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IN VITRO COMPARATIVE STUDIES OF ADIPOCYTE STEM CELLS DERIVED FROM HUMAN ADIPOSE TISSUE AND AMNIOTIC FLUID CELLS

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ABSTRACT

Mesenchymal stem cells (MSCs) represent an archetype of multipotent somatic stem cells that hold promise for application in regenerative medicine. The present study aimed to isolate MSCs from adipose tissue and amniotic fluid (hAFSCs) and re-differentiation into either adipose. Adult human adipose tissue (hATSCs) contains a population of mesenchymal stem cells, which can be harvested readily, safely, and in relative abundance by modern liposuction techniques. MSCs were separated from adipose tissue liposuction and amniotic fluid, cultured in dullbecus modified egal media (DMEM) for two weeks for proliferation of MSCs, which recollected and regrowing in specific media for differentiation of adipocyte cells (ACs). ACs were determined by staining with oil-red O and RT PCR assessments of adipocyte and adiponectin genes.Our findings revealed that the MSCs derived from amniotic fluid cells showed high capacity of differentiation into adipocytes comparing with that derived from adipose tissue. The ACs derived from hAFSCs were more prominent and characterized by reddish brown-droplets following staining with oil red O. Both types of adipocyte stem cells derived from either hATSCs or hAFSCs showed similar expression of molecular bands of adponectin and adipocyte gene. The authors concluded that adipocytes derived from MSCs of hAFSCs were markedly growing and expanded comparing with that hATSCs for application in regenerative medicine.

Keywords: Human adipose tissue, Amniotic fluid cells, Mesenchymal stem cells, Adipocyte stem cells, adipocyte gene, adiponectin gene.

INTRODUCTION

Adipose tissue, like bone marrow, is derived from the mesenchyme and represents a source of stem cells that have previously identified a putative stem cell population within human lipoaspirates [1]. Lipoaspirate (PLA) cells, can be isolated from adipose tissue in significant numbers and exhibits stable growth and proliferation kinetics in culture. Adipose tissue derives from the mesodermal layer of the embryo and develops both pre- and postnatally [2]. There was further evidence supporting the existence of adipose-derived stem cells within lipomas and liposarcomas [3] and obesity [4]. In vivo, models of adipogenesis suggest that, the mature adipocyte is a terminally differentiated cell, with limited capacity for proliferation and replication [5]. *In vitro*, adipose-derived stem/stromal cells display a cell doubling time of 2 to 4 days, depending upon the culture medium and passage number [6]. However, adipose-derived stem cells have high proliferation capacity *In vitro* and the ability to undergo extensive differentiation into multiple cell lineages [7]. Excessive caloric intake without a rise in energy expenditure promotes adipocyte hyperplasia and adiposity. The rise in the adipocyte number is triggered by signaling factors that induce conversion of mesenchymal stem cells (MSCs) to preadipocytes that differentiate into adipocytes [8].

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Kunisaki et al. [9] reported the successful isolation and expansion of unfractioned mesenchymal stem cells (AFMC) from human samples between 20 and 37 weeks of gestation, confirming the presence of multipotent mesenchymal cell types over the progression of gestation. A fully characterization of amniotic fluid pluripotent cell population has first been reported by De Coppi et al. [10]. Mesenchymal stem cells (MSC) are multipotent precursors who are capable of differentiating into various cell types of mesodermal origins, including chondrocytes, osteocytes, adipocytes and stromal cells [11].

Therefore, characterization of genes associated with adipocyte development is the key to understanding the pathogenesis of obesity and developing treatments for this disorder [12]. Northern blotting and gene expression profiling results showed that adipocyte-specific genes and lipogenesis-related genes are highly induced in PPARα$^{-/-}$ livers with PPARγ1 overexpression. These include among of them adipsin, adiponectin and malic enzyme among others, implying adipogenic transformation of hepatocytes [13].

We aimed to describe the differentiation capacity of mesenchymal and adipocyte stem cells from human adipose tissue and amniotic fluid cells, besides expression of adipocyte and adiponectin gene.

MATERIALS AND METHODS

Cell Culture

Human adipose Tissue

Human adipose tissue (fat) was separated during plastic surgery of seven cases of breast reduction. The fat tissue was already dissociated, by placed in a sterile specimen container at the time of the harvest. The obtained sample was stored at ambient temperature and processed within 8 h of operation. Refrigeration was avoided if possible because the lipid hardens at low temperatures. Fifty grams of adipose tissue was processed using eight 15-ml sterile, disposable conical centrifuge tubes.

