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Synovial fluid of patients with rheumatoid arthritis induces α-smooth muscle actin in human adipose tissue-derived mesenchymal stem cells through a TGF-β1-dependent mechanism

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Abbreviations: hASCs, human adipose tissue-derived mesenchymal stem cells; LPA, lysophosphatidic acid; MSCs, mesenchymal stem cells; RA, Rheumatoid arthritis; RA-SF, synovial fluids from patients with RA; SF, synovial fluid; shRNA, short hairpin RNA; siRNA, small interfering RNA; SPC, sphingosylphosphorylcholine; α-SMA, α-smooth muscle actin

Abstract

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disorder that causes the immune system to attack the joints. Transforming growth factor-β1 (TGF-β1) is a secreted protein that promotes differentiation of synovial fibroblasts to α-smooth muscle actin (α-SMA)-positive myofibroblasts to repair the damaged joints. Synovial fluid from patients with RA (RA-SF) induced expression of α-SMA in human adipose tissue-derived mesenchymal stem cells (hASCs). RA-SF-induced α-SMA expression was abrogated by immunodepletion of TGF-β1 from RA-SF with anti-TGF-β1 antibody. Furthermore, pretreatment of hASCs with the TGF-β type I receptor inhibitor SB431542 or lentiviral small hairpin RNA-mediated silencing of TGF-β type I receptor expression in hASCs blocked RA-SF-induced α-SMA expression. Small interfering RNA-mediated silencing of Smad2 or adenoviral overexpression of Smad7 (an inhibitory Smad isoform) completely inhibited RA-SF-stimulated α-SMA expression. These results suggest that TGF-β1 plays a pivotal role in RA-SF-induced differentiation of hASCs to α-SMA-positive cells.

Keywords: mesenchymal stem cells; rheumatoid arthritis; synovial fluid; transforming growth factor β1; α-smooth muscle actin

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by destruction of articular cartilage and adjacent bone tissues (Feldmann *et al*., 1996) and disordered synovial microenvironment, including infiltration of inflammatory cells, hyperplasia of stromal cells, and tissue scarring (Buckley, 2003). Synovial fluid (SF), which nourishes articular cartilage and lubricates articular joint surfaces (Ghosh and Guidolin, 2002), contains various growth factors and cytokines (Szekanecz *et al*., 1998). The pathological events of RA are mediated by a complex interplay of pro-inflammatory cytokines and mediators produced in the joint tissues or synovium of patients with RA (Scrivo *et al*., 2007).

Transforming growth factor-β1 (TGF-β1), a member of the TGF-β super family of cytokines, is involved in diverse cellular responses, such as proliferation, differentiation, apoptosis, and production of extracellular matrix (Wahl, 2007). TGF-β is important for the induction of fibrosis associated with RA (Pohlers *et al*., 2009). Alpha-smooth muscle actin (α-SMA)-positive myofibroblasts reportedly play a key role in the fibrosis and pathogenesis of RA (Kasperkovitz *et al*., 2005) and TGF-β1 is involved in the differentiation of synovial fibroblasts to α-SMA-positive cells (Mattey *et al*., 1997; Steenvoorden *et al*., 2006). TGF-β ligand binding induces the heterodimeric association of TGF-β type I and II serine/threonine receptor kinases (Shi and Massague, 2003; ten Dijke and Hill, 2004). Activated TGF-β type I receptor recruits and phosphorylates Smad2 and Smad3, which in turn associate with the protein Smad4 (Shi and

