SPANDIDOS PUBLICATIONS

TGF-ß1-treated ADSCs-CM promotes expression of type I collagen and MMP-1, migration of human skin fibroblasts, and wound healing *in vitro* and *in vivo*

JAE-WE CHO[1,2], MIN-CHUL KANG[1] and KYU-SUK LEE[1,2]

[1]Department of Dermatology and [2]Institute for Medical Science, Keimyung University School of Medicine, 194 DongSan-Dong Jung-Gu, Daegu 700-712, Korea

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Abstract. Conditioned medium from adipose-derived stem cells (ADSCs) stimulates both collagen synthesis and migration of dermal fibroblasts. However, it is still unknown whether conditioned media from tumor growth factor (TGF)-ß1-treated ADSCs (TGF-ß1-treated ADSCs-CM) induces increased expression of type I collagen, matrix metalloproteinase-1 (MMP-1), and migration as well as cell cycle regulatory proteins in fibroblasts, compared to non-treated ADSCs-CM. Our data showed that TGF-ß1-treated ADSCs-CM promoted effectively the proliferation and migration of human skin fibroblasts, compared to non-treated ADSCs-CM. In addition the expression of MMP-1 were markedly increased by treatment of TGF-ß1-treated ADSCs-CM in fibroblasts, compared to non-treated ADSCs-CM. Expression of type I collagen protein were slightly increased by treatment of TGF-ß1-treated ADSCs-CM in fibroblasts. The expression of cell cycle regulators of G1/S phase transition were not markedly altered by treatment of TGF-ß1-treated ADSCs-CM. Finally, artificial wounds were made using a 4-mm punch biopsy in hairless mice and TGF-ß1-treated ADSCs-CM were injected into the wound area. The injection of TGF-ß1-treated ADSCs-CM promoted the wound healing process in hairless mice. Taken together, our data indicated that TGF-ß1-treated ADSCs-CM induced up-regulation of type I collagen and MMP-1, promoted the migration of skin fibroblasts, and thereby promoted the wound healing process *in vivo*. Our data indicate that TGF-ß1-treated ADSCs-CM will be a component for a wound healing accelerating agent.

Correspondence to: Dr Kyu-Suk Lee, Department of Dermatology, Keimyung University School of Medicine, 194 DongSan-Dong Jung-Gu, Daegu 700-712, Korea
E-mail: janylove@dsmc.or.kr

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Introduction

Adipose-derived stem cells (ADSCs) are a population of pluripotent mesenchymal cells and secrete various growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and transforming growth factor-ß1 (TGF-ß1) (1,2). These secreted proteins play important roles in wound healing, anti-aging, and anti-oxidants (2).

The wound healing process is a complex process, which is mediated by overlapping stages such as inflammation, formation of granulation tissue, re-epithelialization, matrix formation and remodeling (3). The success of wound healing depends on growth factors, cytokines, and chemokines involved in a complex integration of signal networks. Major growth factors and cytokines involved in wound healing are epidermal growth factor (EGF), TGF, and fibroblast growth factor (FGF) family. The FGF, TGF-ß1, and platelet derived growth factor (PDGF) promote the synthesis, deposition, and organization of a new extracellular matrix (ECM) (4).

TGF-ß1 regulates various cell functions such as proliferation, differentiation, apoptosis, cell adhesion, cell motility, and production of extracellular cell matrix (ECM) (5). TGF-ß1 also has been recognized as a key regulator of stem cell renewal and differentiation (6). However, it is still unknown whether the conditioned media from TGF-ß1-treated ADSCs (TGF-ß1-treated ADSCs-CM) induces increased expression of type I collagen, matrix metalloproteinase-1 (MMP-1), and migration as well as cell cycle regulatory proteins in fibroblasts, compared to non-treated ADSCs-CM.

Human dermal skin fibroblasts play key roles in wound healing by secretion of type I collagen and cytokines (7,8). Proliferation rates of fibroblasts are increased during the acute wound healing process. Proliferation of fibroblasts are regulated positively by cell cycle regulatory proteins such as cyclin D1, cyclin E, cyclin dependent kinase 2 (CDK 2), and CDK4 (9-11). However, little has been reported regarding the effect of TGF-ß1-treated ADSCs-CM on proliferation and cell cycle regulatory proteins in fibroblasts.

