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Anti-fibrotic effect of adipose-derived mesenchymal stem cell conditioned medium in muscle fibrosis

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Abstract. – OBJECTIVE: Recent investigations have demonstrated that the administration of MSC in mouse model of diseases provided beneficial effects. On the other hand, human adipose-derived MSC condition medium (ADSC-CM) is reported as containing beneficial secreted factors, but its role in muscle fibrosis has not been identified. The aim of this study was to investigate the inhibitory effects of MSC-CM in muscle fibrosis *in vitro* using the C2C12 murine muscle, myoblast cell line.

MATERIALS AND METHODS: C2C12 cells were cultured overnight in 0.1% albumin-Dulbecco's Modifies Eagle's Medium (DMEM). The cells were then pre-incubated in ADSC-CM for 20 min, treated with 2.5-10 ng/mL human TGFβ1 for 8-72 hours and analyzed using RT-qPCR, Western blot and immunofluorescent staining.

RESULTS: Treatment with 20% ADSC-CM for 3 days suppressed αSMA protein expression in TGFβ1 treated C2C12 cells. ADSC-CM stimulated the proliferation of C2C12 cells in a dose-dependent manner. Furthermore, TGFβ1 induced Acta2/αSMA mRNA expression which was inhibited by ADSC-CM treatment for 8 hours. Decorin, one of the dermatan sulfate proteoglycans and an endogenous inhibitor of TGFβ1, was expressed in ADSC-CM, but not in TGFβ1 pre-incubated ADSC-CM.

CONCLUSIONS: Our studies provide useful information for establishing anti-fibrotic mechanism(s) of ADSC-CM, thus facilitating potential application to prevent muscle fibrosis.

Key Words:

Human adipose-derived mesenchymal stem cells, Conditioned medium, Murine muscle cells, TGFβ1, Anti-fibrotic effect.

Abbreviations

MSC: mesenchymal stem cells, MSC-CM: mesenchymal stem cell conditioned medium, ADSC-CM: adipose-derived mesenchymal stem cell conditioned medium, TGFβ1: transforming growth factor β1, RT-qPCR: reverse transcriptase quantitative polymerase chain reaction, αSMA: alpha smooth muscle actin, Acta2: actin A2, TSG-6: tumor necrosis factor-stimulated gene-6, CCL-2: chemokines C-C motif chemokine ligand, Siglec-9: sialic acid-binding IgG-like lectin 9, IDO-1, PGE-2: prostaglandin E2, STC-1: secretion tumor cell 1, HLA-G: human leukocyte antigen G, ECM: extracellular matrix, EGFR: epidermal growth factor receptor, IGF-IR: insulin growth factor 1 receptor, EC: endothelial cell, DMED: Dulbecco's Modified Eagle's medium, FBS: fetal bovine serum, PBS: phosphate buffer saline.

Introduction

Mesenchymal stem cells (MSC) are somatic stem cells originated from bone marrow, umbilical cord, adipose tissue, and dental pulp or else. MSC are self-renewing and capable of differentiating into at least three lineages, namely adipocytes, osteocytes and chondrocytes[1]. A number of MSC-based clinical trials are available at the website of clinicaltrials.gov[2,3]. In general, disorders targeted by MSC-therapies included mainly inflammatory diseases, brain infarction, autoimmune diseases and fibrosis including liver, renal and muscle fibrosis. Originally, MSC-based therapy has been mainly studied by intravenous transfusion of cells. Thereafter, conditioned medium (CM) derived from MSC culture has also attracted much attention as an alternative way of treatment[4,5]. Several *in vitro* studies[6,7] have suggested that the immune suppression of T cell and M1 activation of macrophage occur by paracrine mechanisms of MSC. The paracrine factors could be found in human MSC, including ADSC. These are reported as tumor necrosis factor-stimulated gene-6[8], chemokines C-C motif chemokine ligand/sialic acid-binding IgG-like lectin 9[9,10], indoleamine-(2, 3)-dioxygenase inhibitor 1[11], prostaglandin E2[12], secre-

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tion tumor cell 1[13], human leukocyte antigen G and extracellular vesicles[14]. Subsequently, MSC which secrete various factors, including immunomodulatory factors is reported to be useful for treating inflammatory bowel disease[3], spinal cord injury[15], diabetes mellitus[16], and muscle atrophy[17].

Intramuscular fibrosis is an abnormal wound-healing response to muscular injury, characterized by the excessive accumulation of extracellular matrix (ECM) proteins in the muscle. The pathogenesis of intramuscular fibrosis involves quiescent satellite cell, myoblast and fibroblast differentiated by TGFβ1 into activated myofibroblasts. Activated satellite cells proliferate and migrate to the site of injury and repair muscle cells, while excess collagen accumulates leading to fibrosis. Skeletal muscle fibrosis impairs muscle function, negatively affects muscle regeneration after injury and increases muscle susceptibility to re-injury. Therefore, it is considered a major cause of muscle weakness[18].

On the other hand, decorin is a small leucine-rich proteoglycan of the ECM. It is involved in a number of cellular processes, including matrix assembly, fibrillogenesis, and the control of cell proliferation[19,20]. Core protein of decorin also serve as a ligand for receptor tyrosine kinases, including EGFR, IGF-IR, and Met[21]. Decorin modulates degradation of the extracellular matrix and plays a protective role against fibrogenesis. Decorin is also an endogenous inhibitor of TGFβ1[22] and inhibits macrophage proliferation and apoptosis[23]. Furthermore, Acta2 or alpha smooth muscle actin (α-SMA) is an established marker of smooth muscles[24] and Atrogin-1 promotes muscle protein degradation by the ubiquitin proteasome pathway[25].

