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Original Article

Enhancement of individual differences in proliferation and differentiation potentials of aged human adipose-derived stem cells

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ABSTRACT

Background: Adipose-derived stem cells (ASCs) are a robust, multipotent cell source. They are easily obtained and hold promise in many regenerative applications. It is generally considered that the function of somatic stem cells declines with age. Although several studies have examined the effects of donor age on proliferation potential and pluripotency of ASCs, the results of these studies were not consistent.
Objective: This study tested whether the donor age affects the yield of ASCs from adipose tissue, as well as the proliferation and differentiation potentials of ASCs.
Methods: This study used ASCs obtained from adipose tissues of 260 donors (ages 5–97 years). ASCs were examined for individual differences in proliferation, and adipogenic, osteogenic and chondrogenic differentiation potentials *in vitro*. Characteristics of ASCs from each donor were evaluated by the principal component analysis (PCA) using their potential parameters.
Results: Analyses on ASCs demonstrated that adipogenic potentials declined with age, but proliferation, osteogenic and chondrogenic potentials were not correlated with age. Interestingly, in all ASC potentials, including adipogenesis, individual differences were observed. Principal component analysis (PCA) revealed that individual differences became evident in the elderly, and those variations were more prominent in females than in males.
Conclusions: This study demonstrated age-related changes in the potentials of ASCs and revealed that the individual differences of ASCs become significant in people over 60 years of age (for females over 60, and for males over 80). We believe that it is important to carefully observe ASC potentials in order to achieve effective regenerative medicine treatments using ASCs.

1. Introduction

Mesenchymal stem cells (MSCs) are adult stem cells capable of differentiating into mesenchymal cells such as osteoblasts, adipocytes, chondrocytes and muscle cells, and are thought to hold great promise in the field of regenerative medicine. MSCs can be obtained from several tissues such as bone marrow, adipose, muscle, skin and umbilical cord, and MSCs from any of these tissues have similar

Abbreviations: αMEM, α minimum essential medium; CFU-F, colony-forming unit-fibroblast; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; GAGs, glycosaminoglycans; PCA, principal component analysis; PDL, population doubling level.

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morphological characteristics and immunophenotypes [1–3]. Among these stem cells, bone marrow-derived stem cells (BMSCs) have been studied over many years; a large number of studies including clinical data have been published [4–11]. However, BMSCs are not always easy to obtain because of the donor's health condition. Furthermore, the percentage of BMSCs contained in bone marrow is only 0.001–0.01% [12] of all nucleated cells.

On the other hand, as many as 600,000 to 800,000 stromal vascular fraction (SVF) cells can be isolated per gram of adipose tissue, and a great number of adipose-derived stem cells (ASCs) are contained in the SVF cells [13]. More specifically, approximately 500-times more MSCs are available from adipose tissue than from the same amount of bone marrow, thus, adipose tissue makes it easier to obtain MSCs from all donors with a minor risk. As ASCs are characterized by excellent proliferation potential, and can be cultured in large quantities and cryopreserved [14], ASCs have gained attention in recent years as a source of cells for application in cellular treatments [15,16].

It is generally considered that the function of somatic stem cells declines with age. Some studies have suggested that proliferation and osteogenic potentials of BMSCs also decline with age [17–19]. Although several studies have demonstrated the effects of donor age on proliferation potential and pluripotency of ASCs, the findings were not consistent across these studies [20–30]. In our previous study, we examined age-related changes using mouse ASCs and found that the number of ASCs obtained, as well as their adipogenic and osteogenic potentials decline with age [31]. In this study, we evaluated age-related changes in the numbers and potentials of human ASCs obtained from 260 donors (ages 5–97 years, mean 61.8).