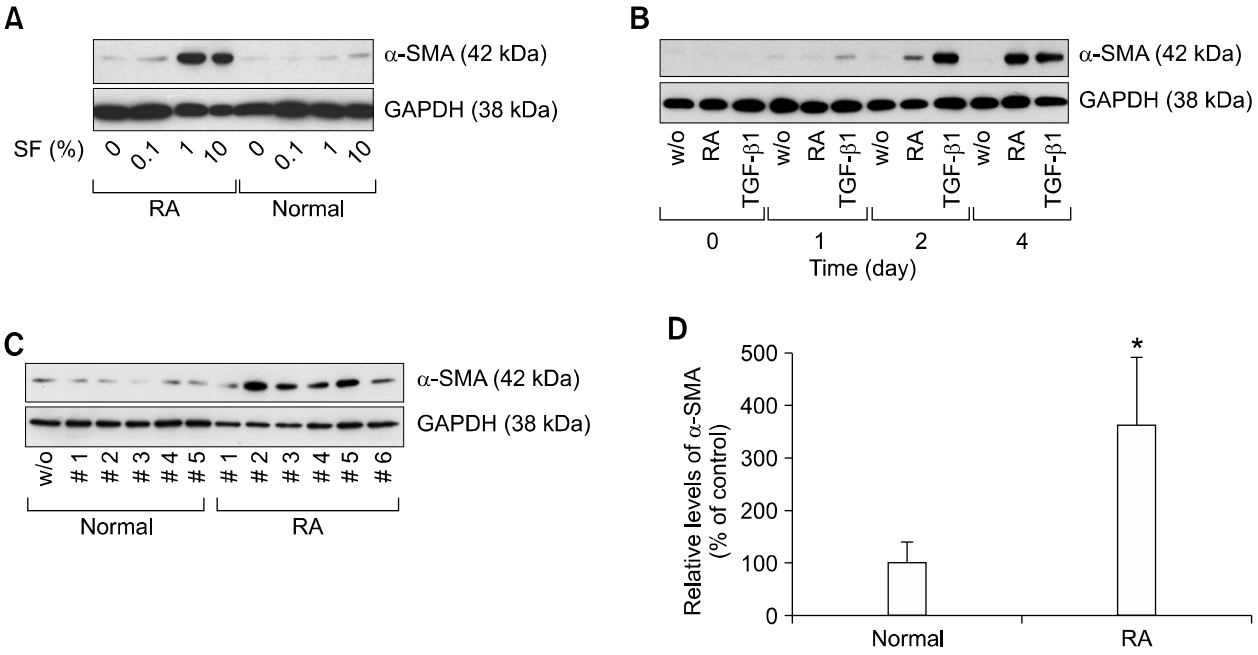

Figure 1. Effects of RA-SF on the expression of α-SMA in hASCs. (A) Serum-starved hASCs were treated with indicated concentrations of SF with RA patients or normal patients for 4 days. (B) Serum-starved hASCs were treated with vehicle, 1% RA-SF or 0.2 ng/ml TGF-β1 for the defined times. (C) Serum-starved hASCs were treated with vehicle (w/o), 1% SF of five normal donors, or 1% SF of six RA patients for 4 days. (D) The densities of α-SMA were quantified from three independent experiments, and the expression levels of α-SMA were normalized to total GAPDH levels in the samples. The data are presented as a percentage of control. *, $P < 0.05$.

Massague, 2003; ten Dijke and Hill, 2004). Complexed Smads translocate to the nucleus, where they regulate transcription of TGF-β-responsive genes, whereas Smad7 inhibits intracellular signaling by the TGF-β superfamily proteins, mainly by interacting with activated type I receptors for the TGF-β superfamily proteins (Attisano and Wrana, 2000; de Caestecker, 2004).

Mesenchymal stem cells or multipotent stromal cells (MSCs) possess self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages (Prockop, 1997; Pittenger *et al*., 1999; Short *et al*., 2003; Barry and Murphy, 2004), suggesting a potential application of MSCs for regenerative medicine. MSCs have been suggested to play a key role in rheumatology, based on their differentiation potential to cartilage/bone and their capacity to modulate host immune responses, angiogenesis, and fibrosis (Djouad *et al*., 2009). Originally discovered in the bone marrow, MSCs have now been identified in various joint tissues, including joint fat, periosteum, synovial membrane, SF, and cartilage (Bouffi *et al*., 2009; Djouad *et al*., 2009). MSCs have been reported to reside in the SF of patients with arthritis (Jones *et al*., 2004) and their migration to the synovium has been demonstrated in a collagen-induced arthritis animal model (Marinova-Mutafchieva *et al*., 2002). We recently reported that synovial fluids from patients with RA (RA-SF) stimulates migration of human bone marrow-derived MSCs (hBMSCs) through a lysophosphatidic acid (LPA)-mediated mechanism *in vitro* (Song *et al*., 2010). Furthermore, human adipose tissue-derived mesenchymal stem cells (hASCs) can differentiate to α-SMA-positive cells in response to treatment with LPA or TGF-β1 (Jeon *et al*., 2008). These results raise the possibility that differentiation of MSCs to α-SMA-positive cells can be regulated by the RA-associated synovial microenvironment. To address this, we examined the effects of RA-SF on the expression of α-SMA in hASCs as a model system for tissue-resident MSCs. Herein, we report on the identification of TGF-β1 as a key factor of RA-SF that induces α-SMA expression.

