Adipose-derived stem cells as a new therapeutic modality for ageing skin

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Abstract: Stem cells are undifferentiated cells, which have the important properties of self-renewal and differentiation. Adipose-derived stem cells (ADSC) have relative advantages in accessibility and abundance compared to other kinds of stem cells. Regeneration therapy using ADSC has received attention in the treatment of various dermatologic diseases. In previous studies, ADSC were shown to have antioxidant, whitening and wound-healing effects in the skin through secretion of growth factors and by activating fibroblasts. In this study, we investigated whether ADSC could be used as an anti-ageing therapy, especially by dermal collagen synthesis and angiogenesis. Subcutaneous injection of ADSC significantly increased collagen synthesis in hairless mice, and dermal thickness, collagen density and fibroblast number also increased. In addition, procollagen type I protein and mRNA expression increased, which accounts for the increased dermal collagen density. Angiogenesis, which was visualized by CD31 and NG2 immunofluorescence stains, also increased in ADSC-treated skin. Our results suggest that ADSC therapy may be useful in ageing skin. Its effects are mainly mediated by stimulating collagen synthesis in dermal fibroblasts and increasing angiogenesis.

Key words: adipose-derived stem cell – anti-ageing – regeneration

Introduction

Cell therapy using reproductive cells such as stem cells restores organs damaged by disease or trauma. This new therapeutic modality has come into forefront of all fields of medicine as a new paradigm for the future. Adult stem cells have been the focus, as they do not have the ethical problems of embryonic stem cells nor the potential problem of carcinogenesis. Among adult stem cells, adipose-derived stem cells (ADSC), which is a kind of mesenchymal stem cell from human adipose tissue, have essentially the same properties as stem cells derived from bone marrow (1). Moreover, they have relative advantages in accessibility and abundance compared to other types of adult stem cells. Therefore, experiments using ADSC have been recently very active.

In dermatology, there are several studies on the effective application of stem cells, such as improved retention in autologous fat transplantation, antioxidant action and whitening effects (2–4). Among the recent reports, the wound-healing and anti-ageing effects of ADSC in photo-damaged aged skin have come into attention (5). However, there are few reports on anti-ageing effects of ADSC in ageing skin. Therefore, we carried out this study to evaluate the effects of ADSC on dermal components and angiogenesis.

Method

Preparation of ADSCs from adipose tissue and human fibroblasts

Subcutaneous adipose tissue was obtained by tumescent liposuction (6) from healthy women donors (n = 2, mean age 27) with informed consents as approved by the institutional review boards. The crude lipoaspirates were washed three times with phosphate-buffered saline solution (PBS) to remove debris and red blood cells. Washed aspirates were digested with 0.075% collagenase (Type 1; Sigma, St Louis, MO, USA) for 45 min at 37°C with constant shaking. Mature adipocytes were separated from the pellets by centrifugation at 250 g for 10 min. Pellets were resuspended in PBS, passed through a 100-μm mesh filter to remove connective tissue and washed twice with PBS. The cell pellets containing ADSCs were resuspended in Dulbecco modified Eagle medium–low glucose (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (Gibco) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) and placed at a density of 2–3 × 10^5 cells/cm² in T75 flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 5–7 days, non-adherent cells were removed by replacing the medium and the adherent cells were cultured for another 2–3 days. After reaching 70–80% confluency, the adherent cells were detached with trypsin containing EDTA (Gibco) and replated at a density of 4–5 × 10^3 cells/cm² in new flasks. The cells were serially subcultured up to passage five for animal experiment. On the day of injection, the ADSCs were harvested using trypsin and EDTA, washed twice with PBS and once with Hanks’ balanced salt solution (HBSS) and resuspended to a final concentration of 1.6 × 10^7 cells/ml in HBSS. Cell viability was >90% when assessed using a trypan blue exclusion assay. Flow cytometric analysis revealed that the expression of CD73 and CD105 was more than 90% of the cells, and the expression of haematopoietic markers CD14, CD34 and CD45 was <3% of the cells (data not shown).

Human dermal fibroblastic MRC-5 cell was used as a control cell, obtained from ATCC (ATCC No. CCL-171) and cultured in Dulbecco modified Eagle medium supplemented with 10% foetal bovine serum (Gibco) and 100 U/ml penicillin/100 μg/ml streptomycin (Gibco). On the day of injection, MRC-5 cells were harvested using trypsin and EDTA, washed twice with PBS and once...
with HBSS and resuspended to a final concentration of 1.6 × 10⁶ cells/ml in HBSS.

