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Research article

Differentiation of adipose-derived adult stem cells into epithelial-like stem cells

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SUMMARY

Adipose-derived adult stem (ADAS) cells can be easily obtained in large quantities. Previous studies have suggested that all-trans-retinoic acid (ATRA) plays an important role in the differentiation of mesenchymal stem cells toward an epithelial lineage. In order to verify that ADAS-cells can differentiate into an epithelial lineage retaining most of the characteristics of stem cells, ADAS-cells were isolated and cultured. They were induced to differentiate toward an epithelial lineage in vitro. Differentiated epithelial cells were assayed as to whether they retain characteristics of stem cells by RT-PCR and cell cycle stage analysis, and were further induced to differentiate toward an osteogenic lineage. RT-PCR analysis revealed that no CK5, CK10 or CK19 mRNA was detected in ADAS-cells, CK19 but not CK5 or CK10 mRNA was detected in differentiated cells at passage 1, CK10 and CK19 expression but not CK5 mRNA was detected in differentiated cells at passage 10. After induction, the expression of CK19 was observed by immunofluorescent staining. Positive staining with alkaline phosphatase (ALP) and Von Kossa staining verified that differentiated epithelial cells still had potential to further differentiate toward an osteogenic lineage. These experiments provide proof that ADAS-cells can differentiate into an epithelial lineage retaining most of the characteristics of stem cells.

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1. Introduction

The loss of tissue due to trauma, tumor resection or vascular insult represents a significant clinical problem with few solutions. For patients with such problems, autologous tissue transfers have been applied to reconstruct the tissue defects, but donor site morbidity can be both cosmetically and functionally limiting. Regenerative medical therapy may well be useful in treating those suffering from tissue defects, and the use of stem cells may be promising for tissue regeneration and engineering (Mizuno and Hyakusoku, 2003).

Although embryonic stem cells (ESCs) are highly beneficial, they are limited by cell regulation and ethical considerations. Bone marrow mesenchymal stem cells have been shown to possess multiple differentiation potential in vitro. However, bone marrow procurement is extremely painful for donors, and only few cells can be harvested (Mizuno and Hyakusoku, 2003).

Stem cells in human adipose tissue have been studied in the past few years. The common terminology used for these cells is processed lipoaspirate (PLA) cells or adipose-derived adult stem (ADAS) cells (Gimble and Guilak, 2003; Schwarz et al., 2011; Zhang and Shao, 2011). Zuk et al. have shown that these cells have surface antigens and differentiation potential similar to those of mesenchymal stem cells (MSC) from human bone marrow stroma (Brzoska et al., 2005). ADAS-cells can be procured easily from the donor with little discomfort and donor site morbidity, which makes clinical application more feasible. Furthermore, these cells are derived from adults and therefore circumvent the ethical ambiguities of using embryonic stem cells. Once obtained and then modified in vitro, these cells can be reintroduced autologously, which has the advantage of reducing the probability of an immune reaction (Brzoska et al., 2005).

ADAS-cells have been proven to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells in vitro in the presence of lineage-specific induction factors (Zuk et al., 2001). All-trans retinoic acid (ATRA) can induce cytokeratin (CK) 18 expression in ADAS-cells and nearly abolish vimentin expression, indicating that ADAS-cells also have epithelial potential (Brzoska et al., 2005).

In this study, we induced ADAS-cells to differentiate toward an epithelial lineage in vitro and further induced differentiated epithelial cells to differentiate toward an osteogenic lineage.

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Table 1
Primer pairs used for PCR.

Primer pairs	Nucleotide sequences	Size of products (bp)
CK5	F: 5′-CCCAGTATGAGGAGATTGCCAACC-3′ R: 5′-TATCCAGAGGAAACACTGCTTGTG-3′	475
CK10	F: 5′-GCTGACCTGGAGATGCAAATTGAGAGCC-3′ R: 5′-GGGCAGGATTCATTTCCACATTCACATCAC-3′	131
CK19	F: 5′-GCTGGCCTACCTGAAGAAGA -3′ R: 5′-CCGCTGGTACTCCTGATTCT-3′	441
α-Tubulin	F: 5′-CACCCGTCTTCAGGGCTTCTTGGTTT-3′ R: 5′-CATTTCACCATCTGGTTGGCTGGCTC-3′	528

2. Materials and methods

2.1. ADAS-cells isolation, culture and assay

2.1.1. ADAS-cells isolation and culture

Lipoaspirates from patients undergoing cosmetic liposuction were processed according to methods first described by Zuk et al. (2001) and modified by Guan et al. (2006). Briefly, raw lipoaspirate (~300 g) was digested with 0.2% collagenase type I plus 1% bovine serum albumin (BSA) for 45 min at 37 °C. The stromal-vascular fraction was separated from remaining fibrous material and the floating adipocytes by centrifugation at 800 g for 4 min. The sedimented SVF-cells were filtered through an 80 μm pore filter and then a 200 μm pore filter, followed by an incubation step in an erythrocyte lysing buffer (160 mM NH_4Cl) for 10 min. For initial cell culture and expansion of the ADAS-cells, low-glucose DMEM medium (L-DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin was used. Cultures were washed with phosphate-buffered saline (PBS) 24–48 h after plating to remove unattached cells and then fed with fresh medium. Cultures were maintained at 37 °C with 5% CO_2 and medium was changed 3 times per week.

