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Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture

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ABSTRACT

Adipose tissue-derived mesenchymal stem cells (ASCs) have been reported to be multipotent and to differentiate into various cell types, including osteocytes, adipocytes, chondrocytes, and neural cells. Recently, many authors have reported that ASCs are also able to differentiate into vascular endothelial cells (VECs) in vitro. However, these reports included the use of medium containing fetal bovine serum for endothelial differentiation. In the present study, we have developed a novel method for differentiating mouse ASCs into VECs under serum-free conditions. After the differentiation culture, over 80% of the cells expressed vascular endothelial-specific marker proteins and could take up low-density lipoprotein in vitro. This protocol should be helpful in clarifying the mechanisms of ASC differentiation into the VSC lineage.

1. Introduction

Recently, adipose tissue is an important source of adult stem cells [1]. Adipose-derived mesenchymal stem cells (ASCs) can be obtained in high yield with minimal discomfort under local anesthesia [2,3]. After the reports of Zuk et al. [4,5], many studies have examined the plasticity, induction ability, and individual characteristics of ASCs. Derived from the embryonic mesoderm, adipose tissue is a heterogeneous cell population that includes smooth muscle cells, fibroblasts, adipocytes, mast cells, and endothelial cells [6–8]. ASCs are an adherent cell population in vitro and maintain their mesenchymal phenotype and plasticity towards the mesenchymal lineage even after they propagate in culture for several passages. These cells can differentiate into several cell types in vitro, including adipocytes, chondrocytes, osteoblasts, cardiomyocytes, and endothelial cells [5,9–13]. Moreover, ASCs are reported to have positive effects on patients who received bone marrow transplantation and suffered from GVHD (graft versus host disease), suggesting that they have an immuno-modulatory function [14].

In the present study, we focused on whether ASCs are able to differentiate into vascular endothelial cells (VECs) in a chemically defined medium after expanding them. Although mouse [15], rat [16], and human [13,17–19] ASCs have already been reported to differentiate into VECs, all of the differentiation methods have utilized fetal bovine serum (FBS). When considering the clinical applications for regenerative medicine in the future, possible contamination by animal serum is a negative factor for safety. Unknown factors in FBS also prevent researchers from accurate analysis of the differentiation mechanism. Therefore, we attempted to develop a new method for differentiating ASCs into functional VECs without serum.

2. Materials and methods

2.1. Isolation of ASCs from mice

Inguinal adipose tissue was isolated from 12- to 14-week-old adult female and GFP-transgenic C57BL/6J mice. The tissue was minced into 2–3 mm pieces in DMEM (Gibco) containing 10% FBS, and incubated at 37 °C in 5% CO_2 incubator for 1 h. Then the suspension was centrifuged at 1300 rpm for 6 min at room temperature. To dissociate the cells, they were treated with 0.12% collagenase type I solution and incubated at 37 °C for 30 min and then centrifuged at 1300 rpm for 6 min at room temperature. The cells were cultured in DMEM containing 5% FBS, 10 units penicillin,

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and 10 μg/ml streptomycin (GIBCO) in a CO_2 incubator at 37 °C. After continuous culture for five passages, they were used for the differentiation experiments. To eliminate any intact VECs, CD31 positive cells were removed using anti-CD31 antibody-conjugated beads (MACS) after 2 h incubation of the preparation. The CD31 negative cells were cultured and serially passaged. Animal experiments were approved by the Animal Care and Use Committee of National Center for Global Health and Medicine.

2.2. Examination of ASCs differentiation capacity into adipogenic and osteogenic differentiation

To confirm the multipotency of our cultured ASCs, we tested whether they could differentiate into adipocytes and osteoblasts, as reported previously. Passage 5 cells were cultured in adipogenic medium for 2 weeks; hMSC Adipogenic Induction SingleQuots (Cambrex) supplemented with indometacin, IBMX, insulin, dexamethasone, NCGS, and L-glutamine. The cells were fixed in 10% formalin for 10 min and stained with Oil red-O solution (Merck) to detect lipid droplets. To confirm osteogenic differentiation, the cells were cultured in osteogenic medium: hMSC Osteogenic SingleQuots (Cambrex) supplemented with ascorbate, MCGS, β-glycerophosphate, and L-glutamine. After 2 weeks, the alkaline phosphatase activity of the cells was measured by Alkaline Phosphatase Kit (Takara Bio) and the expression of an osteogenic protein marker, osteopontin, was examined by reverse transcriptase-polymerase chain reaction (RT-PCR).

2.3. Differentiation into vascular endothelial cells

To initially determine whether the ASCs could develop the characteristics of VECs or not, they were cultured in a commercially available vascular cell maintaining medium, EBM-2 (CAMBREX) containing 2% FBS and EGM-2 BulletKit (mixture of FGF2, VEGF, heparin, IGF-I, EGF, hydrocortisone, and ascorbic acid, CAMBREX) on collagen type IV coated dish, for 12 days; then their gene expression was verified. Next, we surveyed supplements that are able to replace FBS. We tested 2% KSR, B27, N2, G5, or ITS (Invitrogen); each candidate supplement was added in EBM-2 medium instead of FBS. And we also examined the other culture medium such as DMEM, IMDM, and DMEM/F12 instead of EBM-2. Finally, to determine the optimal concentration of FGF2 or VGEF, different concentrations (0, 5, 10, and 20 ng/ml) was tested for induction of endothelial cells. When the optimal culture medium for the VEC induction from ASCs was determined to be DMEM/F12 medium containing 10 ng/ml FGF2, 2% ITS, and EGM-2 BulletKit (without FGF2), further experiments for functional assay and transplantation employed this medium.