2. Materials and methods

2.1. Collection and processing of adipose tissue-derived mesenchymal stem cells

This study was conducted with an ethical approval from the Research and Ethics Committee of Fujita Health University (approval No. 15-235). After obtaining informed consent, subcutaneous adipose tissue was obtained from excess normal skin of total 260 patients aged 5 to 97 with median age of 68 (male: n = 115, 5–97 years old, median age: 66; female: n = 145, 5–96 years old, median age: 69) who underwent a dermatological surgery. Subcutaneous adipose tissue (0.1–11 g, median 0.64 g) from a single part of body of each patient was used to prepare a specimen. The tissues were collected from various body parts (abdomen: 29.2%, groin: 15.8%, lower limb: 14.2%, back: 10.4%, buttock: 7.3%). The subcutaneous adipose tissue was shredded and incubated for 1 h at 37 °C in 0.5% collagenase L (Nitta Gelatin Inc., Osaka, Japan), followed by filtration using 100 μm nylon cell strainer (Falcon®, Coning, NY, USA). After centrifugation, cell precipitates were suspended by tris-buffered ammonium chloride (ACTB) to eliminate red blood cells and washed by PBS. The cells separated in this way are defined as SVF cells, and among SVF cells, only cells which were able to adhere and multiply are referred to as ASCs in this study. The separated SVF cells and ASCs were cultured in complete medium (D/α medium) consisting of 50% Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA)/50% α minimum essential medium (αMEM, Invitrogen) supplemented with 1% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1 × ITS-X (Invitrogen), 10 ng/mL basic FGF (PeproTech, Rocky Hill, NJ, USA), and 0.4 μg/mL hydrocortisone and 1% Antibiotic-Antimycotic (Gibco BRL, Rockville, MD) at 37 °C in a humidified atmosphere with 5% CO_2 until passage 5. Population doubling level (PDL) and doubling time were determined by counting the number of viable cells in each passage using a hemocytometer. Seeding density was 1–2 × 10^4/cm^2. Culture media were replaced every 2–3 days.

2.2. CFU-F assay

To measure a colony-forming unit-fibroblast (CFU-F), ASCs (passage 0) were seeded at 1 × 10^2 cells/well in triplicate in 6-well plates (Falcon®) and cultured at 37 °C in a humidified atmosphere with 5% CO_2 in D/α medium. After 14-day culture, cells were fixed with methanol and stained with a solution of 0.5% (w/v) crystal violet (Sigma-Aldrich). After the individual colonies were counted, the frequency of CFU-F was expressed as a percentage. The culture medium was replaced once after 6 days.

2.3. Measurement of adipogenesis

ASCs (passage 5) were seeded in triplicate in a 24-well plate (Falcon®) at a density of 4 × 10^4 in D/α medium. After 4 days, differentiation induction was carried out using adipogenic induction medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 33 μM Biotin (Sigma-Aldrich), 10 μg/mL Insulin (Sigma-Aldrich), 1 μM deamethasone (Sigma-Aldrich), 0.5 mM 3-isobuthl-1-methylxanthine (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), and 1% Antibiotic-Antimycotic (Gibco) for 6 days. Additionally, ASCs were cultured using adipogenic differentiation medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 3 μM Biotin (Sigma-Aldrich), 10 μg/mL Insulin (Sigma-Aldrich) and 1% Antibiotic-Antimycotic (Gibco) for 4 days. The medium was replaced every 2–3 days. After adipogenic differentiation was induced, optical densities were determined using Cell Counting Kit-8 (cck8; DOJINDO Laboratories, Kumamoto, Japan) solution mixed with D/α medium. The adipogenic differentiation medium was replaced with this solution, and then the differentiated ASCs were incubated for 1 h at 37 °C under 5% CO_2. Next, the supernatants (100 μl) were transferred into a 96-well plate, and the absorbance values were measured at 450 nm. All analyses were carried out in triplicate.

Adipogenesis was assayed by lipid accumulation in differentiated cells with Oil Red O staining. After staining, Oil Red O was extracted with 100% isopropanol and the optical density (OD) of the solution was measured at 520 nm as well as cck8 [31]. Optical densities of Oil Red O were adjusted by the result of cck8. For comparison of Oil Red O/cck8 values among donors, ASCs were cultured with standard cells (UE7T-13: immortalized bone marrow-derived mesenchymal stem cell) for differentiation induction. Oil Red O/cck8 values of ASCs was adjusted relative to those of UE7T-13, and the adjusted values were used for evaluation of adipogenic potential.

2.4. Measurement of osteogenesis

ASCs (passage 5) were seeded in triplicate in a 24-well plate (Falcon®) at a density of 4 × 10^4 in D/α medium. After 4 days, differentiation was carried out using osteogenic differentiation medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 50 μM ascorbic acid 2-phosphate (Sigma-Aldrich), 0.01 μM Deamethasone (Sigma-Aldrich) and 1% Antibiotic-Antimycotic (Gibco) for 21 days. Culture medium was replaced every 2–3 days. After induction was completed, the cell densities were determined using cck8.

Osteogenesis was assayed by calcium content in differentiated cells and matrix mineralization with Alizarin Red S staining. The total calcium content was determined by a colorimetric assay using Calcium E-Test Wako (Wako Pure Chemical Industries, Osaka,