Skeletal muscle fibrosis is considered a major cause of muscle weakness. It impairs muscle function and regeneration of muscle after injury and increases susceptibility of muscle to re-injury. Myofibers in skeletal muscles are multinucleated cells and are surrounded by satellite cells and multi-potential cells namely fibro-adipogenic progenitor (FAPs)[26]. FAPs are a population of mesenchymal interstitial cells that can differentiate to adipocytes and contribute to increased intra-myocellular fat accumulation[27]. FAPs promote satellite cell differentiation in adult skeletal muscle regeneration but in pathological conditions FAPs are responsible for fibrosis and fat infiltrations.

A hallmark of muscular dystrophies, aging and severe muscle injuries is fibrosis of skeletal muscle. Thus, a better understanding of the mechanisms of muscle fibrosis will help to advance our knowledge of the events that occur in dystrophic muscle diseases and develop innovative anti-fibrotic therapies to reverse fibrosis in such pathologic conditions.

Previous scholars[28] have shown that MSC suppress fibroblast proliferation and reduce skin fibrosis. In addition, MSC-CM could differentiate umbilical cord mesenchymal stem cells into the smooth muscle cells[29]. However, underlying molecular mechanism(s) of MSC-CM therapy for muscle atrophy has not yet been fully clarified. In this study, we aimed to investigate human ADSC-CM for use in muscle fibrogenesis and muscle atrophy and provide clues to clarify the anti-fibrotic mechanism(s) of MSC. We investigated the inhibitory effects of ADSC-CM in muscle fibrosis *in vitro* using the C2C12 murine muscle myoblast cell line. The rationale for choosing mouse myoblast cell line was due to unavailability of commercial human muscle cell lines and also inherent heterogeneity of primary human skeletal muscle cells.

Materials and Methods

Cell Line

Murine C2C12 murine muscle and myoblast cell line (RIKEN BRC, Tsukuba, Japan) were cultured in Dulbecco's Modifies Eagle's Medium (DMEM; Sigma-Aldrich, St. Lois, USA) supplemented with 10% FBS (Gibco BRL, Rockville, MD, USA), and 10 mg/mL gentamycin. The cells were maintained in an incubator at 37°C and 5% CO_2.

Mesenchymal Stem Cell Culture and CM

Human MSC were isolated from commercially available adipose tissue and grown using an animal origin-free (AOF) medium (sf-DOT; BioMimetics Sympathies Inc., Tokyo, Japan) and characterized as previously reported[30,31]. Cells were used for experiments during passages 3-7 and stored in liquid nitrogen prior to use. When the ADSC were reached, approximately 80% confluent by proliferating in sf-DOT medium with or without TGFb1 (Miltenyi Biotec., San Diego, CA, USA), ADSC were incubated for 3 days in collecting serum-free medium DMEM/F12 without phenol red (Gibco BRL, Rockville, MD, USA).

The harvested medium was centrifuged at 3000 × g for 5 min, then, the resulting supernatant was collected as conditioned medium.

Experimental Conditions

C2C12 cells were seeded at 4 × 10^4-1 × 10^5 cells/well in 12-well-plate (non-coat) using 10% Fetal Bovine Serum (FBS)/DMEM. Adhesive cells were cultured overnight in DMEM medium containing 0.1% recombinant albumin (Sigma-Aldrich. St Louis, Mo, USA). C2C12 cells were pre-incubated in ADSC-CM for 20 minutes, then treated with 2.5-10 ng/mL human recombinant TGFb1 (Miltenyi Biotec, San Diego, CA, USA). After 8 ~ 72 hours, each analysis was performed (Figure 1).

Western Blot Analysis

After 72 hours from start of test, C2C12 cells were washed by PBS(-), then, suspended in RIPA Buffer (Wako, Tokyo, Japan), and disrupted by sonication for a total of 1 min on ice. After centrifugation of the cell lysate at 15,000 rpm at 4°C for 5 min, the resulting supernatant was used as a cell extract. Protein was determined for this sample as described above. Cell extracts were subjected to 5-15% SDS polyacrylamide gel electrophoresis using NextPage II (Gellnex, Tokyo, Japan), then blotted onto a PVDF-membrane at 15 V for 30 min. The membrane was washed in TBST containing 1% skim milk, then incubated in TBST containing 3% skim milk for 30 min. and probed with 2000-fold dilution of specific antibodies against αSMA (A5228, Sigma-Aldrich. St Louis, Mo, USA) or β-actin (PM053, MBL Co., Ltd., Japan) at 4°C overnight. Thereafter, the membrane was incubated in 2000-fold dilution of HRP-labeled secondary antibodies (GE Healthcare, Tokyo, Japan), and the bands were detected with the luminol/H_2O_2 solution by chemiluminescence analyzer (Multilabel Reader ARVO X3, Perkin Elmer, Waltham, MA, USA).

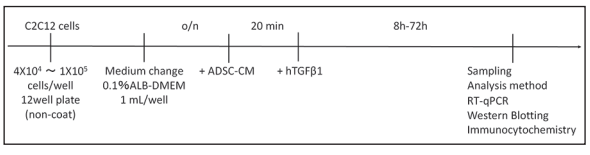

Figure 1. Experimental design. C2C12 cells were seeded 4×10^4-1×10^5 cells/well in 12 well-plate (non-coat) using 10% FBS-DMEM. Adhesion cells were cultured overnight in 0.1% albumin-DMEM medium. C2C12 cells were pre-incubated in ADSC-CM for 20 minutes, then treated 2.5-10 ng/mL human TGFβ1. After 8-72 hours, each analysis was performed.