2.4. RT-PCR and real time PCR

Marker gene expression of VECs was determined by RT-PCR. After ASCs were cultured for 12 days in endothelial differentiation medium on a collagen type IV dish, total RNA was extracted by the use of Isogen (Nippon gene) as described by the manufacturer, and was treated with Superscript III (Invitrogen) to generate cDNA using oligo(dT) adaptor primer (Sigma). Then PCR amplification was performed for mouse *flk1, flt1, VE-cadherin*, and *CD31*. PCR cycles were as follows: 95 °C for 5 min, 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 1 min (25–30 cycles), and 72 °C for 3 min. The RT-PCR products ware analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide. Primers for PCR were follows: *flk1* (5′-GCC AAT GAAGGG GAACTGAAGAC-3′, 5′-TCTGGCT GCTGGTGATGCTGTC-3′), *flt1* (5′-TGTGGAGAAACTTGGTGACCT-3′, 5′-TGGAGAACAGCAGGACTCCTT-3′), *ve-cadherin* (5′-TTGCCCAGCCC TACGAACCTAAAG-3′, 5′-ACCACCGCCCTCCTCATCGTAAGT-3′), *CD31* (5′-GGTGACACTGGACAAAAAGG-3′, 5′-CAGCTTCACTGCTTTGCTT G-3′), *gapdh* (5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, 5′-CATG TAGGCCATGAGGTCCACCAC-3′). For the real-time PCR, primers are follows: *tie2* (5′-GTGAAGATCAAGAATGCTACC-3′, 5′-GTGAAGATC AAGAATGCTACC-3′), *CD31* (5′-GTTTGTCAAGCGAAGGATAGATA A-3′, 5′-TCCTGCACGGTGACGTATTCACT-3′), *von Willebrand factor* (*vWF*, 5′-AACGGAAGTCCATGGTTCTG-3′, 5′-CCCCATTGAAGGCAT ACTCC-3′). Reactions were performed using SYBER Premix ExTaq (Takara Bio) and a MyiQ thermal cycler (BIORAD).

2.5. Immunocytochemistry

Differentiated endothelial-like cells from ASCs were fixed with 4% paraformaldehyde for 30 min at room temperature and then treated successively with 0.3% Triton X-100 (Wako Chemical) in PBS (Sigma) for 15 min followed by 3% bovine serum albumin (Sigma) for 30 min to reduce nonspecific reactions. The cells were reacted overnight with each of the following anti-endothelial marker antibodies at a 1:300 dilution at 4 °C; anti-flk1 (Becton Dickinson), anti-CD34 (Becton Dickinson), and anti-tie2 (Santa Cruz Biotechnology) antibodies. Then the cells were stained by Alexa Fluor 488 or 594 conjugated antibody (Molecular Probes) as the secondary antibody for 1 h at room temperature. Their nuclei were stained with DAPI for 10 min. The photographs were taken with a DP70 digital camera (Olympus) and analyzed by MetaMorph software (Molecular Devices).

2.6. Examination of cell function in vivo and in vitro

For the examination of tubular formation, the cells were seeded on Matrigel (Becton Dickinson) at 5×10^4 cells/35 mm dish. After 24 h, the morphology of the cells was examined, and phase-contrast images were photographed (Olympus IX70). For in vivo examination, the femoral muscle of a mouse was injured by liquid nitrogen and injected with the differentiated vascular endothelial-like cells (1×10^6 cells) from ASCs of GFP-transgenic mice. Two weeks after cell injection, we investigated whether the donor cells had formed vessel-like structures.

LDL uptake was assessed by incubating cells for 4 h at 37 °C with 2.5 μg/ml Alexa Fluor 488 conjugated acetyl-LDL (Molecular Probes). Cells were analyzed by fluorescence microscopy and a flow cytometer (EPICS XL, Beckman Coulter).

3. Results

3.1. In vitro differentiation of ASCs

To prove that the cultured cells from adipose tissue had retained their multipotent differentiation potential, we first confirmed that they differentiated into adipogenic and osteogenic lineages. When ASCs (Fig. 1A) were cultured in adipogenic medium for 2 weeks, more than 40% of the cells became lipid-retaining cells that stained by Oil-red O (Fig. 1B). In osteogenic medium, more than 50% of the cells were induced into an osteogenic lineage confirmed by alkaline phosphatase staining (Fig. 1C). The gene expression of *osteopontin* was also detected (Fig. 1D).

3.2. ASCs cultured in growth factor mix changed their gene expression pattern to closely resemble that of vascular endothelial cells

It was reported that the early passages of ASCs can contain small amounts of VECs and express VEC marker proteins [20]. Therefore, we used anti-CD31 antibody to remove any CD31 positive cells during the preparation of ASCs. To examine whether the ASCs could differentiate into VECs in "vascular endothelial maintaining