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Evaluation of Biochemical Markers in Hepatocyte like Cells Differentiated from Adipose Derivation Stem Cells

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Abstract: Recent evidence proposes that cells with the properties of mesenchymal stem cells (MSCs) can be extracted from adult peripheral tissues, including muscle, dermis and adipose tissue. Adipose tissue is ubiquitous and easily obtainable in large quantities. We separated Adipose derived stem cells (ADSCs) from part of subcutaneous adipose tissue from rat. These cells could be readily differentiated into cells of the chondrocyte, osteocyte, hepatocyte and adipocyte lineage demonstrating their multipotency. It is necessary to describe the ADSCs surface expression of the typical markers by using flow cytometric and survey the results. Finally, these cells transformed into hepatocyte like cell. Immunocytochemical analysis demonstrated that ADSCs are capable to express Albumin and α-fetoprotein during differentiation. Moreover, we determined biochemical markers of hepatocyte like cells such as SGPT, SGOT, urea Production and glycogen synthesis. At last we isolated total RNA from hepatocyte like cells at shown days after differentiation and performed RT-PCR by using the specific primers (albumin, α Fetoprotein and Alpha 1-Antitrypsin).

Key words: Differentiation · Mesenchymal stem cell · Hepatocyte-like cell · Adi pose derived stem cell and Biochemical markers

INTRODUCTION

Not only mesenchymal stem cells (MSCs) display multipotency, but also under perfect conditions, they have the ability to differentiate into lineages of mesenchymal tissues including muscle, bone, cartilage and fat [1]. First MSCs were characterized in bone marrow, later a wealth of investigations proved the presence of independent MSCs progenitor cells in connective tissue of several organs including muscle, adipose tissue and trabecular bone [2]. The function of these cells is not absolutely obvious; however researchers believe that they constitute a cellular fraction reserve for maintenance and repair of tissues. Although several investigators have proved that adipose tissue hMSC is capable of transforming into morphologically recognizable cell types [3], few studies have demonstrated that the functional properties of hMSCs which differentiate *in vitro* are equivalent to those primary cell cultures extracted the same organ.

Adipose tissue may supply an alternative origin of stem cells in order to regenerate and engineer mesenchymal tissue. Adipose derived stem cells (ADSCs) have similar specificities with Bone marrow-derived mesenchymal stem cells (BMSCs) *in vitro* and *in vivo* [4]. This property represents them fascinating as a pattern in order to examine differentiation pathways and is potentially useful for cell and gene therapy.

On this manuscript we focused on the isolation of ASCs from rat adipose tissue and Evaluated of biochemical markers in hepatocyte like cells differentiated of them.

MATERIALS AND METHODS

Reagents: Antibodies for the flow cytometric assay, mouse anti-human monoclonal antibodies for albumin and αFP and the goat anti-mouse FITC-conjugated immunoglobulin G (IgG) were obtained from DAKO (Denmark) and Oxford Biomedical Research, Inc

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(UK).Dulbecco's Modified Eagles Media (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS), 0.25% EDTA solution was obtained from Gibco Bio Cult (Paisley, Scotland, UK). Stem Span media were purchased from Stem Cell Technology (USA). L-glutamine, Hepatocyte growth factor, dexamethasone, oncostatin M, Alizarin red staining and Oil red staining kits and other reagents were purchased from Sigma Aldrich Co (USA). 1% periodic acid (Sigma), Periodic Acid Schiff's (PAS) reagent (Sigma), Mayer's hematoxylin (Sigma). The RNA extraction, cDNA synthesis, hot start PCR master mix and plasmid extraction kits were purchased from Qiagene (USA).

Isolation, Culture and Expansion of ADSCs: Adipose tissue is washed extensively with sterile phosphate-buffered saline to remove blood cells and debris. The extracellular matrix is digested with 0.075% collagenase I at 37°C for 30 minutes to release the cellular fraction. Collagenase I is inactivated with an equal volume of DMEM containing 10% FBS. The infranatant is centrifuged at 250 g for 10 minutes to obtain a high density cell pellet. The pellet is resuspended in proliferation medium consisting of low glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin and 100 ìg of streptomycin, at 37°C, 5% CO_2 and humidified atmosphere containing 95% air. After 3 days, the nonadherent cell fraction was removed by washing with PBS. These cells are maintained in control medium, until they reached 70%-90% confluence. Cells were passage 4 times prior to further analysis so as to ensure removal of contaminating hematopoietic cells [5].