Immunostaining and Quantitative Image Analysis

After 72 hours from the beginning of test, we fixed cells in 4% para-formaldehyde for 5 min, then, we washed the cells in 3 changes of 0.01% TritonX-100/PBS(-) wash solution. The fixed cells were permeated by 0.2% TritonX-100/ PBS(-) for 5 min; then, we washed the cells in three changes of wash solution. We blocked the cells in 0.01% TritonX-100/ PBS(-) containing 3% BSA. We incubated cells with a 1:2000 dilution of anti-αSMA (A5228, Sigma-Aldrich. St Louis, Mo, USA) in 0.01%TritonX-100/PBS(-) at 4°C for overnight and then, we washed the cells in three changes of wash solution. We incubated cells with a 1:2000 dilution of Alexa Fluor ®488 secondary antibodies (Invitrogen, Thermo-Fisher, Tokyo, Japan) at RT for 1 hour. The cells were washed in three changes of wash solution, then stained using Phalloidin (Invitrogen, Carlsbad, CA, USA) and DAPI (Molecular Probes®, Eugene, OR, USA) and observed using a fluorescence microscope. Quantitative image analysis of the expression of aSMA was performed using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA)[32] and the percentage of positively immunostained aSMA cells in each group of before and after treatment with TGFb1 was determined and compared.

Cell Proliferation Assay

After 48 hours from start of test, cells were harvested by trypsinization and suspended in PBS(-). Cell viability was assessed via trypan blue exclusion cell counts. Briefly, cells were stained by 0.2% trypan blue (Gibco BRL, Rockville, MD, USA). In brief, a 4% trypan blue stock solution was diluted with PBS to 0.2% and filtered with 0.2 micron filter. The cell suspension was mixed at 1:1 ratio with 0.2% trypan blue and loaded onto a hemocytometer for counting.

RT-qPCR Analysis

For real-time quantitative PCR (qPCR) analyses, total RNA was prepared using Relia prep RNA Cell Miniprep System (Promega, Tokyo, Japan) according to the manufacturer's instruction. Briefly, cells were lysed in RNA lysis buffer (BL) plus 1-Thioglycerol (TG) by vortexing. Next, 100% isopropanol was added, mixed for 5 seconds and the lysate was transferred into the ReliaPrep™ Minicolumn on collection tube and centrifuged at 12,000 × g for 30 seconds. The liquid from collection tube was discarded and

RNA wash solution was added and centrifuged at 12,000 × g for 30 seconds. Next, 500 µl of RNA Wash Solution was added to the ReliaPrep™ Minicolumn and centrifuged at 12,000-14,000 × g for 30 seconds. Subsequently, DNase I incubation mix was added and incubated for 15 minutes at room temperature followed by 200µl of Column and Centrifuging at 12,000-14,000 × g for 15 seconds. Then, RNA wash solution (with ethanol) was added and centrifuged at 12,000-14,000 × g for 2 minutes. Finally, the ReliaPrep™ Minicolumn was transferred from the Collection Tube to the Elution Tube, and 15 µl nuclease-free water was added and centrifuged at 12,000-14,000 × g for 1 minute. The Elution Tube containing the purified RNA was capped and store at -70°C until use. The cDNA was synthesized using a PrimeScript™ RT master mix (Takara Bio, Shiga, Japan), according to the manufacturer's recommendations. The expression level of Acta2 and Atrogin-1 for qPCR was analyzed using a Thunderbird SYBR qPCR mix (TOYOBO, Osaka, Japan) on Stratagene Mx3000P (Stratagene Corp., La Jolla, CA, USA). The primer pairs used were Acta2: Forward (5'-3') TAACCCTTCAGCGTTCAGC, Reverse (5'-3') ACATAGCTGGAGCAGCGTCT and Gapdh: Forward (5'-3') TTCACCACCATGGAGAAGG, Reverse (5'-3') CACACCCATCACAAACATGG.

Analysis of ADSC-CM

ADSC-CM was incubated in proteinase K (Takara Bio, Shiga, Japan) for 2 h at 37 °C, then inactivated for 13 min at 95°C. Proteinase K treated ADSC-CM was resolved on SDS-PAGE and stained with Coomassie brilliant blue to confirm protein degradation. Decorin levels of ADSC-CM were measured by ELISA and Western blot, using human decorin ELISA Kit (Abcam, Tokyo, Japan) and decorin antibody (R&D Systems, Tokyo, Japan). For ELISA all steps of the procedure were conducted at room temperature. Each experiment was done in triplicate. From each sample, 100 µl per well was added to pre-coated 96-well microplate and incubated 2.5 hours. After washing, plate was incubated with biotinylated antibody for 1 hour. After rinsing excess antibodies, 100 µl of streptavidin-conjugated HRP solution was added to each well and incubated for 45 min. Color development was done by incubation in 100 µl of TMB Substrate for 30 min and then 50 µl of H2SO4 was added to stop the reaction. Measurement of the absorbance at 450 nm was done immediately on microplate reader (Multilabel Reader ARVO X3, Perkin Elmer, Waltham, MA, USA). For Western blot, samples were subjected to 5-15% SDS polyacrylamide gel electrophoresis using NextPage II (Gellnex, Tokyo, Japan), then, blotted onto a PVDF-membrane at 15 V for 30 min. The membrane was washed in TBST containing 1% skim milk, then incubated in TBST containing 3% skim milk for 30 min. and probed with 2000-fold dilution of specific antibody against decorin (R&D Systems, Tokyo, Japan) at 4°C overnight. Thereafter, the membrane was incubated in 2000-fold dilution of HRP-labeled secondary antibodies (GE Healthcare, Tokyo, Japan), and the bands were detected with the luminol/H_2O_2 solution by chemiluminescence analyzer (Multilabel Reader ARVO X3, Perkin Elmer, Waltham, MA, USA).

