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Isolation of adipose tissue mesenchymal stem cells without tissue destruction: A non-enzymatic method

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ABSTRACT

The conventional enzymatic method is widely used for mesenchymal stem cells (MSCs) isolation from adipose tissue. The method holds major drawbacks; it is costly, time-consuming and results in a heterogeneous cell population. Besides, digestion of extracellular matrix causes cell injury and compromise proliferation and differentiation of the cells. Also, because of over handling the samples are also prone to contamination. Here, we introduce a non-enzymatic method for MSCs isolation without disturbing the cells habitat. Small pieces of adipose tissue obtained from animal or human liposuction were explanted into a culture flask, immobilized by fetal bovine serum (FBS) and incubated overnight. The explants were then irrigated with DMEM containing FBS. Within few days, the fibroblast-like cells migrated from the tissue and proliferated rapidly. When subconfluent, the cells were harvested, expanded through 3 passages and used for immunophenotyping and differentiation assays. As judged by flow cytometric analysis of surface markers ($CD44^+$, $CD105^+$, $CD34^-$, $CD45^-$), Oil Red O and Alizarin Red staining, the MSCs isolated by our non-enzymatic method were pluripotent and exhibited the potential for differentiation into adipocyte and osteoblast. Great isolation yields, homogeneity of isolated cells, brief procedure, and high economy are the advantages of our method over the conventional protocol.

1. Introduction

Mesenchymal stem cells (MSCs) are highly proliferative fibroblast-like cells with pluripotent capacity. Their ability to differentiate into different cell types, including adipocyte, osteocyte, chondrocyte, myocyte and neuron, make them attractive source for cell therapies (Schaffler and Buchler, 2007). Therefore, MSCs are considered as a new therapeutic tool for repair or regeneration of damaged organs. The MSCs can be isolated from several tissues such as bone marrow, skin, adipose tissue, tendon, synovial membrane, periodontal ligament and nervous system (Orbay et al., 2012). Subcutaneous adipose tissue is a readily available and abundance source for MSCs isolation. Currently, basic enzymatic digestion is a widely used method for isolation of the MSCs from adipose tissue samples obtained by liposuction or excision. The method has a lengthy procedure and involves digestion of the tissue with collagenase, centrifugation, washing of the differentiated adipocytes and collection of stroma-vascular fraction (SVF). The fraction is then hemolysed by ammonium chloride and has to go through nylon mesh filtration. The isolated cells are then seeded for expansion and characterization (Aguena et al., 2012; Ahmadi et al., 2012). This procedure is not only time-consuming and costly but also results in a heterogeneous cell population that may compromise proliferation and differentiation of the MSCs. Moreover, it increases the risk of culture contaminations because of multistep handling (Francis et al., 2010). Finally, when the sample size is small, the yield of the method is so poor that it is almost impossible to collect enough SVF to be able to expand the isolated MSCs for characterization.

In our previous report, we showed that when a tiny fragment of adipose tissue is explanted into a tissue culture flask, fibroblast-like cells start to migrate from the tissue within few days and proliferate with a considerable rate (Ghorbani, 2010; Ghorbani et al., 2011). In this study we used the same tissue culture protocol to characterize these fibroblast-like cells and determine if they have pluripotent potential.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagles Medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco. Penicillin–streptomycin, type-II collagenase and β-glycerol phosphate were provided from

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Sigma. Indomethacin and human insulin were kindly provided by EXIR Company (Iran). Dimethyl sulfoxide and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Fluka. Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD34, CD44, CD45 and CD105 were purchased from AbD Serotec. All other materials were of analytical grade and obtained commercially.

2.2. Preparation of adipose tissue sample

All experiments were carried out according to the ethical guidelines of Mashhad University of Medical Sciences, Iran. Animal samples were prepared from male albino Wistar rat under ether anesthesia. Briefly, subcutaneous adipose tissue was excised, sliced into small pieces and washed with phosphate-buffered saline (PBS) and used for MSC isolation. Human samples were obtained as waste materials from patients going through cosmetic abdominal liposuction. Upon receiving, oil droplets were removed and the samples were washed with PBS. The slices of the oil-free tissue were then used for the cell isolation.

2.3. Isolation of MSCs by classical method

The tissue samples were incubated at 37 °C for 60 min in PBS containing 2 mg/ml of collagenase and shacked (60 cycles/min) for 60 min. After centrifugation, the floated lipid layer was discarded and the SVF was collected, washed and resuspended in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin and seeded into tissue culture flask. After 24 h, the non-adherent cells were discarded by changing the medium and the anchorage-dependent cells were preserved. When subconfluent, the cells were harvested and expanded further through 3 passages.