Isolation of MSCs from adipose tissue

Processed lipoaspirate (PLA) cells were obtained from raw human lipoaspirates and cultured as described by Zuk *et al.*[1]. Briefly, raw lipoaspirates were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Washed aspirates were treated with 0.075% type IA collagenase (C-2674; Sigma-Aldrich, St. Louis, MO) in PBS for 30 min at 37°C with gentle agitation. The digested adipose tissue was centrifuged at 1,200 *g* for 5 min to obtain a cell pellet. The pellet was resuspended and passed through a 100-Ìm filter to remove debris. The concentration of nucleated cells was determined using a Coulter Counter. Cells were plated in medium at a density of 51106 nucleated cells/100-mm tissue culture dish. The plating density used was based on previous experience (Zuk *et al*., 2001) and designed to result in subconfluent plates within 2 weeks of initial plating. The number of adherent cells was estimated by multiplying the number of plates isolated by the cell density. The yield was determined by calculating the number of adherent cells per gram of tissue harvested. Isolation of processed lipoaspirate (PLA) Cells from adipose tissue: PLA cells were isolated using a modification of a technique described by Zuk *et al*.[1]. Adipose tissue was weighed, extensively washed with phosphate-buffered saline (PBS), minced for 10 min with fine scissors, and enzymatically digested at 37° C for 30 min with 0.075% type IA collagenase (C- 2674; Sigma®; St. Louis, Mo., USA) in PBS. The digested adipose tissue was centrifuged at 1,200 *g* for 5 min to obtain a cell pellet. The pellet was resuspended and passed through a 100-Ìm filter to remove debris. The concentration of nucleated cells was determined using a Coulter Counter. Cells were plated in medium at a density of 5 ! 106 nucleated cells/100-mm tissue culture dish. The number of adherent cells was estimated by multiplying the number of plates isolated by the cell density. The yield was determined by calculating the number of adherent cells per gram of tissue harvested.

Human amniotic fluid mesenchymal stem cells (hAFMSCs)

Mesenchymal stem cell from the human amniotic fluid (AF) of 15 of 48 women who underwent a cesarean delivery for breech presentation were isolated . The mean ± SD of pregnancy duration was 38±1 weeks and the mean volume of the AF samples was 11.2±4.7 ml. The samples were collected by puncturing the membranes after the uterine muscle was opened for the cesarean delivery. Cells were isolated rapidly for more than 4 hours prior to use. The samples were centrifuged at 1100 g for 5 minutes and all the cells isolated were plated in six 35-mm petri dishes containing low-glucose Dubelco modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL of penicillin, 0.1mg/mL of streptomycin, 10 ng/mL of basic fibroblast growth factor, 10 ng/mL of epidermal growth factor (all from Peprotech, Rocky Hill, NJ, USA), and 20% of fetal bovine serum (Invitrogen). The medium was renewed after incubation of the cells at 37 °C with 5% humidified carbon dioxide for 7 days and the nonadhering AF cells were removed.

The medium was replaced twice weekly until the cells reached 70% confluence, when they treated with 0.25% trypsin and 1 mM EDTA (Invitrogen) for 3 minutes. The MSCs were released, collected and replated in a split ratio of 1:3 under the same culture conditions.

Harvesting and Culturing of MSCs

The *MSCs* of both tissue types are usually cultured in CO2 incubator (36°±1 C°) containing 5% regular media (DMEM, 10% % FBS and 1% penicillin/streptomycin, Sigma) freshly prepared media was carried out every 3

days to remove non-sticky cells and twice weekly thereafter at least 3 weeks Cells were passaged with 0.25% trypsin/0.1% EDTA upon reaching 90% confluency and expanded until passage 4, whereupon they were analyzed. After this time, the cells are collected for characterization and isolation of stem cells.

***In vitro* assessment of viability and colony forming units-fibroblast (CFU-F)**

The viability of stem cells was checked by Trypan blue according to the method of Maclimans *et al.* [14]. Equal volume of both solution 0.04% trypan blue and stem cells were mixed and incubated for 10 minutes at 37^{o}C then the number of viable cells (unstained) was counted using a haemocytometer by light microscope. The number of viable stem cells/ml was then calculated according to the following equation.

$$\text{Viable cells (\%)} = \frac{\text{Number of viable cells / ml}}{\text{Total number of cells / ml}} \times 100$$

Concerning *colony forming units-fibroblast,* the growth was evaluated on 75ml flask containing DMEM media. After 7 days, the capability of mesenchymal stem cells to form fibroblast-like colonies was investigated by contrast-phase microscope.