Immunophenotyping of ADSCs by Flow Cytometry: To characterize the ADSCs surface expression of the typical marker proteins, approximately 20×10^5 cells were incubated with fluorescent isothiocyanate(FITC) and phycoerythrin labeled antibodies for CD_{90},CD_{29} and CD_{45},CD_{31},CD_{11} and CD_{34} Isotype-matched irrelevant monoclonal antibodies were used as negative controls. For the purpose of cell-surface staining, cells were incubated in the dark for 30 minutes at 4°C in PBS supplemented with 1% BSA. After washing, cells were resuspended in PBS and measured using a Coulter Epics-XL flow cytometer.

Adipogenic Differentiation of ADSCs: 3×10^3 ADSCs cells were plated in 24-well culture plates. The cultured cells were incubated in the adipogenic medium for 14 days. Fat droplets within differentiated adipocytes derived from hMSCs were observed using the oil red O-staining method. Cell monolayers were fixed in 10% (v/v) formaldehyde solution in aqueous phosphate buffer, washed in 60% isoporpanol and stained with a 0.6% (w/v) oil red O-solution for 10 min at roomtemperature. This was followed by extensive washing with distilled water prior to destainig in 100% (v/v) isoporpanol for 15 min. The stained material was examined with phase constrast microscopy [6].

Osteogenic Differentiation of ADSCs: The potential of ADSCs to differentiate into osteogenic lineages was examined. To induce osteogenesis, hMSCs were incubated at 3×10^3 cells/cm^2 in an osteogenic medium for 2 weeks, with a medium change every third day [7]. To assess osteogenic differentiation, the cells were fixed with 90% methanol for 10 min at room temperature and identified by specific histochemical staining for calcium, using the Alizarin red staining kit. The stained material was examined with phase constrast microscopy.

Hepatic Differentiation Protocol: When ADSCs treated with Hepatocyte growth factor, oncostatin M and dexamethasone, it has been shown that they have the potential to differentiate in to hepatocyte-like phenotype by expressing albumin. Hepatic differentiation was performed using a two-steps protocol. Briefly in the first step which lasted for seven days, the cells were cultured in medium consisting of low glucose DMEM supplemented with 15% FBS, 20ng/ml of dexamethasone and 10^{-7} mol/L of dexamethasone, followed by 20 ng/mL of oncostatin M for 2 weeks [8].

Immunocytochemical Staining: After 3 weeks of cell culture under hepatocyte condition medium, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature, washed with PBS and then permeable with 0.4 % (v/v) Triton X-100 for 20 min. Corresponding primary antibodies including the mouse anti-human albumin (1:1000) and mouse anti-human αFP (1:500) were then added to the cells and incubated overnight at 4°C. The cell were subsequently washed three times with PBS and incubated with a second fluorescence-labeled antibody and FITC-labeled goat anti-mouse IgG at 37°C, for 3 h in the dark. After washing with PBS the cells were incubated with 4, 6-diamidino-2-phenylindole (DAPI) (1:1000) for the purpose of nuclear staining. The cells were then visualized using a fluorescence microscope.

Determination of SGPT and SGOT: Cultured cells were harvested with 0.25% trypsin-EDTA solution on day 21 of differentiation. After centrifugation of cells at 1000 rpm for 5 min, the pellet was resuspended in 300 μM lysis buffer containing 50 mM Tris-HCl, 1% Triton X-100, 150 mM

NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF) (pH ~7.5) on ice. The cell lysate was then homogenized with a short sonication and centrifugation at 12 000 rpm for 10 min. SGOT and SGPT levels in supernatant media derived from 10^5 cells per well were measured with quantitative kits. The methods were based on kinetics UV-test [9].

Urea Production: Differentiated cells were incubated with medium containing 5 mM NH_4Cl for 24 h in 5% CO_2 at 37°C on day 21 of differentiation. Following incubation, obtained supernatant from 1×10^5 cells per well was collected and urea concentration was measured by a colorimetric assay kit. This assay is based on reduction of ammonia produced via urea hydrolysis [9].