In this study, we have investigated the effect of TGF-ß1-treated ADSCs-CM on proliferation, migration, expression of type I collagen, MMP-1, and cell cycle regulatory proteins in skin fibroblasts. Furthermore we studied the effect of TGF-ß1-treated ADSCs-CM on wound healing of hairless mice.

Materials and methods

Materials. Antibodies against type I collagen, MMP-1, ß-actin and cell cycle regulatory proteins (CDK2, CDK4, cyclin A, cyclin D1, cyclin E) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-RB antibody was purchased from PharMingen (BD Biosciences, CA). TGF-ß1 was purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of ADSCs and human skin fibroblasts. Human subcutaneous adipose tissue samples were acquired from elective liposuction of 33 healthy females with informed consent 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, and centrifuged at 1,200 rpm for 10 min. The pellet was filtered with 70 mm nylon mesh filter, and resuspended in phosphate-buffered saline (PBS). Cell suspension was centrifuged at 1,200 rpm for 10 min. Supernatant was discarded, and retrieved cell fraction was cultured overnight at 37˚C with 5% CO_2 in control medium [Dulbecco's modified Eagle's media (DMEM), 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 mg/ml of streptomycin]. ADSCs were cultured and expanded in control medium, and used for experiments at passages 1-5.

Human skin fibroblasts were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's Minimum Essential Medium supplemented with 10% heat inactivated FBS, 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells ($5x10^4$ cells/ml) were seeded in culture dish, and maintained in the tissue culture incubator.

Preparation of TGF-ß1-treated ADSCs-CM. ADSCs ($5x10^5$ cells) were plated on an 100-mm dish and cultured in DMEM (Invitrogen Gibco BRL, Grand Island, NY) serum free media with or without TGF-ß1 (5 ng/ml). CM was collected after 72 h of incubation, centrifuged at 2,000 rpm for 5 min, and filtered through 0.22 μm syringe filter. TGF-ß1-treated ADSCs-CM or non-treated ADSCs-CM (ADSCs-CM) were applied to skin fibroblasts at varying dilution folds from 50 to 100% in DMEM/F12 with FBS concentrations adjusted to 1%.

Flow cytometric characterization of ADSCs. ADSCs of passage 3 cultured in a control medium for 48 h prior to analysis were incubated with FITC-conjugated antibodies for CD49d and CD90 (Chemicon, Temecula, CA) and CD105 (BD Pharmingen) for 30 min at room temperature. As control, cells were stained with isotype control IgG. Cells were subsequently washed with PBS, fixed with 4% formaldehyde, and analyzed on FACScan flow cytometer (Beckton Dickson, San Jose, CA) using CellQuest Pro software.

Migration assay. For the measurement of cell migration, confluent fibroblasts kept in serum-free medium for 24 h were wounded with a plastic micropipette tip with a large orifice. After washing, medium was replaced by control medium with TGF-ß1 (5 ng/ml), ADSCs-CM (100%), or TGF-ß1-treated ADSCs-CM (100%). Photographs of wounded area were taken every 24 h by phase-contrast microscopy under crystal violet staining.