Oil Red O staining

Cells were cultured in petri dishes for 4,7,14 and 21 days in DMEM plus 0.5 m*M* isobutyl-methylxanthine, 1 Ì*M* dexamethasone, 10 Ì*M* insulin, and 200 Ì*M* indomethacin [15,16]. Dishes were washed in PBS and cells were fixed in 3.7% formaldehyde for 0.5 h, followed by staining with Oil-Red-O for 0.5 h. Oil-Red-O was prepared by diluting a stock solution (0.5 g of Oil-Red-O (Sigma-Aldrich) in 100 ml of isopropanol) with water (6:4) followed by filtration. After staining, plates were washed twice in water and photographed [17]. Adipogenic differentiation from either adipose tissue or amniotic fluids were quantified by counting adipocytes in five randomly selected visual fields at magnification (1000) for each specimen (n=5) and calculating the percentage of adipogenic cells (number of cells with oil red O-positive intracellular vesicles/total number of cells).

Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of adiponectin and adipocyte genes

Extra samples of differentiated adipocytes at 4,7,14 and 21 days derived from both tissues. Total RNA was extracted from the cultured cells by using RNeasy Minikit (QIAGEN Inc., Valencia, CA) and treated with deoxyribonuclease I to remove contaminating genomic DNA. Following the manufacturer's instructions, in order to display adiponectin expression, we used primers forward TCCTGCCAGTAACAGGGAAG and reverse GGTTGGCGATTACCCGTTTG. For adipogenic differentiation, the primers used were adipocyte lipid-binding protein (product size 274 bp) forward, 5'-TACCTGGAAACTTGTCTCCAGTGAA -3' and reverse, 5'-CCATTTCTGCACATGTACCAGGACA -3'. The RT-PCR procedure was performed using the One Step RT-PCR kit, beginning at 50°C for 30 min and 95°C for 15 min for reverse transcription, then followed by 35 cycles, with each cycle consisting of denaturion at 94°C for 1 min, annealing at 57°C for 1 min, elongation at 72°C for 1 min, and the final extension at 72°C for 10 min. The amplified DNA fragments were visualized through 2% agarose gel electrophoreses and photographed under UV light [18]. Reverse transcription was performed with 1 µg RNA in a total volume of 20 µl per reaction. Real-time PCR was performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Inc., Hercules, CA). Twenty-five-microliter amplification reactions contained primers at 0.5µM, deoxynucleotide triphosphates (0.2 mM each) in PCR buffer, and 0.03 U *Taq* polymerase along with SYBR-green (Molecular Probes, Inc., Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 5- to 5000-fold to generate relative standard curves with which sample cDNA was compared. Standards and samples were run in triplicate. PCR products from all species were normalized for the amount of 18S amplicons in the reverse transcription sample, which was standardized on a dilution curve [19].

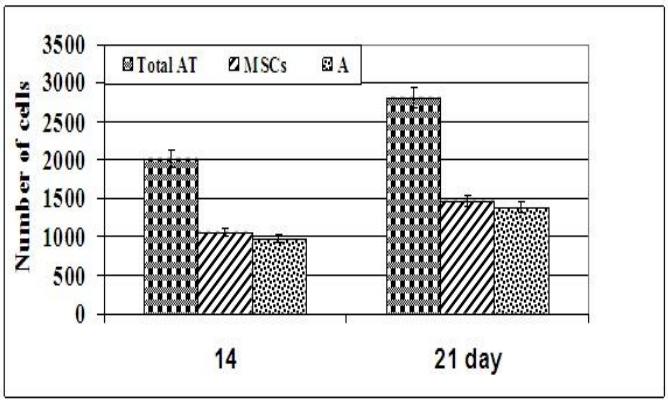
RESULTS

Adipogenic cells isolated from mesenchymal stem cells of either human adipose tissue or amniotic fluid were shown to be able to proliferate in culture. During propagation, the cell morphology changed markedly, from spindle-shaped cells to flat ones. This morphological change was accompanied by a change in cell proliferation ability. Lipid vesicles could be observed. Adipocyte differentiation from human adipose tissue was markedly flourished at 14 days post *In vitro* culturing then slightly increased (Fig. 1). However these cells derived from human amniotic fluid cells showed highest proliferation. The MSCs derived earlier from either both types of human tissues were firstly appeared dedifferentiated and spindle-shaped semi-like fibroblast cells. However, at 4 days, where adipocyte was still less differentiated, a population of both fibroblastoid and non-fibroblastoid cell types were identified. Fibroblastoid cell population was still existed after enzymatic digestion (Fig. 3).

