Antiwrinkle effect of adipose-derived stem cell: Activation of dermal fibroblast by secretory factors

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1. Introduction

Skin regeneration has become the focus of cosmeceuticals and dermatologists treating an aging population overexposed to the sun, and various noninvasive treatments and topical cosmeceuticals have been used to treat some symptoms of photo-aged skin, including wrinkles [1–3]. Despite numerous claims of the reversal of wrinkles, solid scientific evidence regarding this issue is limited. Aging can be divided into two categories: intrinsic and extrinsic. Extrinsic aging refers to components mediated by environmental factors, which include smoking, chemical exposure and primarily ultraviolet-B (UVB) exposure [1,4,5]. Extrinsic aging is characterized by fine and coarse wrinkling, roughness, dryness, laxity and pigmentedary lesion. This type of aging causes a decrease in epidermal thickness and atypia of keratinocytes. In the dermis, UVB exposure has been shown to stimulate collagenase production by human dermal fibroblasts (HDF) and to up-regulate collagenase gene expression. This induces degeneration of collagen and deposition of altered elastic tissue which is prominent as wrinkles and yellow discoloration of skin [6–8]. Lasers and several cosmeceuticals including derivatives of retinol, vitamin C and topical growth factors, all of which are well-known for inducing collagen synthesis from HDF and have been used for the treating skin texture and wrinkling [3].

Mesenchymal stem cells within the stromal-vascular fraction of subcutaneous adipose tissue, adipose-derived stem cells (ADSC), display multi-lineage developmental plasticity and are similar to bone marrow-derived mesenchymal stem cells (BM-MSC) with respect to surface markers and gene profiling [9–12]. In addition, BM-MSC and ADSC produce various cytokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF),
insulin-like growth factor (IGF), platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF-β). Recently, the production and secretion of cytokines has been reported as an essential function of ADSC, and diverse pharmacological actions of ADSC have been demonstrated especially in skin biology [13–17]. For example, it was reported by our group that ADSC has a wound-healing effect through the production of diverse growth factors. These growth factors activated HDF, which increase the proliferation/migration of HDF and mediate the secretion of collagen from HDF. The antioxidant effect of ADSC also has been demonstrated by showing that the secretory factors of ADSC protect HDF from oxidative stress.

The application of topical growth factors stimulated the repair of facial photo-aging resulting in new collagen synthesis, epidermal thickening and the clinical appearance of smoother skin with less visible wrinkling [3,18]. As several growth factors involving skin regeneration are secreted from ADSC, it is hypothesized in this study that ADSC may improve UVB-induced photo-damage. Therefore, wrinkles were induced in hairless mice after an eight-week regimen of UVB irradiation and the antiwrinkle effect was investigated by the subcutaneous injection of ADSC. In addition, mechanisms for improving wrinkling via paracrine routes were investigated further using a conditioned medium of ADSC (ADSC-CM) in cultured HDF.

2. Materials and methods

2.1. Isolation and culture of ADSC

Human subcutaneous adipose tissue samples were acquired from elective liposuction of healthy females with informed consents as approved by the institutional review boards. The obtained samples were digested with 0.075% collagenase type II (Sigma–Aldrich, St. Louis, MO) under gentle agitation for 45 min at 37 °C. The digestion was performed at 37 °C and centrifuged at 300 × g for 10 min to obtain the stromal cell fraction. The pellet was filtered with 70 μm nylon mesh filter, and resuspended in phosphate buffered saline (PBS). The cell suspension was layered onto histopaque-1077 (Sigma–Aldrich, St. Louis, MO), and centrifuged at 840 × g for 10 min. The supernatant was discarded, and the cell band buoyant over histopaque was collected. The retrieved cell fraction was cultured overnight at 37 °C/5% CO2 in control medium (Dulbecco’s modified Eagle media: DMEM, 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin). The resulting cell population was maintained over three to five days until confluence, which were represented as passage 1. ADSCs were cultured and expanded in control medium, and used for the experiments at passages 4 through 5.