Results

RA-SF induces expression of α-SMA in hASCs

To explore whether RA-SF can induce differentiation of hASCs to α-SMA-positive cells, hASCs were treated with different concentrations of SF from RA patients or normal donors. As shown in

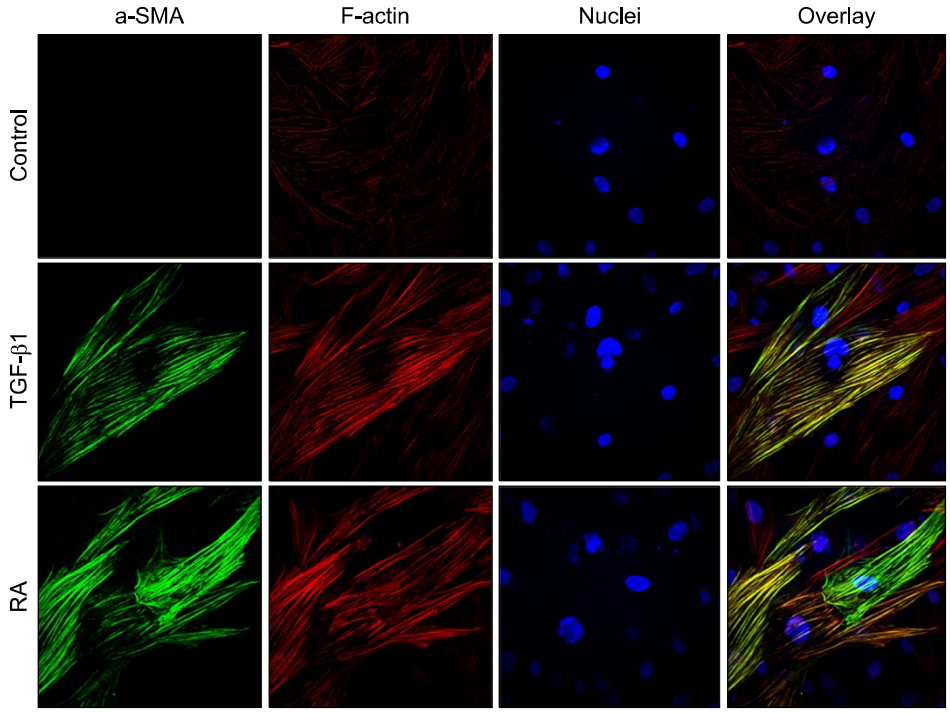

Figure 2. Effects of RA-SF on the formation of actin stress fibers and the localization of α-SMA in hASCs. Serum-starved hASCs were treated with vehicle, 1% RA-SF, or 0.2 ng/ml TGF-β1 for 4 days, and immunofluorescence staining was performed. α-SMA was stained with anti-α-SMA antibody and probed with Alexa Fluor 488-conjugated anti-mouse secondary antibody. F-actin was detected with Alexa Fluor 568 phalloidin and the double stained images were analyzed by confocal microscope (400× magnification). The merged images of α-SMA (green) and F-actin (red) are shown. Representatives of three independent experiments are shown.

Figure 1A, RA-SF induced α-SMA expression in hASCs with a maximal stimulation at 1% concentration. However, SF from normal donors had no significant impact on α-SMA expression. Because TGF-β1 is known to stimulate expression of α-SMA in hASCs (Jeon *et al*., 2006), we compared the effects of RA-SF and TGF-β1 on α-SMA expression. α-SMA expression was apparent on day 2 after treatment of the cells with RA-SF and was maximally induced on day 4 as potent as TGF-β1-induced α-SMA expression (Figure 1B). To evaluate whether RA-SF specifically increased α-SMA expression, we next compared the effects of SF from different RA patients or normal donors. As shown in Figures. 1C and 1D, RA-SF exhibited more potent stimulatory effects on α-SMA expression in hASCs than SF from normal donors, suggesting that RA-SF stimulates expression of α-SMA in hASCs.

We next examined the effect of RA on intracellular distribution of α-SMA and actin filaments by double staining for α-SMA and actin stress filaments. As shown in Figure 2, treatment of hASCs with RA-SF or TGF-β1 for 4 days increased the expression level of α-SMA, which was localized in actin filaments. These results support the idea that RA-SF induces α-SMA expression and formation of intracellular actin filaments in hASCs.