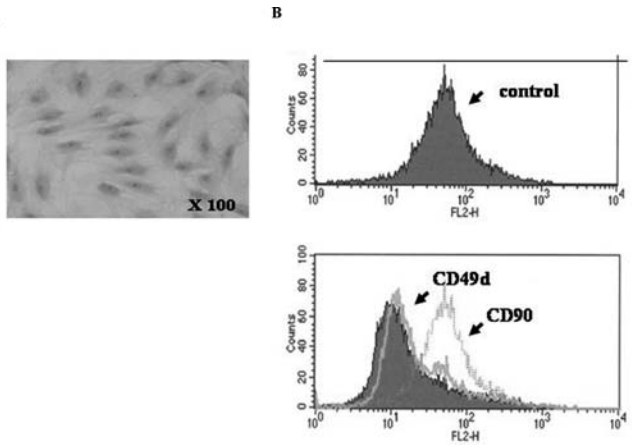

Figure 1. Expression of surface marker CD90 and CD49d in ADSCs. Fibroblastic morphology was observed under inverted microscope (A, x100). Flow cytometric histograms of human ADSCs (B); control, CD49d, and CD90 are indicated by arrows.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNAzol™ B (Biotecx laboratories, Houston, TX) according to the manufacturer's instructions and quantitated by spectrophotometer. Total RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison, WI). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Takara Co., Otsu, Japan). Briefly, 50 μl of a reaction mixture including 2.5 units of Taq polymerase (Takara Co., Otsu, Japan), 5 μl of 10x buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μl of first-strand cDNA, and 25 pmol of each primer, was subjected to 28 PCR cycles (denaturation at 94˚C for 1.5 min, annealing at 58˚C for 1 min, and polymerization at 72˚C for 1 min). The PCR products were analyzed on 1.5% agarose gel. The primer sequences and product sizes were as follows, i) GAPDH (forward, 5'-CGT CTT CAC CAC CAT GGA GA-3', reverse, 5'-CGG CCA TCA CGC CAC AGT TT-3'), 300 base pair (bp); ii) MMP-1 (forward, 5'-ATG CAC AGC TTT CCT CCA CTG-3', reverse, 5'-CAG CCC AAA GAA TTC CTG CAT T-3'), 240 bp. iii) Type I collagen (forward, 5'-CTC CGG CTC CTG CTC CTC TTA-3', reverse, 5'-GCA CAG CAC TCG CCC TCC C-3'), 400 bp.

Western blot analysis. Whole-cell extracts were prepared in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, phenylmethylsulphonyl fluoride (PMSF, 10 mg/ml), aprotinin (10 mg/ml), leupeptin (10 mg/ml), 5 mM phenanthroline and 28 mM benzamidine-HCl]. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 6.5-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as type I collagen, MMP-1, CDK 2, CDK 4, cyclin E, cyclin D1, RB, and