2.2. Animal experiment

Five-week-old female hairless mice (Hos: HR-1) were provided from SLC Inc. (Shizuoka, Japan). All mice were housed in climate-controlled quarters (22 ± 1 °C at 50% humidity) with a 12/12 h light/dark cycle. Animals were allowed free access to water and a chow diet and were observed daily.

The mice were irradiated dorsally using the UVB-emitting system RMX-3W (Handok Biotech, Seoul, Korea) for eight weeks, five times a week. A bank of 10 Toshiba SE lamps was used without any filtering for UVB (peak of emission near 312 nm, the irradiance between 290 and 320 nm corresponding to 55% of the total amount of UVB). The distance from the lamps to the animals’ backs was 89 cm. During exposure, the animals could move around freely in their cages. The irradiation dose was one MED (minimal erythemal dose; 60 mJ/cm²) in the first two weeks, two MED (120 mJ/cm²) in the third week, three MED in the forth week (180 mJ/cm²), and four MED (240 mJ/cm²) in the fifth through eight weeks. The total UVB dose was approximately 115 MED (6.9 J/cm²).

After wrinkle induction, ADSCs (1 × 10⁶, 1 × 10⁷, and 1 × 10⁸) were subcutaneously injected into the restricted area of the mice. ADSCs were suspended in 100 μl HBSS and injected three times in a seven-day interval. One hundred μl of HBSS was injected into each number of the control group.

2.3. Skin replica and image analysis

At the time of wrinkle induction and one week after the final injection of ADSC, negative replicas of the dorsal skin surface were taken by using a silicon-based impression material, Flexitime® (Heraeus Kulzer, New York, NY). To obtain replicas of the wrinkles from the same skin area, the skin was marked using an oil-based marker pen. For ease of measurement, all replicas were cut into circular pieces with a diameter of 1 cm, and the back of each replica was processed into a flat plane using the same impression material.

Light was directed at a 20° angle, and images were incorporated from replica using a CCD. The image of the negative replicas was observed using a wrinkle analysis system skin visiometer SV 600 (Courage & Khazaka, Cologne, Germany). The parameters used in the assessment of the skin wrinkles are listed in Table 1.

2.4. Survival of ADSC

Red fluorescent-labeled ADSCs were transplanted to examine the survival of ADSC, as described previously [19]. Suspended ADSCs (1 × 10⁵ cells) were labeled with fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit, Sigma, Saint Louis, MO.). Three minutes after labeling, PBS was added for 1 min to stop the reaction and the cells were washed by PBS. The sensitivity and specificity for cell labeling with PKH26 was almost 100%. Then, PKH26-labeled ADSCs were subcutaneously injected into the skin of hairless mouse. Two weeks after experiment, frozen sections of the skin appendages were prepared and counterstained with green-fluorescent nucleic acid stain (CYTO®, Molecular Probes, Eugene, OR).

2.5. Histology

Dorsal skins (1 cm × 1 cm) were fixed with a 10% formalin neutral buffered solution, embedded in polyester wax and sectioned at 6 μm. The sections were subjected to Hematoxylin & Eosin (H&E) and Masson’s trichrome staining.

2.6. HDF culture and UVB irradiation dose

HDFs were cultured in a DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO₂ at 37 °C. After starvation with serum-free medium for 24 h, cells were washed with PBS and exposed to UVB with 3–4 drops of PBS. UVB irradiation was carried out using a UV lighter (Waldmann, Schwenningen, Germany). Immediately after the irradiation, the PBS was aspirated and replaced with complete medium. UVB irradiation doses were tested in 50–250 mJ/cm² and finally fixed to be 70 mJ/cm² for further experiment.

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2.7. Preparation of ADSC-CM

ADSCs (4 × 10^5 cells) were cultured in DMEM/F12 (Invitrogen-Gibco-BRL, Grand Island, NY) serum-free medium. Conditioned medium of ADSCs was collected after 72 h of culture, centrifuged at 300 × g for 5 min and filtered using a 0.22 μm syringe filter.

