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Stemness and transdifferentiation of adipose-derived stem cells using L-ascorbic acid 2-phosphate-induced cell sheet formation

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ABSTRACT

Cell sheet technology has emerged as an important tissue engineering approach. Adipose-derived stem cells (ASCs) have valuable applications in regenerative medicine, but their stemness and differentiation capabilities in the cell sheet format have not been well investigated. In this study, we found that L-ascorbate 2-phosphate (A2-P), a stable form of ascorbic acid, significantly enhanced ASC proliferation and induced ASC sheet fabrication in 7 days with abundant extracellular matrix deposition. Importantly, A2-P treatment significantly enhanced expression of pluripotent markers Sox-2, Oct-4 and Nanog, but treating ASCs with antioxidants other than A2-P revealed no stemness enhancement. Moreover, ASC treatment with A2-P and a collagen synthesis inhibitor, L-2-azetidine carboxylic acid or cis-4-hydroxy-D-proline, significantly inhibited the A2-P-enhanced expression of stemness markers. These findings demonstrated that A2-P enhances stemness of ASCs through collagen synthesis and cell sheet formation. We also showed that A2-P-stimulated collagen synthesis in ASCs may be mediated through ERK1/2 pathway. By culturing the ASC sheets in proper induction media, ASC transdifferentiation capabilities into neuron and hepatocyte-like cells were significantly enhanced after cell sheet formation, while adipogenic and osteogenic differentiation capacities were still maintained. Using a murine model of healing-impaired cutaneous wound, faster wound healing was noted in the group that received ASC sheet treatment, and we observed significantly more engrafted ASCs with evidence of differentiation toward endothelial and epidermal lineages in the cutaneous wound tissue. Therefore, A2-P-mediated ASC sheet formation enhanced ASC stemness and transdifferentiation capabilities, thereby representing a promising approach for applications in regenerative medicine.

1. Introduction

Mesenchymal stem cells (MSCs), which are capable of self-renewal and multi-lineage differentiation, have been regarded to have great potential for their application in regenerative medicine. As a potential autologous cell source, the use of MSCs circumvents the complications associated with allogeneic transplantation. Moreover, clinical application of MSCs exhibits virtually no ethical issue that encountered with the use of embryonic stem cells. Among the various sources of MSCs, adipose-derived stem cell (ASC) represents an abundant source of multipotent adult stem cells that are easily obtained from subcutaneous adipose tissue via minimally invasive procedures, such as liposuction [1,2]. As many as 1% of adipose cells are estimated to be stem cells, compared to the 0.001–0.002% found in bone marrow, currently a common source of MSCs [3]. In addition to the ability of self-renewal, ASCs can differentiate into multiple lineages when cultivated under lineage-specific conditions, including osteogenic, adipogenic, and chondrogenic lineages [4]. This ability, together with their easy accessibility and low donor site morbidity, has made ASCs good candidates for a broad range of cell-based therapeutics.

Cell sheet technology has been applied to enhance the regenerative effects of tissue-engineered products in recent years [5,6]. With a cell sheet approach, cell-to-cell connections are not disrupted and cells are harvested as a contiguous cell sheet, thus preserving cell-to-cell junction proteins and native extracellular matrix (ECM) secreted by the cells [6]. ECM has been shown to play an important role in improving propagation of stem cells and stem

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cell differentiation [7,8]. Previous investigators have adapted different techniques for cell sheet formation, such as the development of a temperature-responsive culture dish that could be used to harvest cultured cells and their deposited ECM non-invasively as intact sheets [9]. However, the entire grafting process has been criticized to be relatively complicated, time-consuming, and requires special materials [10]. Alternative approaches of using ascorbic acid to create cell sheets have also been proposed [5,10,11]. Ascorbic acid is not only a common nutrient vital to human health, it also plays a key role in the biosynthesis of collagen and other ECM constituents [12]. By supplementing ascorbic acid in the culture medium, cell sheets derived from human MSCs of various tissue origin can be fabricated in 7–10 days [10].