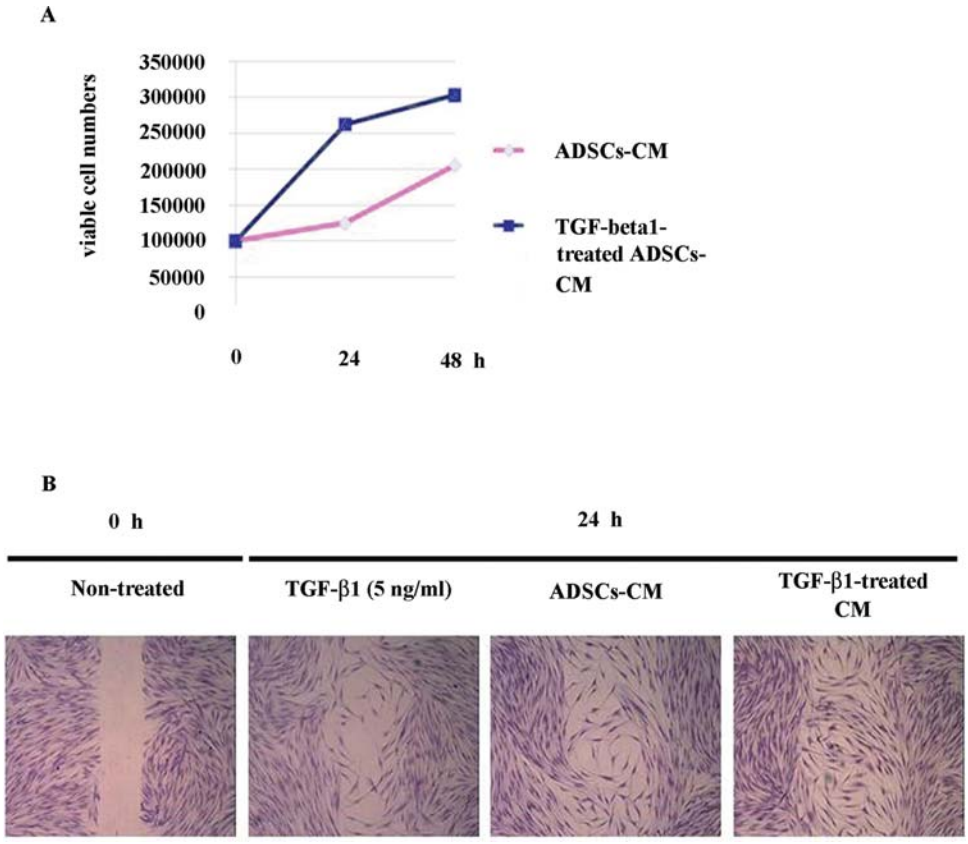

Figure 2. Increased proliferation rates and migration of human skin fibroblasts by treatment of TGF-ß1-treated ADSCs-CM. Human skin fibroblasts ($1x10^5$/ml) were cultured under ADSCs-CM (100%) or TGF-ß1-treated ADSCs-CM (100%) for 2 days. Viable cells were counted using trypan exclusion assay (A). In addition confluent fibroblasts were scratched with plastic micropipette and cells were cultured with control medium with TGF-ß1 (5 ng/ml), ADSCs-CM, or TGF-ß1-treated ADSCs-CM for 24 h and migration of cells were observed by inverted microscope (B).

ß-tubulin protein. The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Mouse wound healing model. Hairless mice SKH-1 (n=3) were housed and treated according to approved animal protocols. On the operation day, mice were anesthetized by subcutaneous injection of a mixture of ketamine (20 mg/kg) and acepromazine (10 mg/kg). Two circular full-thickness wounds of a 4-mm diameter were created on the backs of mice. CM-TGF-ß1-treated ADSCs were prepared before the experiment. Bactroban oint (left side of the back) and bactroban oint with TGF-ß1-treated ADSCs-CM (right side of the back, 0.05 ml/point x 4 point) were injected into the wound base and dressed with transparent Tegaderm (3M Health Care, St. Paul, MN). Wounds were evaluated by digital pictures and histological analyses using H&E staining 10 days after treatment.

Results

Characterization of ADSCs. Human ADSCs have been well characterized with regard to profile of expressed surface cluster of differentiation (CD) markers. As shown in Fig. 1A, ADSCs exhibited a fibroblast-like morphology. ADSCs were strong positive for CD90 and weakly positive for CD49d (Fig. 1B).

Increased proliferation rates and migration activity of TGF-ß1 treated-ADSCs-CM on human skin fibroblasts. Increased proliferation of skin fibroblasts is an important factor for wound healing. It is unclear whether TGF-ß1-treated ADSCs-CM induces highly proliferation rates of skin fibroblasts, compared to non-treated ADSCs-CM. We analyzed the effect of TGF-ß1-treated ADSCs-CM on proliferation of human skin fibroblasts. Fibroblast cells were seeded in culture dish and cultured for 2 days under non-treated ADSCs-CM or TGF-ß1-treated ADSCs-CM. As shown in Fig. 2A, proliferation rates of skin fibroblasts were more increased by treatment of TGF-ß1-treated ADSCs-CM than non-treated ADSCs-CM. In addition migration of fibroblasts plays important roles for wound-healing promotion. It is unclear whether TGF-ß1-treated ADSCs-CM is more effective on migration of human skin fibroblasts, which is compared to non-treated ADSCs-CM. We analyzed the effect of TGF-ß1-treated ADSCs-CM on proliferation of human skin fibroblasts. To examine whether the TGF-ß1-treated ADSCs-CM exhibit more effectiveness to migration of fibroblasts compared to non-treated ADSCs-CM, confluent fibroblasts cells were scratched by plastic micropipette and then cultured for 1 day under TGF-ß1 (5 ng/ml), non-treated ADSCs-CM, or TGF-ß1-treated ADSCs-CM. Migration of fibroblasts were markedly increased by TGF-ß1-treated ADSCs-CM, followed by non-treated ADSCs-CM, and TGF-ß1 treatment (Fig. 2B).