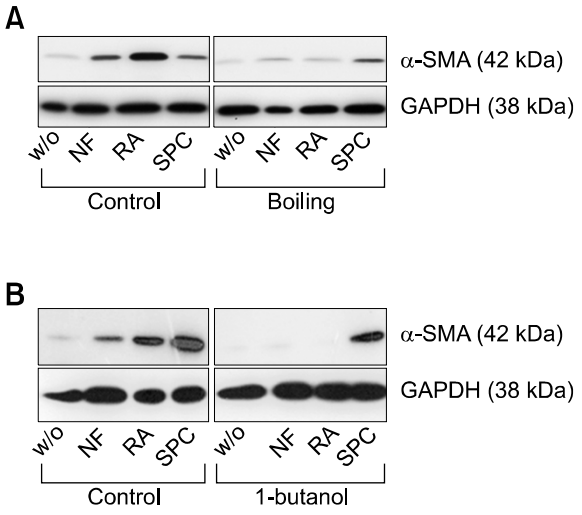

Figure 3. Role of protein components in RA-SF-induced α-SMA expression. (A) To denature protein components, control synovial fluid (NF), RA-SF, or SPC were boiled at 95°C for 5 min. Serum-starved hASCs were exposed to untreated control or boiled samples (1% NF, 1% RA-SF, and 5 μM SPC) for 4 days. (B) Lipid components were extracted from NF, RA-SF or SPC with 1-butanol. Serum-starved hASCs were exposed to untreated controls or 1-butanol fractions (1% NF, 1% RA-SF, and 5 μM SPC) for 4 days. The expression levels of α-SMA and GAPDH were analyzed by Western blot. Representatives of three independent experiments are shown.

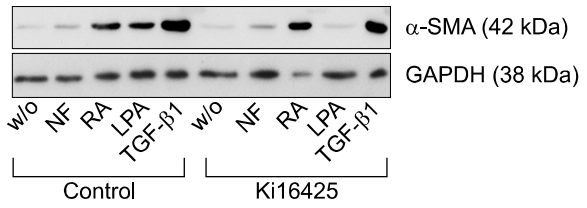

Figure 4. RA-SF induces expression of α-SMA through LPA receptor-independent pathway. Serum-starved hASCs were pretreated with vehicles, 5 μM Ki16425 for 15 min. The cells were then exposed to vehicle, 1% NF, 1% RA-SF, 0.2 ng/ml TGF-β1, or 5 μM LPA for 4 days, and the expression levels of α-SMA and GAPDH were determined by Western blot. Representative of three independent experiments is shown.

Protein components are responsible for RA-SF-induced α-SMA expression

To explore whether protein factors could be responsible for the RA-SF-induced expression of α-SMA, RA-SF and normal SF were heated to 95°C for 5 min to denature protein factors. As shown in Figure 3A, the stimulatory effects of RA-SF or normal SF on the α-SMA expression of hASCs were abrogated by heating. We have reported that the lysophospholipid sphingosylphosphorylcholine (SPC) induces α-SMA expression in hASCs (Jeon *et al.*, 2006). In contrast, SPC-induced α-SMA expression was not affected by heating of SPC. These results support the suggestion that protein factors are likely to be involved in the α-SMA expression induced by RA-SF or normal SF. To support the involvement of protein factors in the RA-SF-induced α-SMA expression, we next examined the effect of lipid fractions extracted from RA-SF with 1-butanol, on α-SMA expression. As shown in Figure 3B, α-SMA expression by RA-SF and normal SF was not recapitulated by the 1-butanol fractions, whereas the stimulatory effect of SPC was maintained in 1-butanol fraction. These results support the suggestion that protein factors are responsible for the RA-SF-induced α-SMA expression.