Ascorbic acid has been used as a supplement for culture of various cell types. In addition to stimulating ECM production, it can also act as an antioxidant to suppress intracellular reactive oxygen species (ROS) levels and exert protective effects on cells [13,14]. Ascorbic acid can also stimulate cellular proliferation and DNA synthesis of MSCs during *in vitro* culture [15]. Moreover, ascorbic acid has been shown to enhance the generation of mouse and human induced pluripotent stem (iPS) cells [16], and it can further enhances the cardiac differentiation of embryonic stem cells or iPS cells [17,18]. For MSCs, a previous report also showed that ascorbic acid can inhibit differentiation and upregulate pluripotent marker expression of *Oct4* and *Sox2* [19]. Therefore, the addition of ascorbic acid during *in vitro* culture of stem cells is considered to be beneficial. However, the use of ascorbic acid is limited by its rapid oxidation [20]. L-ascorbic acid 2-phosphate (A2-P), a more stable oxidation-resistant derivative of ascorbic acid, has been adapted in several studies and stimulated the growth of various cells more effectively [15,21–23].

The application of ASC-based cell sheet technology has been successful in creating tissue-engineered adipose substitute, treatment of myocardium infarction and enhancing skin wound healing [5,24,25]. However, the regenerative mechanism of ASC sheets is not well understood, and the differentiation capabilities of ASC sheets have not been fully investigated [10,26]. In this study, we tested the hypothesis that ASCs in a cell sheet format can maintain a stable undifferentiated status without loss of multipotency. We aimed to develop a practical method of A2-P-mediated ASC sheet formation, and we further examined the stemness and differentiation capabilities of ASCs within the cell sheets. The information is useful in elucidating the importance of ECM within cell sheets in maintaining the regenerative capabilities of stem cells, so it should benefit the future application of cell sheet-based tissue engineering.

2. Materials and methods

2.1. Cell culture

Subcutaneous adipose tissue from abdomen was obtained from 5 female donors undergoing abdominoplasty procedures with an average age of 42 (range 30–57) and an average body mass index (BMI) of 24.8 (range 21.0–26.6). The study protocol was approved by the Internal Ethical Committee of National Taiwan University Hospital. The adipose tissue was placed in a physiological solution (0.9% NaCl), washed twice with phosphate-buffered saline (PBS; Omics Biotechnology, Taipei, Taiwan) and finely minced. The scraped adipose tissue was then placed in a digestion solution: 1 mg/ml collagenase type I (Gibco, Carlsbad, CA) dissolved in PBS, at 37 °C in agitation for 60 min. After digestion, the cell suspension was filtered through 40 μm cell strainers (BD Falcon, Franklin Lakes, NJ). The cells were cultured in expansion medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Hyclone, Logan, UT), 10% fetal bovine serum (FBS; Biological industries, Kibbutz Beit Haemek, Israel), 1% 1% antibiotic-antimycotic (Biological Industries), and 1 ng/ml basic fibroblast growth factor (bFGF; R&D systems, Minneapolis, MN). The cells were cultured at 37 °C in 5% CO_2, and the medium was changed every 2 days. When the cells have reached 90% confluence, the cells were lifted with 0.05% trypsin-EDTA (Biological Industries) and replated.

2.2. Cell sheet formation

Third passage ASCs were harvested or further experiments. To create cell sheets, 5×10^5 ASCs were cultured in a 100 mm culture dish for 7 days. The culture medium consisted of DMEM-HG (Gibco), 10% FBS, 1% antibiotic-antimycotic, and 250 μM A2-P (Sigma). The medium without A2-P was used in the control group. The culture medium was refreshed every 2–3 days. Cells with or without A2-P treatment were also subjected to analyses including reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence, and western blotting. U0126, an inhibitor of ERK1/2, was prepared in DMSO and used in ERK1/2 signaling inhibition assay. The concentration of U0126 used was 5 μM, which did not affect APCs proliferation in our pilot study. The ERK signaling in the long-term culture was checked by treating the cells with A2-P or U0126 for 3 days.

2.3. Flow cytometry analysis

After treatment with or without 250 μM A2-P for 7 days, ASCs were subjected to flow cytometry analysis to determine cell surface antigen expression. The cells were incubated with the following antibodies: human monoclonal antibodies against CD31 (BD pharmingen, San Jose, CA), CD34, CD73, CD90 (all from BioLegend, San Diego, CA), CD105 (eBioscience, San Diego, CA) and CD166 (BioLegend). The samples were analyzed using a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, NJ) which counts 10,000 cells per sample. Positive cells were determined as the proportion of the population with higher fluorescence than 95% of the isotype control.