**Animal experiment**

**(Experiment for the evaluation of effects of ADSC compared with control media–injected group)**

Six female hairless mice (48 weeks old) were purchased from the animal laboratory of Yonsei University. Yonsei University at Wonju Campus Institutional Animal Care and Use Committee (IACUC) approved this animal experiment. Mice were kept under controlled humidity (40%) and temperature (22 ± 2°C). The cell dose (5 × 10⁵ cells) applied in this experiment was based on the preliminary experiment in which we used 1 × 10⁵ cells and 5 × 10⁶ cells, and the latter cell dose was more effective. Thirty microlitres of ADSC (5 × 10⁵ cells/30 μl) was subcutaneously injected with a 26-gauge needle into one side of dorsal skin. For media-injected control group, HBSS was subcutaneously injected into the other side. We marked injection point by marking pen daily. Two weeks after injection, biopsy specimens were taken with a 5-mm punch to evaluate changes in morphology and protein expression. Biopsy specimens taken with a 5-mm punch were divided into two parts. One part used to evaluate dermal thickness, fibroblast, collagen density and immune identification of dermal angiogenesis was fixed with formalin solution. The other part managed to study procollagen type I, MMP-13, and genomic DNA was used with fresh status. So, 5 mm diameter was the minimum diameter for these.

**(Experiment for the evaluation of effects of ADSC compared with control cell injected group)**

Eleven female hairless mice (48 weeks old) were used. Thirty microlitres of ADSC (5 × 10⁵ cells/30 μl) was subcutaneously injected with a 26-gauge needle into one side of dorsal skin. For the control cell injected group, human cultured fibroblast was subcutaneously injected into the other side. One week after injection, biopsy specimens were taken from 5 of 11 with a 5-mm punch and 2 weeks after injection from remained six mice to evaluate changes in morphology.

**Tissue preparation and microscopic measurement**

Biopsied skin specimens were fixed with 10% formalin solution and embedded in paraffin. The specimens were stained with haematoxylin and eosin (H&E), and pictures of each were taken under ×200 magnification. Dermal thickness, which was defined as the length between subcutaneous fat and the dermoepidermal junction, was measured randomly at five sites on each picture. The numbers of dermal fibroblast were also counted on the H&E stained sections. Collagen densities, which were visualized with Masson-trichrome staining, were measured with a histogram created with Adobe Photoshop 7.0 software (Adobe Systems; Mountain View, CA, USA).

**Immunofluorescence stains for CD31 and NG2**

Two vessel markers were used for immune identification of dermal angiogenesis (i.e. the vascular endothelial cell marker): CD31 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) and a pericyte marker, NG2 (Santa Cruz Biotechnology, Inc.).

Formalin-fixed paraffin-embedded sections (6 μm) were de-paraffinized and rehydrated sequentially with 100%, 90% and 70% ethanol and incubated for 5 min in PBS. After rehydration, sections microwave antigen retrieved for 2.5 min in 10 mM citrate buffer (pH 6.2, containing 2 mM EDTA, and 0.05% Tween 20) at 90°C. The tray was removed from the microwave oven and allowed to cool at room temperature (7–9). Slides were then blocked for 10 min with Protein Block solution (DAKO, Carpinteria, CA, USA) and incubated overnight at 4°C with a primary antibody, followed by incubation with Alexa Fluor® 488 donkey anti-goat secondary antibody (Molecular Probes, Inc., Eugene, OR, USA) for 40 min at room temperature. Slides were counterstained with propidium iodide and visualized on a Leica TCS–SPE confocal microscope (Leica Systems GmbH, Watzlar, Germany). Controls without primary antibodies showed no immunolabelling (10).

**Western blot**

Briefly, murine dermal samples were homogenized in mammalian cell lysis buffer from a Qproteome Mammalian Protein Prep kit (Qiagen, Hilden, Germany) containing Benzonase® Nuclease and Protease Inhibitor Solution. Lysates were centrifuged at 14 000 g for 10 min, and supernatants were collected to perform Western blots. Protein concentration was determined by a Bradford assay. Equal amounts of proteins were loaded onto a 10% SDS–polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% skim milk in TBST (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) and incubated with the indicated antibodies. For detection of type I procollagen and MMP-13, polyclonal anti- procollagen type I antibody, polyclonal anti-MMP-13 antibody and polyclonal anti-GAPDH antibody were purchased from Santa Cruz Biotechnology. Blotting proteins were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

**Quantitative real-time RT-PCR analysis**

Total RNA was isolated from skin specimens using Trizol, then resuspended in RNase-free water and quantified with a UV spectrophotometer (Perkin Elmer Ltd, Waltham, MA, USA). Single-strand cDNA was prepared from 1 μg of total RNA in a 20 μl reaction volume using an Oligo-dT primer (Roche Molecular Systems, Inc., Branchburg, NJ, USA), which contains 5 mM MgCl2, 1 mM dNTP mixture, 1 U/μl RNase inhibitor (Roche Molecular Systems, Inc.) and 0.25 U/μl M-MLV reverse transcriptase (Promega, Madison, WI, USA).