Oil red O staining of adipocyte revealed discrete spots of cytoplasmic oil droplet within adipocytes derived from human amniotic fluid cells comparing with human adipose tissue (Fig. 2 & 4).

RT PCR analysis showed similar expression of adipocyte gene and adiponectin in *In vitro* culture cells forming adipocytes at 4,7,14 and 21 days from either human adipose tissue or amniotic fluid cells. Adipocyte gene was expressed at 280 bp, however adiponectin gene was expressed at 100 bp (Fig. 5A & B).

Fig 1. Adipocyte (A) differentiation from human adipose tissue (AT)



Each result represent the mean ± SE of 5 replicates. Total MSCs and AF significant at $P < 0.05$.

Fig 2(A&B). Photomicrograph of *In vitro* differentiation of adipose-derived stem cell (ASC) into adipocytes (A), as shown by positive Oil Red O staining of intra cellular lipid at 14 (A) and 21 days (B). Oil Red O

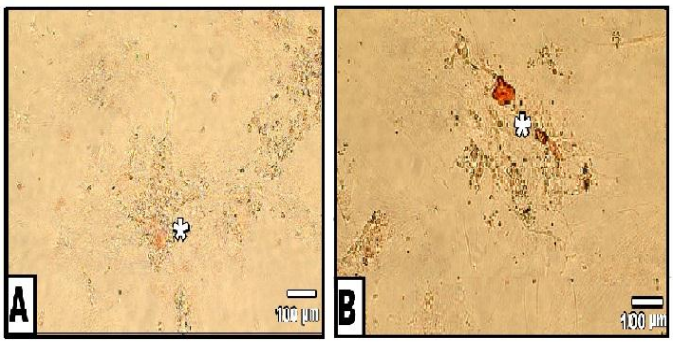
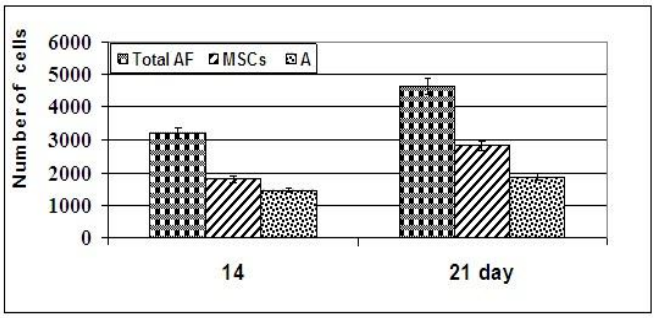


Fig 3. Adipocyte (A) differentiation from human amniotic fluid cells (AF).



Each result represent the mean ± SE of 5 replicates. Total MSCs and AF significant at P $P < 0.05$.

Fig 4 (A & B). Photomicrograph of *In vitro* differentiation of amniotic fluid (AF)into adipocytes (A), as shown by positive oil red O staining of intra cellular lipid at 14 (A) and 21 days (B). Oil Red O

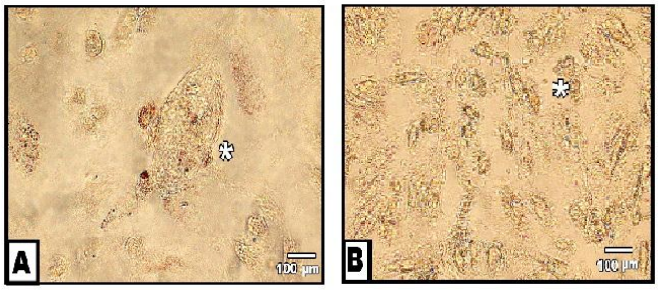
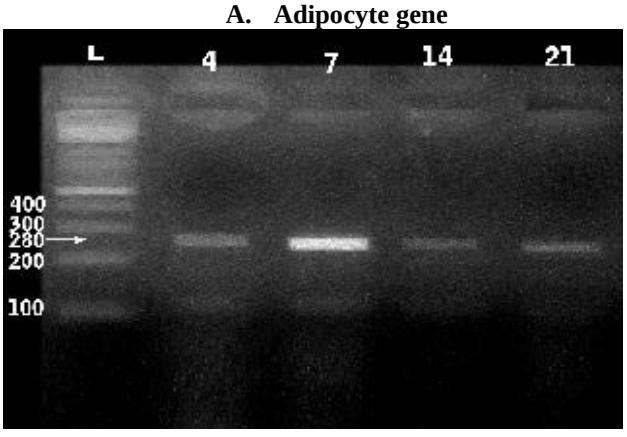
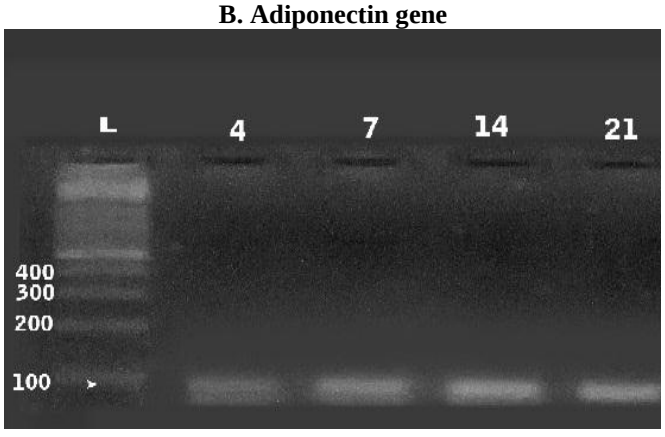