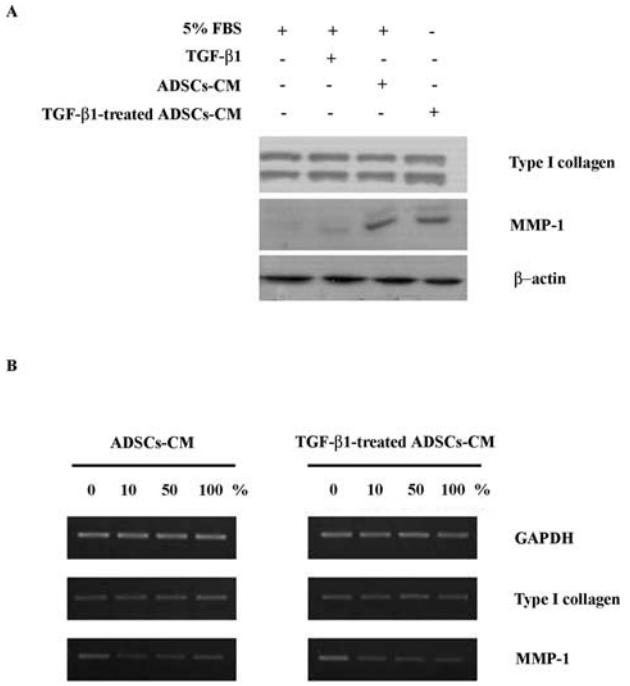

Figure 3. Up-regulation of MMP-1 in human skin fibroblasts by ADSCs-CM. Cells were treated by TGF-ß1 (5 ng/ml), ADSCs-CM (100%), or TGF-ß1-treated ADSCs-CM (100%) for 24 h. Expression of type I collagen and MMP-1 proteins were determined by Western blot analysis (A). Expression of Type I collagen and MMP-1 mRNA were also evaluated by RT-PCR analysis (B).

Increased expression of type I collagen and MMP-1 in skin fibroblasts by TGF-ß1-treated ADSCs-CM. To determine the effect of TGF-ß1-treated ADSCs-CM on expression of type I collagen and MMP-1 in skin fibroblasts cultured in TGF-ß1, non-treated ADSCs-CM or TGF-ß1-treated ADSCs-CM for 24 h. Expression of MMP-1 was markedly increased by treatment of non-treated ADSCs-CM or TGF-ß1-treated ADSCs-CM and expression of type I collagen was slightly increased by TGF-ß1-treated ADSCs-CM, but not by TGF-ß1 or non-treated ADSCs-CM (Fig. 3A). Interestingly mRNA expression levels of type I collagen and MMP-1 were not significantly modulated by treatment of TGF-ß1-treated ADSCs-CM (Fig. 3B).

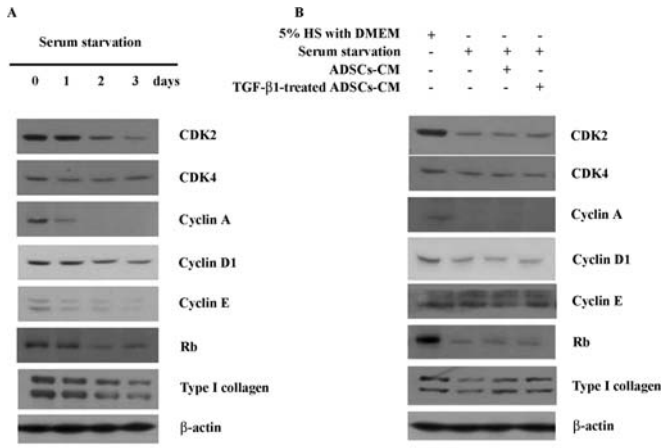

Figure 4. Up-regulation of type I collagen in serum-starved human skin fibroblasts by TGF-ß1-treated ADSCs-CM. Cells were cultured without serum for 3 days and protein extracts were subjected to Western blot analysis using specific primary antibodies (A). Serum starved fibroblasts were also cultured for 24 h under ADSCs-CM (100%) or TGF-ß1-treated ADSCs-CM (100%). Expression of cell cycle regulators and type I collagen were analyzed by Western blot analysis (B).