2.8. Cell proliferation assay

HDFs were plated at a density of 5 × 10^3 cells/well in 96-well plates, and the proliferation of HDF was measured using a CCK-8 Kit (Dojindo, Gaithersburg, MD). After starvation with serum-free medium for 24 h, the cells were continuously cultured for 24 h with or without ADSC-CM and exposed to UVB (70 mJ/cm^2) for 90 s. Then, UVB-irradiated cells were cultured in complete medium for 24 h and harvested. HDFs were added to 10 μl of the CCK-8 solution and incubated for 3 h. The absorbance was measured at 450 nm using a microplate reader (TECAN, Grödig, Austria). OD values of each well were calculated to their relative cell numbers with comparable standard curves.

2.9. Cell cycle analysis by flow cytometry

Apoptosis was scored by assessing the fraction of cells with a sub-G0/G1 DNA content by flow cytometry. HDFs (2 × 10^5) were seeded in 100-mm dishes, incubated and allowed to grow to 60% confluency. After starvation with serum-free medium for 24 h, the cells were continuously cultured for 24 h with or without ADSC-CM and exposed to UVB (70 mJ/cm^2) for 90 s. Then, UVB-irradiated cells were cultured in complete medium for 24 h, harvested, washed twice with PBS, and permeabilized with 70% ethanol at 0 °C before analysis. The cells were then washed once with PBS-treated RNAse (1 h at 37 °C, 500 U/ml). Cellular DNA was stained with 50 μg/ml propidium iodide. The distribution of cell cycle phases with different DNA contents was read in a FACScan flow cytometer (Becton–Dickinson, San Jose, CA).

2.10. Western blot analysis

HDFs (2 × 10^4 cells/well) were seeded in 24-well plates and pretreated as described above. Then, the cells were lysed in a RIPA buffer (50 mM Tris–HCl, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, 50 mM NaF, 1 mM Na3VO4, 5 mM dithiothreitol, 1 μg/ml leupeptin and 20 μg/ml PMSF, pH 7.4). Fifty micrograms of proteins were separated on an 8% SDS-polyacrylamide gel by electrophoresis. The proteins were transferred to PVDF membranes. The membranes were incubated with antibodies of collagen type I (Santa Cruz, Saint Louis, MO), matrix metalloproteinase1 (Calbiochem, Darmstadt, Germany), and β-actin (1:10,000 Santa Cruz, Saint Louis, MO). Then, the membranes were washed and incubated with horseradish peroxidase-conjugated anti-goat IgG antibody (1:10,000 Santa Cruz, Saint Louis, MO). The blots were reacted with immunobilon western reagent and exposed to X-ray film.

2.11. Statistical analysis

In vitro data are representative of three or more independent experiments. A one-way ANOVA test, followed by paired t-test, was used for statistical analysis of animal experiment, and p < 0.05 was considered significant.

3. Results

3.1. Characterization of ADSCs

ADSCs expanded easily in vitro and exhibited a fibroblast-like morphology. In flow cytometry, characteristic expressions of stem cell-related surface markers were confirmed [15,16]. ADSCs expressed CD73, CD90 and CD105, and were lacking in CD34 and CD49d. Adipogenic, osteogenic, and chondrogenic differentiation was also confirmed by the conventional method [15,16].

3.2. ADSC reduced UV-induced wrinkles

During the study period, the body weight of the hairless mice was measured regularly. All groups were homogeneous in body weight during experiment Table 1. During the period of UV exposure, the mice were observed for fine wrinkling of the skin. However, the ADSC-treated group appeared to have fewer wrinkles than the control group during the ADSC treatment (n = 8 for each group). In a replica analysis, Fig. 1 shows that repeated ADSC treatment improved the fine wrinkles induced by UVB irradiation. When our group measured the parameters for the wrinkles of replicas with the skin visiometer SV 600, injection of mid-level and high doses (1 × 10^4, 1 × 10^5 cells) of ADSC significantly reduced all parameters for wrinkles listed in Table 2 (Fig. 2).