Data Analysis

All quantified data are expressed as mean ± standard error of the mean (SEM) of triplicate experiments. The significance of differences between the two groups was tested by unpaired *t*-test and Fisher's exact test at the 0.05 significance level.

Results

Anti-TGFβ1 Effect of ADSC-CM

We investigated whether fibrotic signaling could be activated in C2C12 cells in response to TGFβ1 (2.5-10 ng/ml). After 3 days treatment with TGFβ1, C2C12 cells expressed αSMA proteins by Western blot analysis and immunofluorescent staining (Figures 2A, B), indicating transformation into myofibroblast. Expression of αSMA protein was suppressed by application of ADSC-CM (Figures 2A, 2B and 2C).

Proliferation of C2C12 Cells by ADSC-CM

We examined the effects of ADSC-CM on the proliferation of murine C2C12 cells (Figure 3). MSC-CM (0%-20%) induced the proliferation of C2C12 cells dose-dependently (Figures 3A, B). Compared with the control group, ADSC-CM-treated C2C12 cells showed a more than 4-fold increase in cellular proliferation (Figure 3B). In addition, the expression level of Atrogin-1 mRNA, a marker of muscular atrophy was significantly decreased in C2C12 cells at 20% ADSC-CM application (Figure 3C).

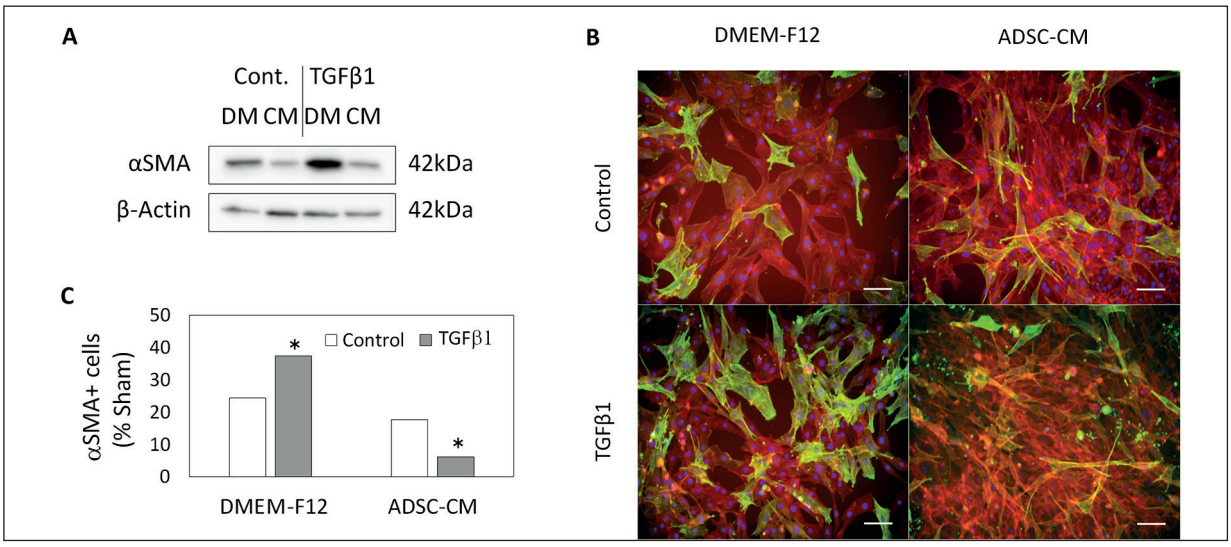

Figure 2. The change in C2C12 cells treated TGFβ1 and ADSC-CM for 3 days. **A,** TGFβ1 (10 ng/mL) treated C2C12 cells were subjected to Western blot analysis using αSMA (Sigma, A5228) or β-actin (MBL, PM053) antibody. Application of ADSC-CM (20%) was able to suppress αSMA expression. **B,** Immunofluorescent staining for αSMA (Green) demonstrated that ADSC-CM (20%) suppressed αSMA expression induced by TGFβ1 (10 ng/mL). **C,** Quantitative image analysis also supported this observation (*p<0.05). Phalloidin (Red) and DAPI (Blue) were used for actin filament and nuclear counterstains. HPF; high power field, DM; DMEM/F12, CM; ADSC-CM, Cont.; Control (no treatment). Scale bar = 50 μm.

ADSC-CM Blocked Expression of TGFβ1 Induced Acta2 mRNA

In order to examine whether ADSC-CM-blocked transformation of C2C12 cells was regulated through the TGFβ1-induced expression, we analyzed the Acta2 gene expression in C2C12 cells treated TGFβ1 (2.5-10 ng/mL) and ADSC-CM (0-20%) for 8-24 hours. After 8 hours, TGFβ1 induced Acta2 mRNA expression was inhibited by pre-incubating C2C12 cells with 20% ADSC-CM.