Real-time PCR was performed on a Rotor-GeneTM 3000 (Corbett Life Science, Sydney, NSW, Australia) using SYBR Green PCR Master Mix (Qiagen). Gene-specific primers based on the complete coding sequence for the mouse procollagen type 1-α1 subunit (Col1a1) in GenBank™ (accession no. NC000077) were designed using Geneious Basic 4.6 software (Biomatters Ltd., Auckland, New Zealand). Gene-specific primers were 5′-TCAGGTGACGAGATGTGAG-3′ (forward) and 5′-GCTTTGGAGCTTCAGTGAC-3′ (reverse). The quantity of PCR products was calculated from the cycle threshold value. The levels of gene expression were normalized with those of the GAPDH gene.

**Genomic DNA extraction and polymerase chain reaction analysis**

Genomic DNA was obtained from skin tissues using the Wizard genomic DNA purification kit (Promega) and quantified by absorbance at 260 nm. Also, genomic DNA was isolated from human ADSCs and used as a positive control. The presence of human-specific DNA within the skin tissues of transplanted mice was analysed by polymerase chain reaction (PCR) amplifying an 850-bp fragment of α-satellite region of the human chromosome 17 (Cr17). Endogenous mouse c-mos oncogene (mMOS) was also
amplified, as an internal control to confirm the quality of genomic DNA. One hundred nanograms of genomic DNA template were used for the PCR analyses of Cr17 and mMOS. Both PCR protocols include an initial denaturing step at 94°C for 10 min (2 min for mMOS). The protocol for CR17 is 35 cycles of denaturation at 94°C for 1 min, annealing/elongation at 60°C for 1 min and a final elongation at 72°C for 10 min. The protocol for mMOS is 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, elongation at 72°C for 30 s and a final elongation at 72°C for 2 min. Amplified DNA fragments were electrophoresed through 1.5% agarose gels and subsequently visualized through ultraviolet light after staining with ethidium bromide.

**Statistical analysis**

Paired t-tests were used for statistical analysis of the results, with \( P < 0.05 \) considered to be statistically significant.

**Results**

**ADSC injection increases dermal thickness and fibroblast number**

On haematoxylin and eosin stains, dermal thickness and the quantity of fibroblasts were significantly increased in the ADSC-injected skin compared with cell media–injected control skin. There was no definite immune response at ADSC-injected skin 2 weeks later histologically (Fig. 1). In addition, the Masson-trichrome stain showed statistically significant increases in dermal collagen density in the stem cell–injected skin (Fig. 2).

**Effects of ADSC injection on dermal thickness and density are similar with control cell (human cultured fibroblast) injection**

Control cell (human cultured fibroblast)–injected skin also increased dermal thickness and collagen density as in the ADSC-injected skin. There were no statistically significant differences between ADSC and control cultured fibroblast (data not shown).

**ADSC injection increases collagen synthesis but not collagenase in the dermis**

Western blot and real-time PCR were performed to evaluate the expression of procollagen type I and MMP-13, which may account

![Figure 1. Dermal thickness (a) and the number of fibroblasts (b) increased in the adipose-derived stem cells–injected group compared to the control group (H&E, x200; Scale bars represent 50 \( \mu m \)).](image)

![Figure 2. The histogram function of Photoshop was used to evaluate collagen density. Collagen density increased in the adipose-derived stem cells–injected group compared to the control group (Masson–Trichrome stain, x200; Scale bars represent 50 \( \mu m \)).](image)

![Figure 3. Western blot was carried out to evaluate the expression of type I procollagen and MMP-13. The expression of type I procollagen was increased in adipose-derived stem cells–injected skin compared to control, but MMP-13 expression showed no difference.](image)
ADSC injection increases dermal angiogenesis

On the CD31 stain, an increase in fluorescence was observed in the ADSC-injected skin compared with the control (Fig. 5). On the NG2 stain, increased fluorescence was also observed in the ADSC-injected skin (Fig. 5). These results indicate that angiogenesis increased in the ADSC-injected skin.

The fate of injected ADSC

To identify the engraftment human cells, we performed PCR experiments using human cell-specific primers (Cr17) and genomic DNAs from ADSCs-transplanted skin tissues. As expected, there was no amplified DNA fragment in skin tissues from non-transplanted mice. PCR analysis using mouse cell-specific primers mMOS showed amplified DNA fragments in all the DNA samples except in DNA samples from ADSCs only, indicating that the isolated genomic DNAs were suitable for PCR analysis. Interestingly, there was no human cell in the skin tissues of the mice transplanted with ADSCs after both 1 and 2 weeks. As the PCR protocol for Cr17 showed the detection of <10 human cells (50 pg of genomic DNA from ADSC) in 1.6 × 10^4 mouse cells (100 ng of genomic DNA from mouse skin) (data not shown), the skin tissues of the mice transplanted with ADSCs nearly had no human cells (Fig. 6).