Cells were grown to confluence after the initial plating ($P = 0$), typically within 10–14 days. Once confluent, the adherent cells were released with 0.5% trypsin–EDTA and then either plated at 2 K/cm^2 or used for experimental analysis. All cells used for analysis were early passage (passages 3–5).

2.1.2. ADAS-cell surface marker detection

Flow cytometric characterization of ADAS-cells ranging from passage 3 to 5 was performed. Cells were suspended in PBS containing 1% BSA, and distributed to 1×10^6 cells per sample. All samples were incubated with antibodies conjugated with either FITC or PE, against the following antigens: CD29 (PE, Serotec), CD34 (PE, Miltenyi Biotec), CD44 (FITC, Serotec), CD45 (FITC, Serotec), CD49d (FITC, Serotec), CD71 (FITC, Serotec), and CD90 (FITC, Serotec), respectively.

The cytometer is FACScalibur (Becton–Dickinson) with a 488 nm krypton–argon laser. Samples were compared with isotype-matched controls.

2.1.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

The total RNA from ADAS-cells was isolated using Trizol reagent (Gibco BRL), and reverse transcribed into complementary DNA (cDNA). PCR was performed on the cDNA using primers to detect CK5, CK10, and CK19 using a GeneAmp PCR System 2400 (Perkin-Elmer), with α-tubulin served as the housekeeping gene control. The primers used are listed in Table 1 (Abiko et al., 2004; Cohen-Kerem et al., 2002; Kamiya et al., 2003). The protocol for PCR was 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C, with a final 7 min extension at 72 °C. Amplified PCR products were analyzed by ethidium bromide staining after gel electrophoresis using a high-performance gel documentation and image analysis system (AlphaImager^{TM}3300, Alpha).

2.1.4. Cell cycle analysis

Duplicate samples were harvested. 2×10^5 cells were fixed for 1 h with 75% ethanol, then incubated with propidium iodide (PI) at a concentration of 20 μg/mL for 30 min at 0 °C. Cell cycle stage was assayed using a FACScalibur cytometer (Becton–Dickinson) with a 351 nm laser.

2.2. Epithelial differentiation of ADAS-cells

2.2.1. Epithelial differentiation protocol

Isolated human ADAS-cells were plated in flasks and start differentiation process at passage 3. The epithelial differentiation culture medium was a mixture of L-DMEM and DF-12 (1:1) with 10% FBS and 1% ITS (Sigma), supplemented with 20 ng/mL epidermal growth factor (EGF, R&D), 5 ng/mL fibroblast growth factor (bFGF, R&D), 5 μM all-trans-retinoic acid (ATRA, Gibco), and 0.1 μM dexamethasone (Dex, Sigma).

The epithelial differentiation duration is 7 days and culture medium was changed every 2 days. Differentiated cells were used for different analysis. The rest of differentiated cells were cultured in keratinocyte growth medium (KGM). The KGM was L-DMEM/DF-12 (1:1) with 10% FBS, supplemented with 10 ng/mL epidermal growth factor (EGF, R&D), 5 ng/mL fibroblast growth factor (bFGF, R&D), 5 μg/mL insulin (Sigma), 0.33 μg/mL hydrocortione (Sigma), and 10 μg/mL transferrin (Sigma). The KGM was changed every 2 days.

2.2.2. Epithelial differentiated cell surface marker detection

Flow cytometric characterization of differentiated cells at passage 10 was performed using FITC or PE labeled antibodies against CD29 (PE, Serotec), CD34 (PE, Miltenyi Biotec), CD49d (FITC, Serotec), CD49f (FITC, Serotec), and CD71 (FITC, Serotec).

2.2.3. RT-PCR analysis

The mRNA level of CK5, CK10 and CK19 of differentiated epithelial cells were checked by RT-PCT at passages 1 and 10.

2.2.4. Cell cycle analysis

Cell cycle stage of differentiated cells was analyzed at passage 1 as described above.

2.2.4.1. Immunofluorescent staining. For immunofluorescence analysis, differentiated cells were first fixed in 4% paraformaldehyde for 15 min at room temperature and rinsed with PBS. Then cells were permeabilized with 0.1% Triton X-100 and 3% H_2O_2 for 10 min. The primary antibodies (Mouse anti Human CK19, ZSGB-BIO) were diluted at a definite concentration in accordance with instructions. Incubation was performed at 4 °C for 16 h. Then cells were incubated with a secondary antibody (FITC-Goat anti Mouse IgG, ZSGB-BIO) for 1 h at 37 °C. The immunofluorescence images were captured using a fluorescence microscope.

2.3. Osteogenic differentiation of differentiated epithelial cells.

2.3.1. Osteogenic differentiation protocol

Osteogenic differentiation was induced by culturing differentiated epithelial cells in an osteogenic medium (OM) for 3 weeks: L-DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 0.2 mM ascorbic acid (all from Sigma) (Cao et al., 2005). The osteogenic differentiation culture medium was changed every 3 days.

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