2.4. Isolation of MSC with non-enzymatic method

Four small pieces (≈5 mg) of each sample were washed thoroughly with FBS. After washing, tissue fragments were explanted into the corner of two separate tissue culture flask. The surface of each specimen was then covered with 50 μl of pre-warmed FBS. Care was taken to avoid specimen floating. The explants were incubated for 24 h under standard condition; 37 °C and 5% CO_2. At the end of the incubation period, the FBS was replaced by DMEM containing antibiotic and 20% FBS and the cultures were monitored daily by microscopic examination until the fibroblast-like cells were appeared around the tissue fragments; usually 3–5 days after seeding. At this time, 5 ml of DMEM containing antibiotic and 20% FBS was added to the flask and the cells incubated under standard condition until they reached confluent. The cells from subconfluent cultures were then harvested and expanded through 3 passages.

2.5. Differentiation of MSCs to adipocyte

Subconfluent (70–80%) MSCs were seeded in 12-well culture plate containing DMEM supplemented with antibiotic and 10% FBS for 24 h. The medium was then changed into differentiation medium consisting of DMEM supplemented with 3% FBS, 66 μM biotin, 250 μM IBMX, 1 μM dexamethasone, 34 μM D-panthothenate, 5 μM indomethacin and 0.2 μM insulin. The cells were maintained in the differentiation medium for 3 days and then exposed to the adipocyte maintenance medium consisting of DMEM supplemented with 3% FBS, 66 μM biotin, 1 μM dexamethasone, 34 μM D-panthothenate and 0.2 μM insulin. The cells were maintained in the adipocyte maintenance medium for 9 days with medium replacement of every 3 days (Ghorbani et al., 2014; Yu et al., 2011).

2.6. Differentiation of MSCs to osteoblast

The isolated MSCs were seeded into the wells of a 12-well culture plate with DMEM supplemented with antibiotic and 10% FBS and allowed to reach 70–80% confluent. Then the culture medium was changed with differentiation medium consisting of DMEM supplemented with 10% FBS, 10 μg/ml ascorbic acid, 5 mM β-glycerol phosphate and 0.1 μM dexamethasone. The cells were maintained in the differentiation medium for two weeks and the culture medium was replaced every 3 days (Hsu et al., 2012).

2.7. Oil Red O and Alizarin Red staining

Adipogenesis was confirmed by Oil Red O, which stains intracellular triglyceride droplets. After 12 days of differentiation start, the cells were fixed with 10% formalin and then incubated for 15 min with Oil Red O solution. Thereafter, the cells were washed three times with distilled water and the dye was eluted from cells using isopropanol. The optical density of the extracted dye was read at 540 nm (Aguena et al., 2012; Yu et al., 2011).

For Alizarin Red staining, after two weeks of starting the osteogenic differentiation, the cells were fixed with 10% formalin and then incubated for 5 min with 2% Alizarin Red solution. Thereafter, the cells were washed three times with distilled water and photographed using inverted microscope (Raynaud et al., 2012).

2.8. Flow cytometric characterization of MSCs

The cells harvested from the passage 3 cultures were suspended in PBS (2×10^5/100 μl for each reaction) and then incubated for 30 min at 4 °C with the FITC-conjugated antibodies against CD34, CD44, CD45 and CD105. Flow cytometric analyses were performed using a FACSCalibur (BD Biosciences) flow cytometer. Acquired data were then analyzed by utilizing the WinMDI 2.9 software.

2.9. Statistical analysis

Statistical comparison was performed using independent-samples *t*-test. The *p*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Tissue explants and primary cell culture

Fig. 1 shows migrating cells moving out of the tissue fragments explanted into tissue culture flasks for 3 days. Cells adhered to the flask and proliferated with a considerable rate. The cells are fibroblast-like and had spindle shape that is consistent with MSC morphology.

3.2. Differentiation of MSCs

To determine whether the cells have pluripotent capacity, they were cultured in differentiating media specific for adipocyte and osteocyte. Oil Red O and Alizarin Red staining showed that the cells isolated by classical and non-enzymatic methods could differentiate into adipocyte and osteoblast lineages. Cells isolated from rat adipose tissue (Fig. 2) and human fat tissue (Fig. 3) respond to the differentiating media in a similar way. As shown in Fig. 4, the adipocytes differentiated from the MSCs isolated by classical method and non-enzymatic method did not show significant differences in the level of lipid content.