As cell transplantation between species mediates immune rejection responses, the survival of ADSC from humans was investigated after injection of ADSC labeled with PKH26 (red color, see Fig. 3 insert). After two weeks of injections, a mouse skin block was made using cryosection and counterstaining with green-fluorescent nucleic acid stain. As shown in Fig. 3, survival of the ADSC was clearly demonstrated two weeks after injection. Although the survival rate of the ADSC-injected mice was not measured, human ADSC may survive and improve skin wrinkling in hairless mice for two weeks.

3.3. Histological observation

UVB-irradiated hairless mice showed great changes in skin appendages and the effect of ADSC on dermal thickness in UVB-irradiated hairless mice was investigated. Fig. 4 shows the histological measurements of the dermal thickness of the hairless mouse skin by H&E staining. Measurement of the dermal thickness showed significant increases in the mid-level- and high-dose administration of ADSC (16 and 28%, respectively). Histological sections of skin were subjected to Masson’s trichrome staining to visualize the extracellular matrix (ECM) components and morphological changes. Fig. 5 shows the Masson’s trichrome staining results of collagen (blue) in the ADSC-treated group and the control group in the mice. A marked increase of collagen bundles was observed in the ADSC-injected group, but not in the control group.

3.4. ADSC increased the proliferation of HDF

To further study the paracrine mechanism regarding the improvement of skin wrinkles with ADSC, a cell proliferation

Table 2

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<td>R1</td>
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<td>R2</td>
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<td>R3</td>
<td>Average roughness</td>
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<td>R4</td>
<td>Smoothness roughness</td>
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<td>R5</td>
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Parameters used in assessment of wrinkles
assay was performed in primarily cultured HDF with a conditioned medium of ADSC. UVB irradiation significantly decreased the proliferation of HDF, but pretreatment of ADSC-CM showed a protective effect on HDF in a dose-dependent manner (Fig. 6). As the ADSC-CM contains diverse growth factors and a unique characteristic of growth factors is their ability to initiate mitosis of quiescent cells, enhanced proliferation by ADSC-CM in this experiment might be mediated by growth factors secreted from ADSC.

3.5. ADSC reduced apoptosis of HDF induced by UVB irradiation

As UVB irradiation induced apoptotic cell death, cell cycle analysis was performed after ADSC-CM pretreatment. UVB irradiation increased the sub-G1 phase of the HDF cells, which was reversed by ADSC-CM pretreatment (Fig. 7). These results indicate that ADSC-CM decreased the apoptotic cell death in HDF induced by UVB irradiation: similar phenomena had been previously demonstrated in tert-butyl hydroperoxide-induced oxidative stress [15].

3.6. Expression of collagen type I and MMP1

Since collagen contents in the dermis were significantly increased in ADSC-treated hairless mice (Fig. 4), protein expressions of collagen type I and MMP1 were examined in HDF after ADSC-CM treatment (Fig. 8). UVB irradiation clearly reduced the expression of collagen type I and induced that of MMP1. However, expression of collagen type I was significantly increased after ADSC-CM pretreatment, while that of MMP1 was decreased after ADSC-CM pretreatment. These results indicate that increased collagen contents in the dermis of ADSC-treated hairless mice were mediated by the stimulation of collagen synthesis and the inhibition of collagen degradation in dermal fibroblasts.

4. Discussion

Photo-aging is a complex process having pathologic similarities to skin wounds [20]. Dermal fibroblasts play key roles in these processes as they interact with keratinocytes, fat cells and mast cells. They also are the source of extracellular matrix proteins,
glycoproteins, adhesive molecules and various cytokines [21]. By supplying these molecules and supporting cell-to-cell interactions, skin fibroblasts contribute to the fibroblast–keratinocyte–endothelium complex that accelerates wound repair and maintains the skin integrity and youthfulness. Conventional treatments of skin aging, such as laser and topical regimens, are mostly based on increasing ECM synthesis via fibroblast activation. In our group’s previous study, it was observed that the ADSC-CM stimulated collagen synthesis of HDF and activated the proliferation/migration of HDF [16]. Therefore, our group investigated the antiwrinkle effect of ADSC in this study. As expected, UVB-induced wrinkling was significantly improved by the ADSC injection in the hairless mice. Dermal thickness and collagen contents in the dermis also increased. To characterize the paracrine mechanism involving the antiwrinkle action of ADSC, ADSC-CM was incubated with cultured HDF. UVB irradiation reduced the proliferation of HDF, but this was reversed by the pretreatment of ADSC-CM. In a cell cycle analysis, ADSC-CM decreased the UVB-induced apoptotic cell death, which was demonstrated by the reduced sub-G1 phase of the cell cycle. In addition, ADSC-CM increased the expression of collagen type I of HDF and decreased the protein level of MMP1 in HDF. Collectively, these results indicate that ADSC and their soluble factors have an antiwrinking effect, and it is mainly mediated by activating dermal fibroblasts, which reduces UVB-induced apoptosis and stimulates collagen synthesis.