In C2C12 cells, Acta2 mRNA expression after treatment with TGFβ1 was observed at highest levels at 8 hours followed by no change at 24

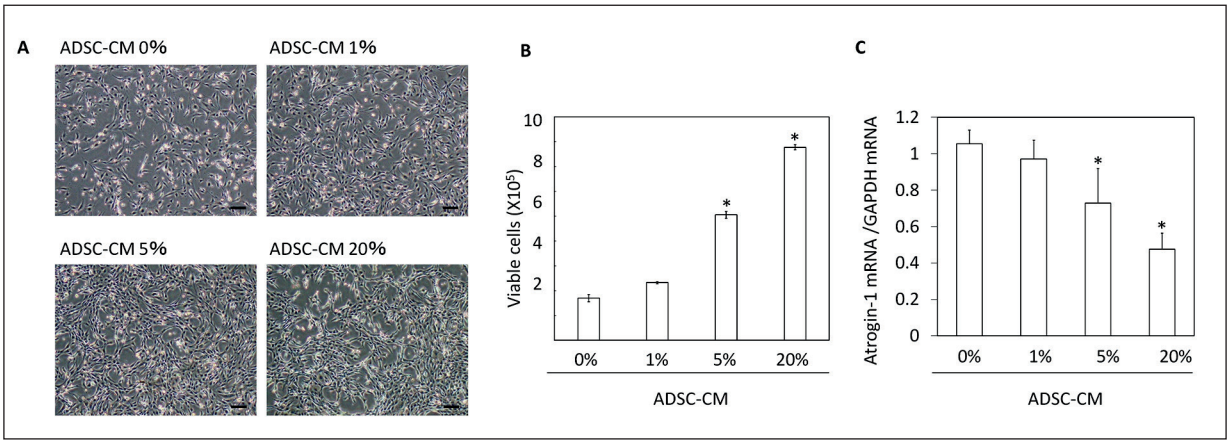

Figure 3. Proliferation of C2C12 cells applied ADSC-CM for 48 h. **A**, The phase contrast images show C2C12 cells treated 0%, 1%, 5% and 20% ADSC-CM for 48h. **B,** Cell viability was determined by 0.2% Trypan Blue exclusion. C2C12 cells were proliferated by ADSC-CM in a dose-dependent manner. **C,** Expression of Atrogin-1 mRNA was decreased by treatment with ADSC-CM for 8 h in C2C12 cells. Data represent the mean ± SD of triplicate measurements; *Bars *vs*. 0%, p<0.05. Scale bar = 100 μm.

hours. At 8 hours after treatment with TGFβ1 and ADSC-CM, TGFβ1-induced Acta2 mRNA expression was blocked by ADSC-CM. After 24 hours, Acta2 mRNA expression was decreased in ADSC-CM treated groups compared with DMEM/F12 treated groups (Figure 4A). We infer that ADSC-CM treatment for 24 hours stimulated the proliferation of Acta2 low C2C12 cells.

Expression of Acta2 mRNA induced by TGFβ1 was inhibited by ADSC-CM treatment. ADSC-CM treatment was dose-dependently decreased Acta2 mRNA levels induced by TGFβ1 as compared with the ADSC-CM no treated control group (Figure 4B). Therefore, these results suggested that application of ADSC-CM suppressed activation of Acta2 gene by TGFβ1, in addition to activation of cell proliferating signals, in C2C12 cells.

Anti-TGFβ1 Activity of Proteinase K Treated ADSC-CM

No band was detected for ADSC-CM protein degradation by proteinase K as resolved by SDS-PAGE (Figure 5A). TGFβ1 treated C2C12 cells showed increase expression of Acta2 mRNA, and this effect was inhibited by ADSC-CM treatment. TGFβ1-pre-incubated ADSC-CM showed no effects.

Proteinase K treatment but not heat treatment of ADSC-CM did not block the Acta2 mRNA expression of C2C12 cells in response to TGF-β1 treatment (Figure 5B), indicating that peptide(s) or protein(s) of ADSC-CM was required for inhibition of TGF-β1-mediated fibrogenesis of C2C12 cells.

Effect of Conditioned Medium from TGFβ1-Pretreated ADSC

We examined the effects of TGFβ1-pretreated ADSC-CM on the proliferation of murine C2C12 cells. To evaluate effect of TGFβ1 pretreatment on ADSC-CM, proliferation of C2C12 cells was measured at absorbance level of A450 by WST-8 analysis. ADSC-CM from TGFβ1-pretreated MSC showed enhanced cellular proliferation similarly to ADSC-CM (Figure 6A). ADSC-CM from TGFβ1-pretreated MSC did not inhibit the Acta2 mRNA expression of C2C12 cells in response to TGF-β1 treatment (Figure 6B). In ADSC-CM, decorins were detected at higher levels as compared with TGFβ1-pretreated ADSC-CM by ELISA and Western blot analysis (Figure 7A, B). These results suggested that decorin, one of the anti-TGFβ1 component of ADSC-CM, could be responsible for inhibition of TGF-β1-mediated fibrogenesis in C2C12 cells.

Discussion

In this study, we aimed to investigate human ADSC-CM for use in muscle fibrogenesis and muscle atrophy and provide clues to clarify the