Fig 5(A&B). Gel electrophoresis for adipocyte differentiation at 4, 7, 14 and 21days showed adipocyte gene band at 280 bp and adiponectin gene at 100 bp.

A. Adipocyte gene



B. Adiponectin gene



DISCUSSION AND CONCLUSION

Adipose tissue is an abundant and accessible source of adult/ somatic stem cells for tissue engineering and regenerative medicine [20].

Taken together, the current status of knowledge is that the amniotic fluid cells harbour the potential to differentiate into cell types of the three germ layers (ectoderm, mesoderm, and endoderm) and can form embryoid bodies, known as the principal step in the differentiation of pluripotent stem cells [21].

In the present study, we confirm the multi-lineage capacity of a population of stem cells, termed PLA cells, isolated from human lipoaspirates. Preliminary studies characterized the heterogeneity and growth kinetics of this

cell population and revealed that PLA cells may have multi-lineage potential [1]. The purpose of this work was twofold: 1) to confirm whether stem cells exist in adipose tissue, and 2) to compare the differentiation potential of these cells to MSCs, a well characterized stem cell population isolated from amniotic fluid cells.

Our study revealed that the human amniotic epithelial cells and adipose tissue claimed that they possess stem cell potential, capable of differentiating into adipocyte cells. These represent the major source of multiline age of cells [22].

From the present findings, the potentiation of proliferation of MSCs and adipocytes from amniotic fluid cells exceeds the capacity of similar cells derived from human adipose tissue. These were confirmed by the marked increase of oil red o staining. The MSCs derived earlier from either both types of human tissues were, firstly, appeared dedifferentiated and spindle-shaped semi-like fibroblast cells. However, at 4 days, where adipocyte was still less differentiated, a population of both fibroblastoid and non-fibroblastoid cell types were identified. Fibroblastoid cell population was still existed after enzymatic digestion. The amniotic fluid cells grow easily in culture and appear phenotypically and genetically stable. They are capable of extensive self-renewal, a defining property of stem cells. Also, RT PCR analysis showed similar expression of adipocyte gene and adiponectin in *In vitro* culture cells forming adipocytes at 4,7,14 and 21 days from either human adipose tissue or amniotic fluid cells. Adipocyte gene was expressed at 280 bp, however adiponectin gene was expressed at 100 bp.

Our study directly compares the adipogenic potential of hATMSCs and hAFMSCs at the level of gene expression. Although the multi-lineage potential of hATMSCs and hAFMSCs was similar according to cell morphology and histology, some minor differences in marker gene expression occurred before and after induction of diverse differentiation pathways. Adiponectin is an adipose-secreted protein that exerts both anti-atherogenic and insulin-sensitizing effects, and a reduced production of adiponectin is closely coupled to insulin resistance [23,24] . The plasma concentration of adiponectin in obese subjects is lower than that in non-obese subjects and inversely correlates with body mass index [25]. Hypoadiponectinemia is associated with reduced endothelium-dependent dilatation in both diabetic and non-diabetic human subjects [26]. So the present findings suggest that adipose tissue can be an important therapeutic target in the protection of vascular dysfunction in diabetes by producing and releasing anti-inflammatory vasoactive hormones, among which adiponectin plays an indispensable role in protecting vascular function.

Finally, the author concluded that adipocyte is an important mediator in many physiologic and pathologic processes regarding energy metabolism. It has an important role in the development of obesity, cardiovascular disease and noninsulin-dependent diabetes mellitus. Differentiation from human adipose tissue and amniotic fluid cells is of critical importance for regenerating medicine.

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