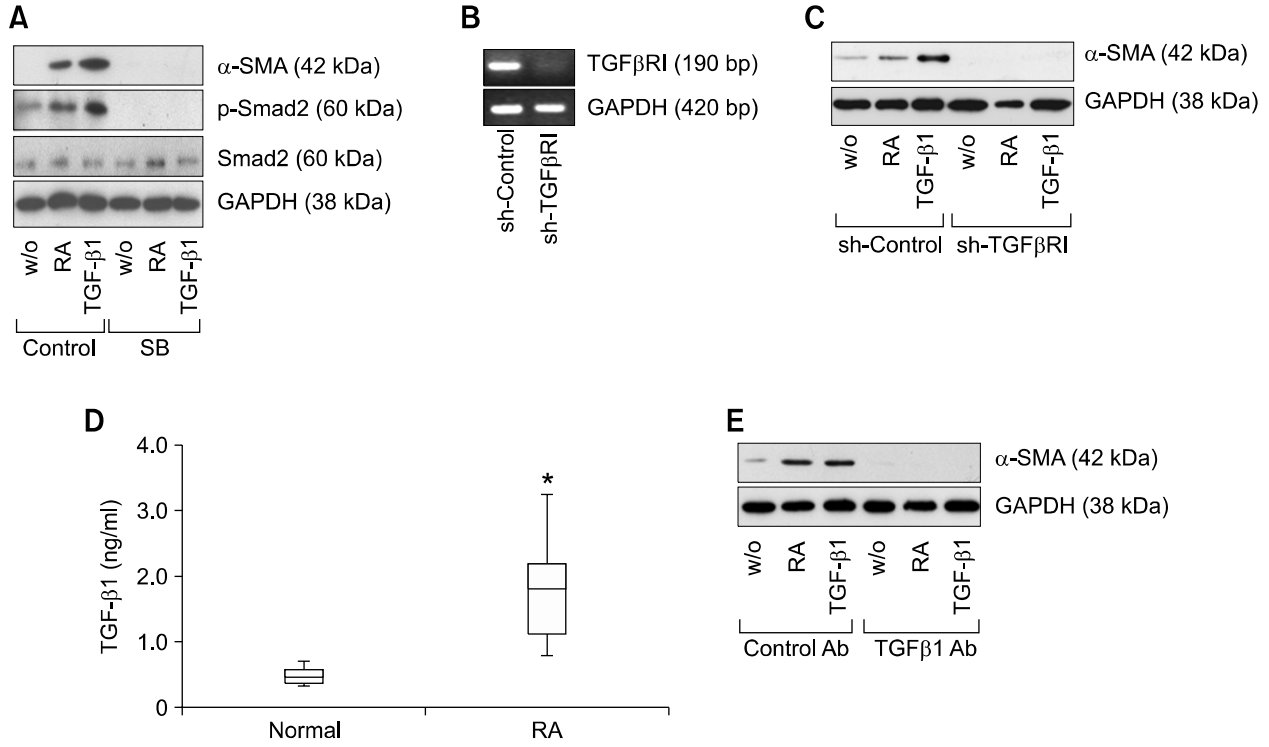

Figure 5. Role of TGF-β1-dependent pathway in the RA-SF-induced α-SMA expression. (A) Serum-starved hASCs were treated with 1 % RA-SF or 0.2 ng/ml TGF-β1 for 4 days in the absence or in the presence of 10 μM SB-431542. (B) Serum-starved hASCs were infected with control or lentiviral TGF-β type I receptor-specific shRNA. The mRNA levels of TGF-β type I receptor (TGFβR1) were determined by semi-quantitative RT-PCR. (C) Lentiviral infected cells were then exposed to vehicle, 1% RA-SF or 0.2 ng/ml TGF-β1. (D) SF of normal donors or RA patients was subjected to ELISA for determination of the concenturations of TGF-β1. In the statistical analysis of TGF-β1 concentrations, box and whisker plots represent median values, 25-75% range, and 10-90% range. *, $P < 0.05$ vs. normal. (E) 1% RA-SF or 0.2 ng/ml TGF-β1 were incubated with 0.2 μg/ml anti-TGF-β1 or control antibodies for 1 h, and supernatants were collected after precipitation of the immune complexes for depletion of TGF-β1. hASCs were treat with the TGF-β1-depleted supernatants for 4 days. The expression levels of α-SMA and GAPDH were determined by Western blot. Representatives of three independent experiments are shown.

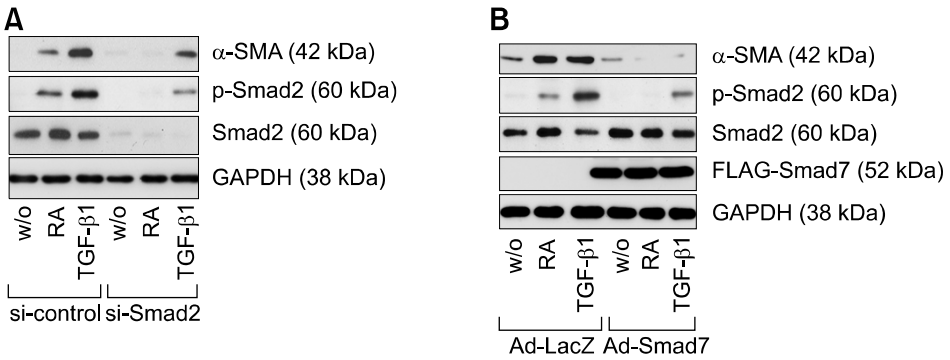

Figure 6. Role of Smad2 in the RA-SF-induced expression of α-SMA. (A) Serum-starved hASCs were transfected with control si-RNA (Control) or Smad2 si-RNA (si- Smad2), and then exposed with 1% RA-SF or 0.2 ng/ml TGF-β1 for 4 days. (B) hASCs were infected with adenoviruses carrying β-galactosidase (Ad-LacZ) or FLAG-tagged Smad7 (Ad-Smad7), and then treat with vehicles or 1% RA-SF and 0.2 ng/ml TGF-β1 for 4 days. The expression levels of α-SMA, Smad2, FLAG-tagged Smad7, and GAPDH were determined by Western blotting with anti-α-SMA, anti-Smad2, anti-FLAG and anti-GAPDH antibodies. Phosphorylation of Smad2 was determined by Western blotting with anti-p-Smad2. Representatives of three independent experiments are shown.