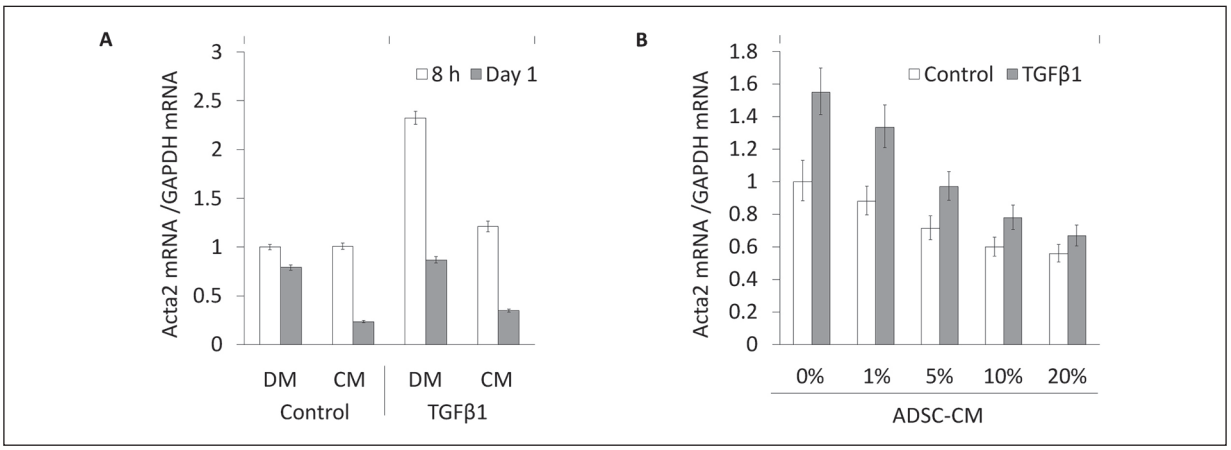

Figure 4. Expression of Acta2 mRNA induced by TGFβ1 was inhibited by ADSC-CM treatment. **A,** TGFβ1 (2.5 ng/mL) stimulated C2C12 cells were treated with 20% ADSC-CM for 8 h or 1 day. Real-time PCR reactions were performed using mouse Acta2 and GAPDH primers. TGFβ1 increased expression of Acta2 mRNA in C2C12 cells, and the effects was inhibited by ADSC-CM treatment. **B,** A dose-dependency of ADSC-CM was evaluated by suppressing Acta2 mRNA expression for 9h in 10 ng/mL TGFβ1 treated C2C12 cells. By application of 5% ADSC-CM, Acta2 mRNA levels were suppressed in TGFβ1 treated C2C12 cells. Data represent the mean ± SD of triplicate measurements. DM; DMEM/F12, CM; ADSC-CM, Control; no treatment.

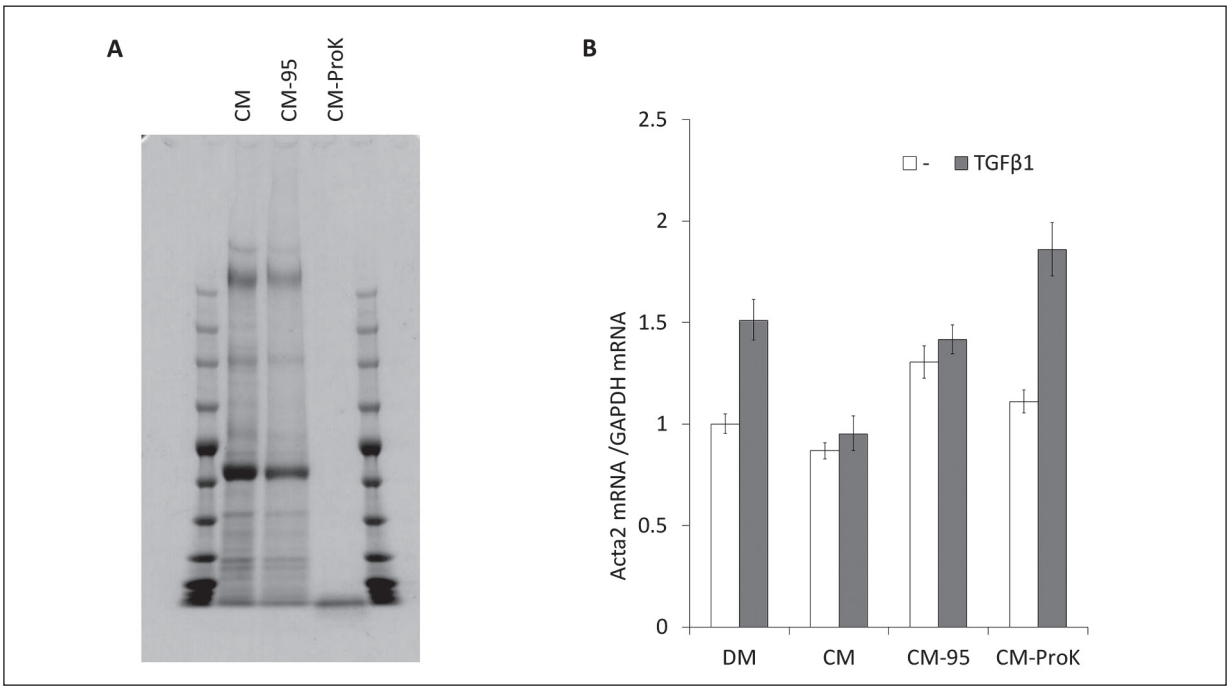

Figure 5. Anti-TGFβ1 activities of proteinase K-treated ADSC-CM. **A,** ADSC-CM was resolved on SDS-PAGE. **B,** C2C12 cells were treated with 10% ADSC-CM for 8 h. Real-time PCR reactions were performed using mouse Acta2 and GAPDH primers. TGFβ1 10 ng/mL treated C2C12 cells showed increase in the expression of Acta2 mRNA, and the effects was inhibited by ADSC-CM treatment. Proteinase K-treated ADSC-CM showed no effects. Data represent the mean ± SD of triplicate measurements. DM; DMEM/F12, CM; ADSC-CM, CM-95; ADSC-CM heat treated for 2 h at 37°C then for 13 min at 95°C, CM-ProK; ADSC-CM of proteinase K treatment for 2 h at 37°C then for 13 min at 95°C.