The pathological agents responsible for UV-induced changes such as wrinkles and roughness are reactive oxygen species (ROS) that deplete and damage the non-enzymatic and enzymatic antioxidative defense mechanism of the skin, leading to oxidative damage of the skin and ultimately premature aging and cancer [22,23]. ROS are believed to activate the cytoplasmic-signaling transduction pathways in the resident fibroblasts, which are related to growth, differentiation, senescence, connective tissue degradation and permanent genetic changes. As was previously demonstrated by chemically induced ROS, ADSC-CM had a protective effect on HDF and inhibited UVB-induced apoptosis in this study [15]. Although the underlying mechanisms regarding the protective effect of ADSC-CM against UVB-induced ROS were not characterized in this study, ADSC-CM might induce the antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase in HDF, which finally inhibit the apoptotic cell death induced by UVB [15].

Several studies on the pathophysiology of photo-aging have found correlations with certain aspects of wound healing [24]. Histologic features of photo-aged skin show marked alterations of ECM composition. The collagenous component of dermal ECM is responsible for the strength and resiliency of skin and is intimately involved in the pathology of photo-aging [25,26]. The dermis in the normal skin contains predominantly type I collagen (85–95%) and a lesser amount of type III collagen (10–15%). However, the precursors
of fibrillar type I and type III collagens are significantly reduced in the papillary dermis, and their reduction has been shown to correlate well with the clinical severity of photo-aging. This reduction results from a combination of reduced procollagen biosynthesis and increased enzymatic breakdown via the actions of MMPs. Fisher et al. showed that UV irradiation induced the synthesis of MMPs in human skin in vivo [27]. Among the MMP family, MMP1, MMP13, and membrane-type MMP14 display collagenolytic activity, and MMP2 and MMP9 have been described as true elastases. MMP-mediated collagen and elastin destruction accounts for a large part of the connective tissue damage that occurs in photo-damaged skin [8,25,27,28]. In this study, our group found that ADSC-CM not only attenuated UVB-induced MMP1 expression in HDF.

Wound healing and skin rejuvenation from photo-damage are a complex but orderly process and are orchestrated via cytokines and growth factors. These cytokines do not operate in isolation, but rather interact with a variety of cytokines as well as other regulatory proteins. Five major growth factor families have been studied with regard to the wound-healing process: TGF, IGF, PDGF, epithelial growth factor, and fibroblast growth factor. Growth factors released from platelets initiate this cascade, and continued epithelial growth factor, and fibroblast growth factor. Growth regulatory proteins. Five major growth factor families have been described as true elastases. MMP-mediated collagen and elastin destruction accounts for a large part of the connective tissue damage that occurs in photo-damaged skin [8,25,27,28]. In this study, our group found that ADSC-CM not only inhibited a UVB-induced decrease of the type I collagen but also attenuated UVB-induced MMP1 expression in HDF.

In summary, it has been demonstrated that ADSC has an antiwrinkle effect, and it is mediated by activating HDF via a paracrine mechanism. In addition, the wound-healing, antioxidant, and skin-whitening effects of ADSC and its secretory factors have been previously reported by our groups [15,16]. Therefore, comparing the clinical efficacy and safety of ADSC, autologous ADSC and its secretory factors have great promise for applications in cosmetic dermatology, especially in treating photo-damaged skin.

References