LPA is not involved in RA-SF-induced expression of α-SMA in hASCs

LPA is responsible for RA-SF-induced migration of hBMSCs (Song *et al*., 2010). Furthermore, LPA treatment stimulates α-SMA expression in hASCs (Jeon *et al*., 2008). To explore whether LPA could be involved in the RA-SF-induced α-SMA expression, we examined the effect of the LPA receptor antagonist Ki16425 on α-SMA expression induced by RA-SF or LPA. Ki16425 completely inhibited LPA-induced α-SMA expression (Figure 4). On the other hand, α-SMA expression stimulated by RA-SF or TGF-β1 was not affected by Ki16425 treatment. These results support the notion that LPA is not responsible for RA-SF-induced expression of α-SMA in hASCs.

TGF-β1 is involved in the RA-SF-stimulated α-SMA expression in hASCs

TGF-β1 stimulates α-SMA expression in hASCs (Jeon *et al*., 2006, 2008). To explore the involvement of TGF-β-dependent mechanism in the RA-SF-stimulated α-SMA expression, we tested the effect of the TGF-β type I receptor kinase inhibitor SB431542. As shown in Figure 5A, pretreatment of hASCs with SB431542 completely abrogated α-SMA expression which was induced not only by TGF-β1 but also by RA-SF. Furthermore, both RA-SF and TGF-β1 elicited phosphorylation of Smad2 through a SB431542-sensitive mechanism. To confirm the involvement of TGF-β type I receptor in the RA-SF-induced α-SMA expression, we depleted endogenous TGF-β type I receptor using lentiviral infection of TGF-β type I receptor-specific shRNA (Figure 5B). Silencing of TGF-β type I receptor expression completely blocked the RA- or the TGF-β1-induced α-SMA expression (Figure 5C). To elucidate the involvement of TGF-β1 in the RA-SF stimulation of α-SMA expression, we next determined the concentrations of TGF-β1 in the SF derived from patients with RA and normal donors using ELISA. As shown in Figure 5D, TGF-β1 levels in SF with RA were significantly increased compared with those of normal controls. Furthermore, TGF-β1- and RA-SF-induced α-SMA expression were abrogated by immunodepletion of TGF-β1 with anti-TGF-β1 neutralizing antibody (Figure 5E). These results support the suggestion that RA-SF induces α-SMA expression in hASCs through TGF-β1-TGF-β receptor-dependent mechanism.

Role of Smad2 in RA-SF-stimulated α-SMA expression in hASCs

Because Smad2 plays a key role in TGF-β1-induced signaling pathways (Shi and Massague, 2003; ten Dijke and Hill, 2004), we examined the involvement of Smad2 activation in RA-SF-induced α-SMA expression using siRNA-mediated depletion of Smad2. As shown in Figure 6A, depletion of endogenous Smad2 markedly attenuated α-SMA expression and Smad2 phosphorylation which were induced by RA-SF or TGF-β1. Adenoviral overexpression of Smad7, an inhibitory Smad isoform, blocked α-SMA expression and Smad2 phosphorylation stimulated by RA-SF or TGF-β1 (Figure 6B), suggesting that Smad2-dependent pathway is involved in the RA-SF induction of α-SMA expression in hASCs.

Discussion

It is conceivable that MSCs are useful for therapy of RA due to their immunoregulatory characteristics, differentiation potential, and paracrine function (Djouad *et al*., 2009; Kastrinaki and Papadaki, 2009). In the present study, we demonstrate that RA-SF can stimulate α-SMA expression in MSCs through a TGF-β1-dependent mechanism. The concentration of TGF-β1 was significantly augmented in RA-SF than normal SF, and immunodepletion of endogenous TGF-β1 from RA-SF using TGF-β1 neutralizing antibody abrogated the RA-SF-stimulated α-SMA expression in human adipose tissue-derived MSCs, implying that TGF-β1 in RA-SF directly stimulates α-SMA expression. Furthermore, pharmacological inhibition or shRNA-mediated depletion of TGF-β type I receptor blocked RA-SF-stimulated α-SMA expression. In addition, siRNA-mediated silencing of Smad2 or adenoviral overexpression of Smad7 suppressed RA-SF-induced α-SMA expression by ablation of TGF-β receptor-Smad2 signaling pathway. TGF-β1 reportedly plays a pivotal role in fibrosis and pathogenesis associated with RA (Pohlers *et al*., 2009). Treatment with TGF-β1 induces differentiation of human synovial fibroblasts to α-SMA-positive myofibroblasts (Mattey *et al*., 1997), which play a key role in RA-associated synovial inflammation (Kasperkovitz *et al*., 2005). Synovial fibroblasts from patients with RA exhibited constitutive activation of the TGF-β signaling pathway due to abundant expression of TGF-β1 and TGF-β type I receptor (Pohlers *et al*., 2007). Moreover, treatment with not only TGF-β1 but also RA-SF stimulates α-SMA expression in synovial fibroblasts (Steenvoorden *et al*., 2006). Because MSCs have been shown to reside in RA-SF (Jones *et al*., 2004), these results support the notion that TGF-β1 plays a pivotal role in the RA-SF-induced differentiation of MSCs to α-SMA-positive cells.