Periodic Acid-Schiff (PAS) Staining for Glycogen: Glycogen storage was evaluated using paraffin-embedded tissues. Briefly, sections were oxidized in 1% periodic acid for 5 min and rinsed three times in deionized water. Slides were then treated with PAS reagent for 15 min, rinsed in deionized water for 5-10 min, stained with Mayer's hematoxylin for 1 min and finally rinsed in deionized water [10].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): RT-PCR was used to determine the expression of albumin, αFP(α Fetoprotein), β-actin (as an internal control) and AAT(Alpha 1-Antitrypsin). Total RNA was isolated from ADSCs and HepG2 cells using the RNase Mini Kit protocol (Qiagene). Single-stranded cDNA was performed using the reverse transcription-PCR protocol of the First Strand cDNA Synthesis Kit. PCR parameters for amplification of the hepatocyte marker genes were as follows: reverse transcribed into first strand cDNA using oligo(dT) primer and amplified by 35 cycles (94 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min) of PCR using 10 pmole of specific primers[11]. The primers sequence used is shown in Table 1.

RESULTS

ADSCs Isolation: 1 g of adipose tissue yields approximately 5×10^3 stem cell, which is 500-fold greater than the number of MSCs in 1 g of bone marrow.

Characterization of ADSCs: Flow cytometry analysis revealed that the ADSCs cells were positive for CD_{90}, CD_{29} and negative for CD_{34}, CD_{45}, CD_{11} and CD_{31} (Figure 1).

Adipogenic and Osteogenic Differentiation of ADSCs: To examine whether ADSCs have potential to differentiate into adipocytes, oil red O-staining was performed after 12 days of culturing in adipogenic medium. Fat droplets were seen in red on the surface of cells (Figure 2A). We further examined whether ADSCs can differentiate into osteoblasts by Alizarin red staining assay. Existing of calcium deposits, characteristic of osteogenic cells, in differentiated cells after 12 days of culturing in osteogenic medium were observed (Figure 2B). ADSCs cultured in normal medium, served as a control and were negative for both staining.

Hepatic Differentiation of ADSCs: During the initiation step of hepatic differentiation, the cells showed a remarkable transition from bipolar fibroblast-like morphology to a round epithelial-like shape [12].

Immunocytochemical Staining: To determine *in vitro* hepatic differentiation of ADSCs, the expressions of albumin (liver specific protein) and αFP (a protein indicative of hepatocyte morphology) were examined. The differentiated cells were positively stained for albumin and αFP on day 21. The percentage of albumin and αFP positive cells were 79.23±2.95 and 69.37±3.79 in the differentiated cells. After washing with PBS, cells were incubated with DAPI (4, 6-diamidino-2-phenylindole; 1:1000) for nuclear staining. The cells were visualized and photomicrographed using a fluorescence microscope.

Table 1: Primers used for Reverse transcription polymerase chain reaction (RT-PCR) of liver specific gene expression

Primer	Sequence	Product size (bp)
Albumin	F 5' CTT TGG CAC AAT GAA GTG GGT AAC 3'	351
	R 5'GCA GTC AGC CAT TTC ACC ATA GG 3'	
α-Fetoprotein	F 5' CCT GTA ACC TGT GAG ACT GG 3'	252
	R 5'ATT CAA GCA CCG AAA TCT GTA G 3'	
AAT	F 5'AGA CCC TTT GAA GTC AAG GAC ACC 3'	400
	R 5'CCA TTG CTG AAG ACC TTA GTG ATG 3'	
β-actin	F 5' CTG GAA CGG TGA AGG TGA CA 3'	185
	R 5' AAG GGA CTT CCT GTA ACA ATG CA3'	

AAT (Alpha-1 antitrypsin)