Expression of cell cycle regulatory proteins in skin fibroblasts by TGF-ß1-treated ADSCs-CM. Cell cycle regulatory proteins play important roles on proliferation and migration in human skin fibroblasts. We have investigated whether TGF-ß1-treated ADSCs-CM promotes the proliferation and migration activity in fibroblasts through up-regulation of G1/S transition regulatory proteins. To determine basal expression levels of cell cycle regulatory proteins in fibroblasts cells, cells were cultured without fetal bovine serum for 3 days. After serum starvation for 3 days, cells were cultured under non-treated ADSCs-CM or TGF-ß1-treated ADSCs-CM (100%) for 24 h. The expression levels of G1/S transition regulatory proteins, such as cyclin D1, cyclin E, Cdk2 and Cdk4, were progressively decreased in serum starved skin fibroblasts (Fig. 4A). Simultaneously Rb protein was markedly dephosphorylated by serum starvation. Interestingly expression of type I collagen was increased by stimulation of TGF-ß1-treated ADSCs-CM in serum starved fibroblasts, but the expression of cell cycle

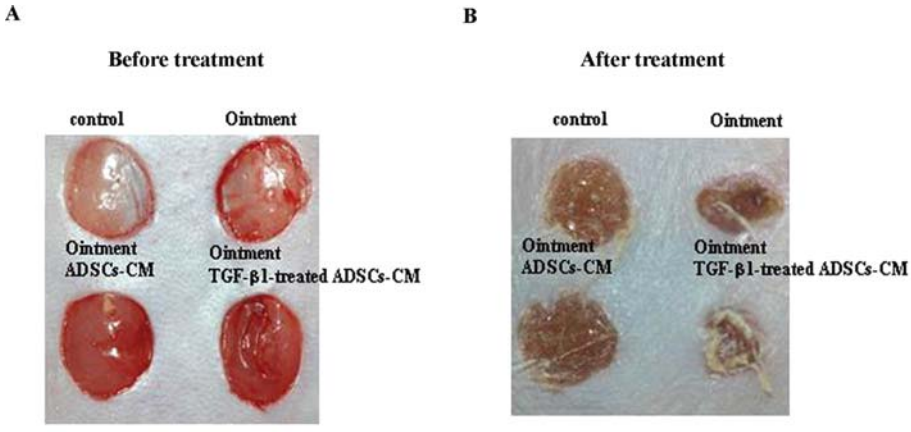

Figure 5. Effects of TGF-ß1-treated ADSCs-CM on wound healing in hairless mouse. Artificial wounds were made using a 4-mm punch biopsy (A) and ADSCs-CM or TGF-ß1-treated ADSCs-CM were injected into dermis on hairless mouse with ointment treatment. Intradermal injection of TGF-ß1-treated ADSCs-CM significantly accelerated wound healing in the right side of the back after 10 days (B).

 proteins were not markedly modulated by treatment, vels of Rb phosphorylation (Fig. 4B).

Effect of TGF-ß1-treated ADSCs-CM on wound of hairless mouse. Finally, we tried to study the effect of TGF-ß1-treated ADSCs-CM on wound healing promotion of hairless mice. Four circular full-thickness wounds of a 4-mm diameter were created on the backs of mice by a 4-mm punch. We injected regularly ADSCs-CM or TGF-ß1-treated ADSCs-CM on backs of hairless mice with ointment for 2 weeks, twice per week. Our data showed injection of TGF-ß1-treated ADSCs-CM promoted wound healing in hairless mice, evidenced by reducing wound size (Fig. 5).

Discussion

ADSCs have potential ability to differentiate into mesodermal cells, such as adipocytes, fibroblasts, myocytes, osteocytes, and cartilagocytes (12). In addition there is increasing evidence for ability of ADSC to differentiate into cells of nonmesodermal origin, such as neurons, endocrine pancreatic cells, hepatocytes, and cardiomyocytes. It is also well known ADSCs secrete various growth factors such as VEGF, IGF, HGF, and TGF-ß1 (1). Thus autologous ADSC therapy might be a promising tool for skin regeneration.