We recently reported that LPA is responsible for the RA-SF-induced migration of hBMSCs (Song *et al*., 2010); Pretreatment of cells with the LPA receptor antagonist Ki16425 or silencing of LPA1 receptor expression abrogated RA-SF-stimulated cell migration. RA-SF induced migration of not only hBMSCs but also hASCs through LPA-dependent mechanism (data not shown). Our present findings suggest that LPA is not involved in RA-SF-induced α-SMA expression. Firstly, Ki16425 treatment had no significant impact on the RA-SF-induced α-SMA expression. Secondly, heat denaturation and lipid extraction experiments demonstrated that protein components, but not lipid factors, were involved in the RA-SF-stimulated α-SMA expression. Therefore, it is likely that LPA and TGF-β1 mediate RA-SF-induced cell migration and α-SMA expression, respectively. RA-SF-induced α-SMA expression was potently stimulated by treatment with 1% RA-SF (Figure 1A), whereas RA-SF-induced cell migration was maximally stimulated at 10% concentration (Song *et al*., 2010). The concentrations of LPA in RA-SF are estimated to be 0.98 ± 0.12 μM (Song *et al*., 2010), therefore, the concentration of LPA in 1% RA-SF can be calculated to be 9.8 ± 0.12 nM. Because LPA treatment elicited α-SMA expression at 2 μM (Jeon *et al*., 2008), these results suggest that LPA concentration in 1% RA-SF is not sufficient to stimulate μ-SMA expression in hASCs.

α-SMA-positive myofibroblasts play pivotal roles in a variety of physiology and pathophysiology, including wound healing, cancer, and tissue fibrosis (Gabbiani, 2003). The present study supports the view that increased levels of TGF-β1 in the synovial microenvironment of RA patients may locally affect the differentiation of tissue-resident MSCs within joint tissues to myofibroblast-like cells. α-SMA is a marker for not only myofibroblasts but also smooth muscle cells, and both myofibroblasts and smooth muscle cells share similar phenotypic characteristics (De Wever *et al*., 2008). The importance of TGF-β1 in RA involves an association with certain vascularization patterns in the synovial membrane (Salvador *et al*., 2006). While RA primarily affects joints, increasing body of evidence demonstrates that RA is associated with the development of various cardiovascular diseases including atherosclerosis, vasculitis lesions, and coronary artery disease (Tanasescu *et al*., 2009). The inflammatory hypothesis of cardiovascular diseases in RA implies that mediators originating from inflamed synovial tissue may have systemic vascular responses by modulating differentiation status of MSCs. More research is needed to clarify the pathophysiological significance of RA-SF-stimulated differentiation of MSCs to myofibroblast-like cells and the involvement of TGF-β1 in the differentiation of MSCs *in vivo*.

Methods

Materials

α-Minimum essential medium, phosphate-buffered saline, trypsin, fetal bovine serum, Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, and Lipofectamine plus™ reagent were purchased from Invitrogen (Carlsbad, CA). Human recombinant TGF-β1, anti-TGF-β1 neutralizing antibody, and enzyme-linked immunosorbent assay (ELISA) kits for TGF-β1 were purchased from R&D

Systems (Minneapolis, MN). 1-Oleoyl-*sn*-glycero-3- phosphate (1-oleoyl-LPA), fatty acid-free bovine serum albumin, Ki16425, SB431542, and anti-α-SMA mouse monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO). Anti-phospho-Smad2 (Ser465/467) and anti-Smad2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Alexa Fluor 488 goat anti-mouse antibody and Alexa Fluor 568 phalloidin were from Molecular Probes (Eugene, OR).