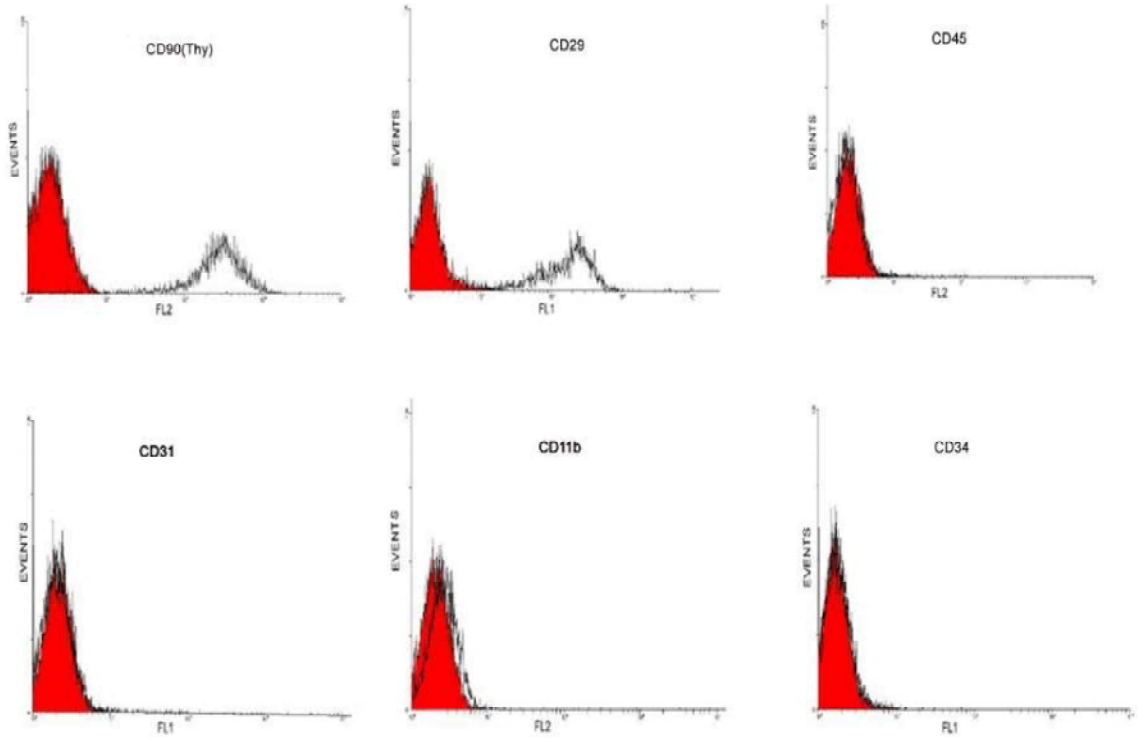

Fig. 1: Flow cytometric analysis of the ADSCs. Flow cytometric analysis was performed for the specific markers of and hematopoitic markers. ADSCs were positive for CD_{90}, CD_{29} and negative for CD_{34}, CD_{45}, CD_{11} and CD_{31}.

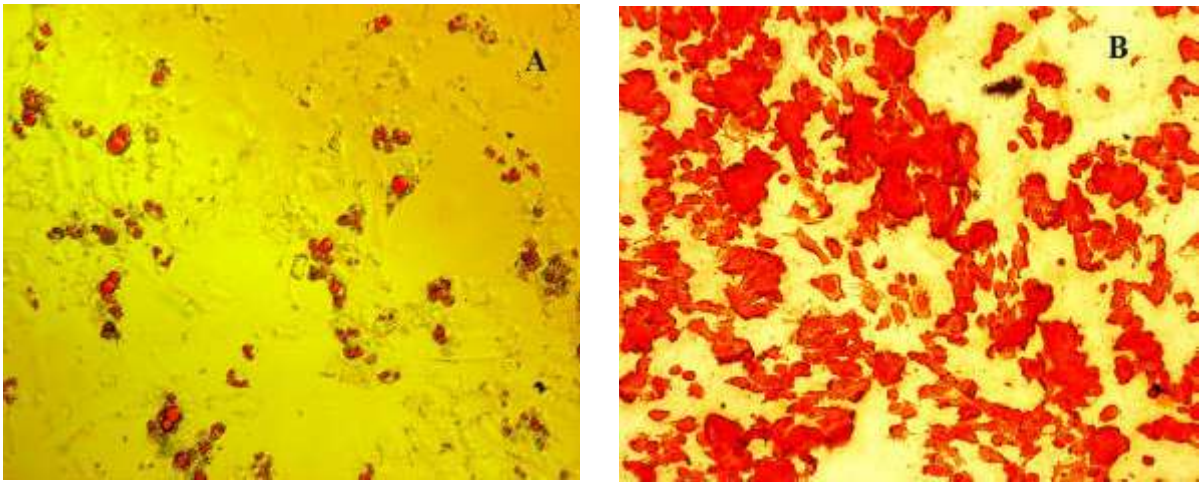

Fig. 2: Differentiation of ADSCs into adipocytes (A) and osteoblasts (B)