TGF-ß1 stimulates proliferation of fibroblasts and expression of type I collagen, implying that downstream of TGF-ß1, including connective tissue growth factor (CTGF), correlates with signal pathways of cell cycle progression and synthesis of type I collagen (13-15). One downstream signal molecule of TGF-ß1 is implicated in the transcriptional regulation of a wide range of genes participating in cell proliferation as well as ECM production. We hypothesized that CM from TGF-ß1-treated cells shows many soluble factors exhibiting growth promotion and modulation of ECM (16). Thus, in this study, we investigated whether the TGF-ß1-treated ADSCs-CM promotes effectively the proliferation, migration, expression of type I collagen and MMP-1 in skin fibroblasts, compared to ADSCs-CM. Our data indicated that TGF-ß1-treated ADSCs-CM promoted more effective the proliferation, migration, expression of type I collagen and MMP-1 than ADCSs-CM in skin fibroblasts. Even though the migration as well as expression of type I collagen and MMP-1 were increased by treatment of ADSCs-CM in skin fibroblasts, our data showed the TGF-ß1-treated ADSCs-CM had more potent properties than ADSCs-CM. In addition, intradermal injection of TGF-ß1-treated ADSCs-CM stimulated the wound healing process *in vivo*.

In this study, proteomic analysis of TGF-ß1-treated ADSCs-CM showed more several high density spots than ADSCs-CM (data not shown). This data supports the hypothesis that secreted proteins from ADSCs were increased markedly by stimulation of TGF-ß1.

The TGF-ß1-treated ADSCs-CM-induced type I collagen and MMP-1 expression may play important roles in modulation of ECM, thereby promoting the wound healing process. MMP-1 is able to cleave components of cell-cell junctions and cell-matrix contacts within the epithelium to promote re-epithelialization (16). MMP-1 is present in human cutaneous wounds during re-epithelialization but turns off once wound closure is completed (17). Furthermore the migration activity was increased markedly by treatment of CM-TGF-ß1-treated ADSCs in human skin fibroblasts, compared to CM-ADSCs. However, it is still unknown which soluble factor is involved directly to induce expression of type I collagen, MMP-1, and migration of fibroblasts.

Cell proliferation and migration are tightly regulated by cell cycle regulatory proteins (18-20). In addition the wound healing process is closely related with cell cycles (21). Arrested population of cells was progressively decreased and some cells were actively proliferated and contributed to wound repair, thereby wound healing is more likely completed. In contrast, senescent fibroblasts were markedly observed in chronic wounds. Cell cycle progression is regulated by two protein classes, the cyclins and their kinase partners, CDKs. Two families of cyclins are successively activated during the G1 phase, and thus also named as G1 cyclins. G1 cyclins are composed of D- and E-type cyclins. D-type cyclins assemble with its catalytic partners, cdk4 and cdk6, followed by cyclin E interacting with its catalytic partner, cdk2. The subsequent cyclin-cdk complexes drive cell cycles from G1 phase into S phase by phosphorylation of Rb. Therefore, cyclin D1 and cyclin E are the key regulatory proteins in progression of G1/S transition phases. In this study the expression of cyclins and cdks were not markedly increased by treatment of CM-TGF-ß1-treated ADSCs. Interestingly, Rb phosphorylation was slightly increased and expression of type I collagen was markedly increased by treatment of CM-TGF-ß1-treated ADSCs in serum starved fibroblasts. Previous findings suggest that TGF-ß1-induced CTGF plays an important role in collagen synthesis as well as proliferation of fibroblasts (22,23). We further studied whether CTGF involving CM-TGF-ß1-treated ADSCs induced up-regulation of type I collagen, migration and proliferation in skin fibroblasts.

In conclusion, our data suggest that TGF-ß1-treated ADSCs-CM play important roles on promotion of wound healing in skin through up-regulation of type I collagen, MMP-1 expression, migration, and proliferation of fibroblasts. Further studies are required to prove that the soluble factor of TGF-ß1-treated ADSCs-CM are involved in wound healing promotion.

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