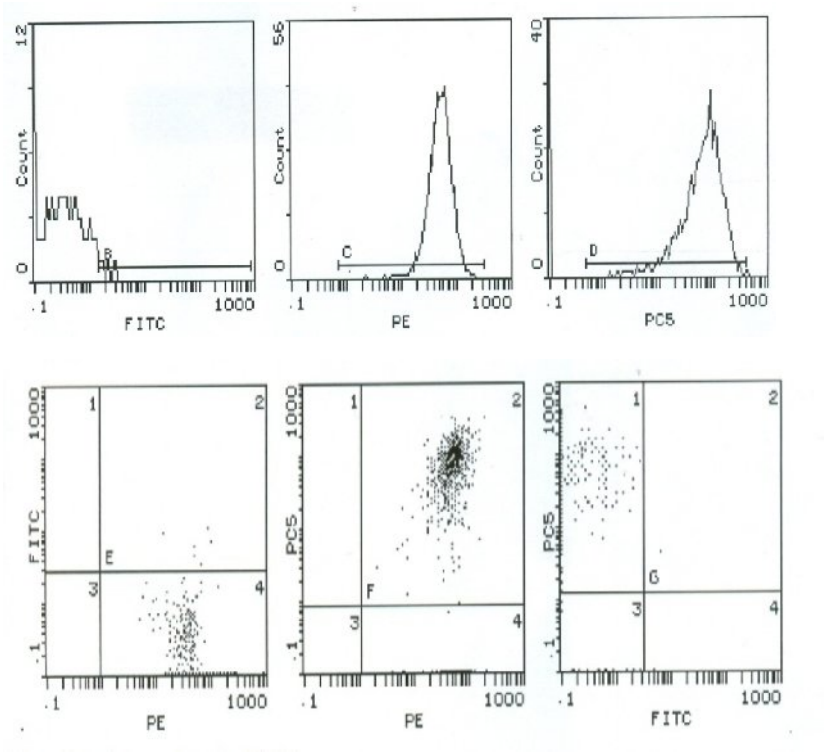

Figure 2. Immunophenotypic analysis of ADSCs. (a,b,c)Histogram representing the flow cytometry performed on the ADSCs The ADSCs were stained with monoclonal antibodies conjugated to FITC, PE and PCY5 against the following markers: CD45, CD 29 and CD90 respectively . (d) 99.2 % of cells positive for CD 29 and negative for CD45 (e) 91 % of cells positive for both CD29 and CD 90. (f) 90.4 % positive for CD 90 and negative for CD45 .

Marker	%
CD90	91
CD29	99.9
CD45	1.55

Table (1) Immunophenotypic characterization of ADSCs

CFU

CFU-F assay is a suitable tool for evaluating the proliferation and colonogenic capacity of the cells (3th passage) expanded in culture. The colony number of 100 ADSCs per 100-mm tissue culture dish is 38 ± 1.

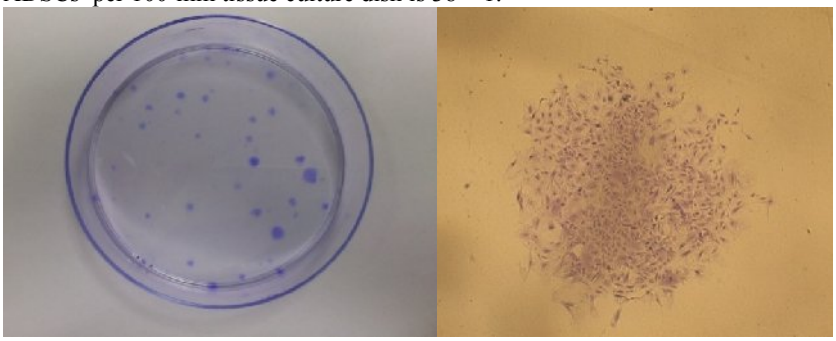

Fig 3 . (A) Crystal violet stained plate of CFU-F assays (B) one colony magnified under microscope

DISCUSSION

In the present study, rat ADSCs were isolated from the inguinal fat pad 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 (Zuk et al., 2002; Peroni et al., 2008) (2,5). Moreover, Greco et al. (2007) (6) showed similarities between mesenchymal stem cells and embryonic stem cells.

In the present study, flow cytometric analysis showed that 91% of ADSCs expressed CD90 and 99.9% of ADSCs expressed CD29, after three passages. CD29 (beta-1 integrin) is a mesenchymal cell marker that is expressed in both BM-MSCs and ADSCs (7). Nevertheless, the presence of hematopoietic cell markers on the human (8) ADSCs has been reported. In the present study, the expression of CD45 was detected in the freshly isolated rat ADSCs (1.95%).

In summary, the results of the present study showed that adipose tissue 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 ADSCs combined with their the proliferation and colony forming capacity suggest that ADSC is a good multipotential cell candidate for the future cell replacement therapy.

CONCULSION

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