2.4. Electron microscopy, histology and immunohistochemistry

For the electron microscopic study, cell samples were washed with PBS twice and fixed with 2.5% glutaraldehyde in PBS for 1 h. After thoroughly washing with PBS, the cells were dehydrated by gradual change of concentrated ethanol and then dried by lyophilization. The specimens were then sputter coated with platinum and examined using a scanning electron microscope (JSM-6700F, JOEL, Tokyo, Japan).

ASC sheets induced by A2-P treatment could be easily detached from tissue culture plates and fixed in 4% paraformaldehyde for paraffin-embedded histological analysis. Sections were cut perpendicular to the surface of the cell sheet into a thickness of 5 μm. Paraffin-embedded sections were rehydrated and stained with hematoxylin and eosin (H&E, Sigma) and Masson's trichrome stain (Sigma). Immunohistochemical analysis was performed using anti-collagen type I (Epitomics, Burlingame, CA), anti-fibronectin (Epitomics) and anti-laminin (Pierce Biotechnology, Rockford, IL). Bovine serum albumin (BSA, Santa Cruz, Santa Cruz, CA) was used on all sections before secondary antibody labeling, followed by subsequent linking to horseradish peroxidase and substrate/chromogen reaction using immunoperoxidase secondary detection kit (Millipore, Billerica, MA). Negative controls without utilizing primary antibodies were also prepared to rule out nonspecific labeling.

2.5. Cell proliferation assay and growth curve

The cell proliferation assay was performed by measuring double-strand DNA (dsDNA) content of the ASCs. Cells were seeded at a density of 5×10^3 cells per well in 24-well plates with or without 250 μM A2-P. On day 1, 4, 7, DNA content was measured fluorometrically using the Quant-iT Picogreen dsDNA Reagent and Kits (Invitrogen) according to the manufacturer's protocol (excitation wavelength, 485 nm; emission wavelength, 535 nm). Moreover, ASCs were seeded at a density of 2.5×10^5 cells per 100 mm culture dishes with or without 250 μM A2-P treatment. Every 7 days, cells were lifted, counted with Scepter™ 2.0 Cell Counter (Millipore), and replated. The process was repeated until cells reached senescence.

2.6. Differentiation of human ASCs

ASCs were pretreated with or without 250 μM A2-P for 7 days before induction of differentiation. Adipogenic differentiation was induced in DMEM-HG supplemented with 10% FBS, 1% antibiotic-antimycotic, 500 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO), 1 μM dexamethasone (Sigma), 10 μM insulin (Sigma) and 400 μM indomethacin (Sigma). After 9 days, the cells were fixed in 4% paraformaldehyde and stained with Oil Red O (Sigma) to observe lipid droplets. Then the dye was eluted by isopropanol and measured by a spectrometer (μQuant, BIOTEK, Seattle) at 510 nm. Osteogenic differentiation was induced by culturing ASCs in DMEM-HG supplemented with 10% FBS, 1% antibiotic-antimycotic, 10 nM dexamethasone, 50 μM A2-P, 10 nM 1α,25-Dihydroxyvitamin D_3 (Sigma), and 10 mM β-glycerophosphate (Sigma). After 9 days, ASCs were fixed in 4% paraformaldehyde and stained with Alizarin red S (Sigma) to observe mineralized matrix apposition. The bound Alizarin red S was extracted by 10% hexadecylpyridinium chloride monohydrate (Sigma), and the absorbance of dye was measured at 550 nm. Quantification of ASC adipogenic and osteogenic differentiation was estimated by normalizing the absorbance of the eluted dye to the dsDNA content within each well.

To induce neurogenic differentiation, the cells were cultured in DMEM-HG containing 1% FBS, 1% antibiotic-antimycotic and 100 ng/ml bFGF (R&D) for 7 days, followed by supplementation with 100 ng/ml bFGF and 10 μM forskolin for another 7 days [27]. At day 14, the expression of neurogenic differentiation markers, Nestin and GFAP, was analyzed by RT-PCR, western blot and immunofluorescence. Hepatogenic differentiation was induced in DMEM-HG medium with 1% antibiotic-antimycotic, 20 ng/ml epidermal growth factor (R&D) and 10 ng/ml bFGF for 48 h. In