anti-fibrotic mechanism(s) of MSC. We found that ADSC-CM could suppress TGFβ1 induced expression of fibrogenesis markers, such as Acta2/αSMA mRNA expression in C2C12 myoblast cells. We also found that decorin, an inhibitor of TGFβ1, was expressed in ADSC-CM and not in

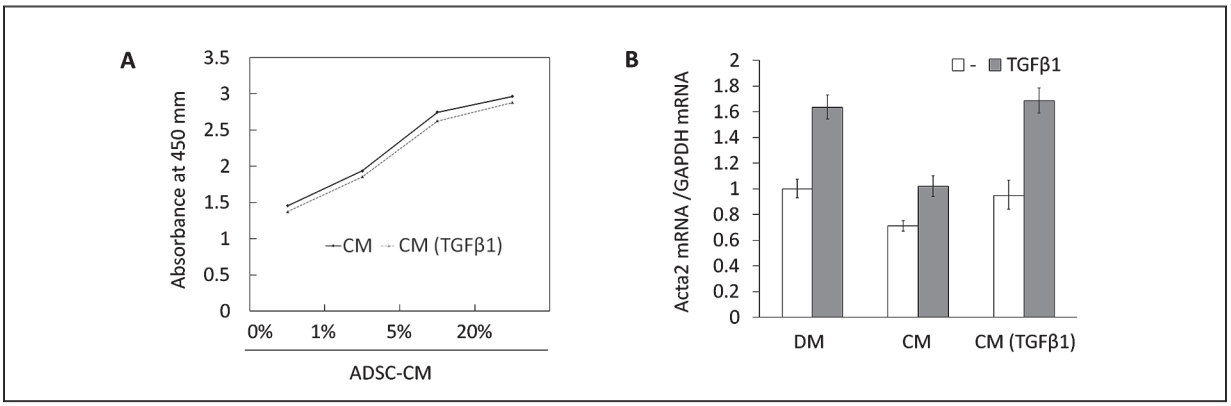

Figure 6. Effect of TGFβ1-pre-incubated ADSC-CM evaluated by WST-8 analysis and expression of Acta2 mRNA in C2C12 cells. **A,** In dose-dependent application of ADSC-CM, the proliferation of C2C12 cells was evaluated by WST-8 assay. No changes were seen in absorbance of between CM and CM (TGFβ1) groups. **B,** C2C12 cells were treated with 5% ADSC-CM for 8 h. Real-time PCR reactions were performed using mouse Acta2 and GAPDH primers. TGFβ1 treated C2C12 cells showed increased in expression of Acta2 mRNA, and this effect was inhibited by ADSC-CM treatment. TGFβ1-pre-incubated ADSC-CM showed no effects. Data represent the mean ± SD of triplicate measurements. DM; DMEM/F12, CM; ADSC-CM, CM (TGFβ1); TGFβ1-pre-incubated ADSC-CM.

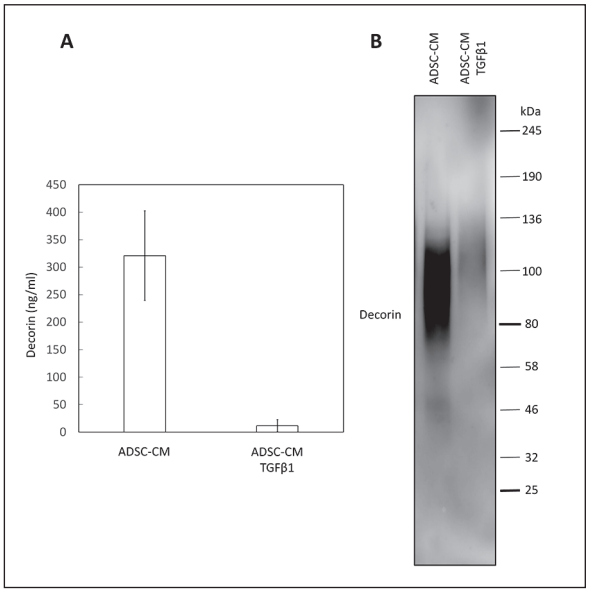

Figure 7. Decorin is one of the biologically active constituents in ADSC-CM. **A,** ADSC-CM contained decorin was analyzed by ELISA. The TGFβ1-preincubated ADSC-CM contained low concentrations of decorin as compared to normal ADSC-CM. Data represent the mean ± SD of triplicate measurements. **B,** Decorin in ADSC-CM was detected. ADSC-CM and TGFβ1-preincubated ADSC-CM 10 μL were subjected to Western blot analysis using decorin antibody.

ADSC-CM pre-incubated with TGFβ1. Thereby, we identified ADSC-secreted decorin as a protective factor against TGFβ1.

In recent years, several cell preparations in the field of regenerative medicine have been approved in pharmaceutical industry. MSC therapy, which involves expanding and culturing stem cells *in vitro* and returning them into body, is highly desirable due to anti-inflammatory and anti-fibrotic effects and regeneration of damaged tissues. MSC is a type of somatic stem cell that is present throughout the body even after birth. Adipose-derived MSC were discovered in 2001[1,33]. Adipose tissue contains abundant progenitor cells and stem cells and is recognized as a source of MSC. MSC can be collected from 1 mL of bone marrow aspirate to 6-60 × 10^3 cells and from 1 g of adipose tissue to 5 × 10^4-2 × 10^5 cells[34]. Separation of MSC depends on the characteristics of the donor such as age, sex, BMI, location and type of adipose tissue, and the production process such as tissue collection and culture method, thus the amount of MSC that can be collected varies.