Functional Assay: The levels of urea excreted in culture media from differentiated cells were 15.2±4 (mg/dL) on day 21 ($P < 0.04$). Moreover, differentiated cells produced higher levels of SGPT (5.2 ± 2 mg/dL) and SGOT (7.3 ± 1 mg/dL) as compared to undifferentiated ADSCs ($P < 0.01$). The levels of urea and SGPT in culture media or cell lysate derived from undifferentiated ADSCs were negligible.

Glycogen Synthesis: Glycogen storage was determined by Periodic Acid Schiff's (PAS) staining in hepatic like cells cultured at day 21. Spontaneously differentiated cells were negative for PAS staining.

Functional Characterization of Differentiated ADSCs into Hepatocyte-Like Cells: To determine whether the cells have functional properties of hepatocytes, total RNA

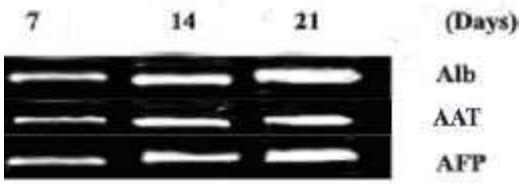

Fig. 3: Determination of hepatic lineage genes by RT-PCR. We isolated total RNA from hepatocyte-like cells at the indicated days after differentiation and performed RT-PCR by using the specific primers.

was isolated at 0, 7, 14 and 21 days after differentiation of the ADSCs into hepatocyte like cell and the expression of several hepatic proteins were examined by RT-PCR. On day 0, ADSCs did not express. Undifferentiated ADSCs did not express albumin. During differentiation expression of AFP was increased and expression of albumin was induced in ADSC-derived hepatocytes cultured with HGF, OSM and Dexamethazone at the 7_{th} day after differentiation. Expression of all the genes was demonstrated by hepG2 (positive control). The relative amount of albumin in differentiated ADSCs to HepG2 cells was 24.7% and 27.7% at 30 and 35 PCR cycles, respectively (Fig. 3). These results indicate that the hepatocyte-like cells have characteristics that closely resemble those of adult hepatocytes.

DISCUSSION

MSC could differentiate into adipocytes, osteocytes, chondrocytes, myocytes and endothelial cells [5,13]. MSC are also capable of ''transdifferentiation'' into ectodermal cells, such as neural cells [14,15]. BMSC can differentiate into hepatocyte-like cells [16]. In this study, we showed that ADSCs can be differentiated into functional hepatocyte like cells by differentiation Media. HGF originally identified and cloned as a potent mitogen for hepatocytes, shows mitogenic and morphogenic activities for a wide variety of cells that express the HGF receptor c-Met. Moreover, HGF plays an essential role in the development of the liver [17]. It has been reported that treatment of the cultures with OSM increased the cell size of hepatocytes and enhanced cell differentiation [8].

In this study, we established the method for differentiation of ADSCs into hepatocytes *in vitro*. When ADSCs were cultured in the media containing HGF and OSM and Dexamethasone was added, cells showed morphology of mature hepatocytes. In liver cell transplantation most important problem is immunorejection. However, autologous stromal cells isolated from adipose tissue are immunocompatible. Different studies have shown that transplanted hepatocytes can repopulate the diseased liver in several mouse models, including a mouse model of childhood metabolic liver disease. Transplantation of hepatocyte-like cells into recipient mice also results in detectable levels of donor cell in the circulation [18,19]. Recent studies have shown that infusion of mesenchymal stem cell through the tail vein can protect against rat liver fibrosis [20]. Typical functional hepatic features such as synthesis and/or secretion of SGPT, SGOT and urea were measured in hepatocyte-like cells derived from ADSCs and positively stained glycogen granules were detected in the cytoplasm of cells.. Therefore, we expect that ADSCs may become very useful source to hepatocyte regeneration or liver cell transplantation. This property renders them interesting as a model for studies of differentiation pathways and potentially useful for cell and gene therapy.

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