Discussion

Cell therapy has come into attention in all medical fields as a new paradigm for future medicine in which reproductive cells such as stem cells are used to restore organ functions after damage by disease or trauma.

Stem cells used in cell therapy can differentiate into various tissues, proliferate continuously and self-renew (11). Embryonic stem cells obtained from foetuses have been thoroughly investigated, but they also have severe ethical problems and carcinogenic potential. Therefore, cell therapies using mesenchymal stem cells as a kind of adult stem cell have come into attention. Adult stem cells, which can be obtained after birth, are different from embryonic stem cells, because they are obtained after the developmental process is completed. Nevertheless, adult stem cells have variable reproductive properties and potentials characteristic of embryonic stem cells. Moreover, adult stem cells have several advantages compared with embryonic stem cells, as they are free of ethical problems and have the possibility of self-transplantation. In addition, these cells are easily applied as they are somewhat differentiated into a specific cell type (12). Mesenchymal stem cells, a kind of undifferentiated adult stem cells, present in differentiated cells in tissues or organs including subcutaneous fat, muscle and cartilage. As with other types of stem cells, mesenchymal stem cells have the possibility of self-renewal and differentiation to various tissues such as adipocytes, osteocytes and chondrocytes (13,14). Among mesenchymal stem cells, adipose-derived stem cells (ADSC) that are easily obtained from subcutaneous fat tissue have the relative advantages of accessibility and abundance. This means that there is less pain to the patient in obtaining the cells and it is easy to obtain a sufficient quantity of cells. In addition, the possibility of an immunologic reaction is few because the cells originate from autologous stem cells. For these reasons, many recent experiments have used ADSCs.

Recently, some studies have reported on the wound-healing effects of adult stem cells by proliferating fibroblasts and secreting cytokines. Re-epithelization and angiogenesis were observed after application of bone-marrow-derived mesenchymal stem cells to wound sites. Additionally, mesenchymal stem cells help in the formation of granulation tissues at wound sites (15). Kim et al. (4) performed an experiment on the wound-healing effects of ADSC and observed that they are mainly mediated by stimulating collagen synthesis of dermal fibroblasts.
As the anti-ageing mechanism is very similar to that of wound healing, the application of ADSC as an anti-ageing treatment is worth investigation. In the ageing process, there is a decrease in procollagen synthesis and an increase in extracellular matrix enzymatic degradation by increased matrix metalloproteinase activity (16). To inhibit the ageing process, fibroblasts play an important role as a source of collagen fibre as in wound healing. Fibroblasts maintain skin integrity and restore the extracellular matrix via matrix synthesis and secretion of variable cytokines (17–19). Kim et al. (5) showed that wrinkles are reduced by increasing dermal thickness and collagen density after ADSC injection into photo-damaged aged skin. In our study, we used 48-week-old mice undergoing accelerated intrinsic ageing. It is generally accepted that markedly aged species mean >75 years in humans and >18 months in mice, near the end of their life expectancy (20,21). And moderately aged species corresponds to 50–80 years in human and 12–15 months in mice (22). Usually, moderately aged people are concerned about increasing in their wrinkling rather than markedly aged people. So, we used moderately aged mice model about 12 months. Dermal thickness was increased in the ADSC-injected skin compared to the control skin. In addition, histopathology showed that the number of fibroblasts and collagen density increased in the ADSC-injected skin compared to the control skin. Control cell (human cultured fibroblast) injection also increased dermal thickness and collagen density as in the ADSC injection. Therefore, we anticipate that this effect was mainly mediated by increased collagen production by dermal fibroblasts. These morphologic changes were compared quantitatively by Western blot and real-time PCR. On Western blot, which was carried out to evaluate the expression of type I procollagen and MMP-13, the expression of type I procollagen was significantly increased in the ADSC-injected skin compared with the cell media-injected control. However, MMP-13 expression did not show statistically significant differences between the ADSC and cell media control. However, MMP-13 expression did not show statistically significant differences between the ADSC and cell media control groups. mRNA of type I procollagen was also compared using real-time RT-PCR, which showed that ADSC increased the expression of type I procollagen and MMP-13, of cells and amount of cellular components from the differentiated ADSCs could not be elucidated with this study.

In this study, we confirm that dermal thickness and collagen density are increased after injection of ADSC in moderately aged mice. This agrees with previous reports on ADSC. From these results, we conclude that ADSC has regenerative or anti-ageing potential on depressed, atrophic or wrinkled skin (especially photo-damaged aged skin) by stimulating collagen synthesis of dermal fibroblasts and increasing dermal angiogenesis through its paracrine effect.

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**Conflict of interest**

The authors have no conflict of interest to disclose.

**References**