MSC secrete various factors that support cell survival, including growth factors, cytokines, and extracellular matrix (ECM). These can promote progenitor cell self-renewal, stimulate angiogenesis, and reduce apoptosis and/or inflammation[35]. Thus, MSC-CM contains the same factors and has shown various biological effects after administration. Both extracellular vesicles including exosomes and soluble component of MSC-CM could promote tissue regeneration, inhibit abnormal immune response and induce angiogenesis in ischemic tissues[36]. Furthermore, MSC-CM has shown immunoregulatory, angiomodulatory and anti-apoptotic effects that resulted in enhanced tissue repair and regeneration[37]. Some studies also suggested that MSC-CM may be more effective than MSC in tissue repair[38].

In our study, we showed that ADSC-CM has the effect of inhibiting the function of TGFβ1 thereby could prevent fibrogenesis. When mouse myofibroblast C2C12 myoblast cells were seeded and TGFβ1 was added, the expression of αSMA, a fibrosis marker, was increased. The ADSC were cultured in a human and animal origin-free, sf-DOT medium, then changed to a basic medium, and the supernatant was collected. When this ADSC-CM was added to C2C12 myoblast cell culture, the cell proliferation was markedly increased and the expression level of Atrogin-1 mRNA, a marker of muscular atrophy was significantly decreased. This result suggested that ADSC-CM enhanced the proliferation of C2C12 cells through a mechanism(s) mediated by Atrogin-1[25]. Addition of ADSC-CM to C2C12 myoblast cells prior to addition of TGFβ1, showed that the expression of αSMA that was induced by TGFβ1 could be suppressed. This observation, was thus provided clues for anti-fibrotic mechanism(s) of ADSC and ADSC-CM and implicated their potential application to prevent muscle fibrosis. It was suggested that some components derived from ADSC might suppress the fibrotic effect of TGFβ1. In a previous study[39], the therapeutic potential of ADSC-CM against experimental rat models of pulmonary hypertension and pulmonary fibrosis was evaluated. It was found that either ADSC or ADSC-CM treatment alone could halt the progression of monocrotaline or bleomycin-induced pulmonary hypertension or pulmonary fibrosis. In our study, we used mouse myoblast cell line as a model due to unavailability of commercial human muscle cell lines. Thus, our results should be confirmed using human myoblast cell line whenever it is available. We found that decorin was expressed in ADSC-CM. Decorin is one of the dermatan sulfate proteoglycans and an endogenous inhibitor of TGFβ1. We

identified ADSC-secreted decorin as a protective factor against TGFβ1. In this respect, we are now studying the role of decorin in more detail by establishing a dose dependent effect of decorin on muscle cells' αSMA expression. Taken together, the above findings suggest that ADSC-CM could be a good candidate for cell-free therapy of muscle fibrosis.

The production of a medium with high cell growth ability and stable performance is a critical step for supplying a stable cell preparation[40] which can also contribute to the quality of CM derived from those cells. In 2013, we developed an animal origin-free (AOF) medium branded "sf-DOT" for culture of MSC, which demonstrated a high cell growth capability[30,31]. The development of this medium made it possible to overcome lot of problems due to animal-derived materials and to stably supply cell preparations and their CM. For example, CM from low serum ADSC culture significantly increased endothelial cell (EC) proliferation and decreased EC apoptosis compared to that obtained from high serum counterpart or control media only[40]. Such ADSC could produce more HGF and VEGF that promoted regeneration by lowering the serum concentration. A secretory element from MSC was reported to be one of the factors showing an anti-fibrotic effect[41]. In fact, the MSC phenotype can be manipulated by stimulation with one factor or a plurality of factors. Unlike serum containing media, human and animal origin-free media with a well-defined composition will deepen understanding of the physiological mechanisms of cells and facilitate control of cell phenotypes. Therefore, it may be possible to prepare ADSC having performance suitable for a specific disease. Taken together, these results point to the importance of stable ADSC preparation with homogenous function for a constant production of quality ADSC-CM. The role of ADSC-CM in muscle fibrosis has not yet been identified. In this respect, the novel findings of our study are that ADSC-CM through suppression of TGFβ1-induced expression of fibrogenesis markers such as Acta2/αSMA mRNA expression and also by secretion of decorin, an inhibitor of TGFβ1, could promote anti-fibrotic effect to prevent muscle fibrosis.

Conclusions

In conclusion, we assessed changes in muscle cell function using ADSC-CM with or without treatment with TGFβ1. We found that ADSC-CM could suppress TGFβ1 induced expression of fibrogenesis markers, such as Acta2/αSMA mRNA expression in C2C12 myoblast cells. We also found that decorin, an inhibitor of TGFβ1, was expressed in ADSC-CM and not in ADSC-CM pre-incubated with TGFβ1. Thereby, we identified ADSC-secreted decorin as a protective factor against TGFβ1. Our studies provide useful information for establishing anti-fibrotic mechanism(s) of ADSC-CM, thus facilitating potential use to prevent muscle fibrosis. Further studies are needed to evaluate the functional mechanism(s) of ADSC-CM in various cell types in health and disease.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Data Availability

The processed data supporting the findings of this study are available from the corresponding author upon request.

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Ethics Approval and Consent to Participate

Not applicable.

Authors' Contribution

KO: conception and design, interpretation of data and manuscript writing; SY: collection of data, data analysis and interpretation; NU: conception and design, provision of study material and final approval; MG: checked collection of data and analysis and interpretation of results, and manuscript writing.

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