Collection of SF

SF was obtained with the patient's consent, as approved by the Institution Review Board of Busan National University Hospital. SF was obtained from patients with RA during therapeutic arthrocentesis as previously described (Song *et al*., 2010). SF from normal donors was obtained postmortem from organ donors without joint diseases. SF was transferred to heparin-treated tubes, transported immediately to the laboratory, and centrifuged at 3,000 × *g* for 10 min at 4°C to remove possible inflammatory cells and blood cells. Aliquots of the supernatants were used immediately or stored at -80°C for future analysis. SF aliquots were either used immediately or subjected to one freeze-thaw cycle.

Extraction of lipid components and denaturation of protein components of SF

To extract lipid components, aliquots (200 μl) of SF were each treated with 1 volume of 1-butanol. After vigorous shaking and centrifugation (5 min at 15,000 rpm), the upper 1-butanol phase was collected and evaporated under nitrogen. The resultant lipids were solubilized in 200 μl of 1 mg/ml fatty acid-free bovine serum albumin. To denature proteins in SF, an aliquot (200 μl) of SF was heated at 100°C for 5 min, centrifuged at 15,000 rpm for 5 min to remove denatured proteins, and the supernatants were collected.

Cell culture

After informed consent, adipose tissues were obtained from patients undergoing elective abdominoplasty. For isolation of hASCs, adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of 0.1% collagenase for 30 min at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation at 300 × *g* for 5 min. The cell pellet was filtered through a 100-mm nylon mesh to remove cellular debris and incubated overnight at 37°C in an atmosphere of 5% CO_2 in α-minimum essential medium containing 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin. Following incubation, the plates were washed extensively with PBS to remove residual nonadherent red blood cells. When the monolayer of adherent cells reached confluence, the primary hASCs were subcultured at a concentration of 2×10^3 cells/cm^2. The primary hASCs were cultured for 4-5 days until they reached confluence and were defined as passage 0. The passage number of hASCs used in these experiments was 3-10. The hASCs were positive for CD29, CD44, CD90, and CD105, all of which have been reported to be mesenchymal stem cells marker proteins. However, these cells did not express c-kit, CD34, or CD14, which are known as hematopoietic markers (data not shown).

Immunocytochemistry and microscopy

Immunostaining and confocal microscopy were used to determine the subcellular distribution and organization of proteins. Cells were fixed in PBS containing 4% paraformaldehyde for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 10 min, and blocked with PBS containing 2% bovine serum albumin. For immunostaining, specimens were incubated with anti-α-SMA antibody for 2 h and Alexa Fluor 488-conjugated anti-mouse secondary antibody for 1 h. To stain F-actin filaments, the specimen was incubated with Alexa Fluor 543-conjugated phalloidin for 30 min followed by confocal microscopy. The images of the specimen were collected with a Leica TCL SP2 confocal microscope system (Leica Microsystems, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using Trizol (Invitrogen). For RT-PCR, aliquots of 2 μg RNA were subjected to cDNA synthesis with 200 U of M-MLV reverse transcriptase and 0.5 μg of oligo (dT) 15 primer (Promega, Madison, WI). The cDNA in 2 μl of the reaction mixture was amplified with 0.5 U of GoTaq DNA polymerase (Promega, Madison, WI) and 10 pmol each of sense and antisense primers as follows: TGF-β type I receptor (190 bp) : sense 5'-GAACTTCCAACTACTGGTTT-3', antisense 5'-GATAAATCTCTGCCTCACGG-3', glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 420 bp): sense 5'-TCCATGACAACTTTGGTATCG-3', antisense 5'-TGTAGCCAAATTCGTTGTCA-3'. The thermal cycle profile consisted of denaturation at 95°C for 30 s, annealing at 52-55°C for 45 s depending on the primers used, and extension at 72°C for 45 s. Each PCR reaction was carried out for 30 cycles, and PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and photographed under UV transillumination.

Transfection with small interfering RNA (siRNA)

siRNA duplexes were synthesized, desalted, and purified by Samchully Pharm. (Siheung, GyeongGi, Korea) using Smad2 sense 5'-GUCCCAUGAAAAGACUUAATT-3' and antisense 5'-UUAAGUCUUUUCAUGGGACTT-3' primers. Nonspecific control siRNA (D-001206-13-05) was purchased from Dharmacon (Lafayette, CO). For siRNA experiments, hASCs were seeded on 6 well dishes at 70% confluence prior to transfection with siRNAs using Lipofectamine plus™ reagent (Invitrogen) according to the manufacturer's instructions. Briefly, Lipofectamine plus™ reagent was incubated with serum-free medium for 15 min, and respective siRNAs were then added to the mixtures. After incubation for 15™min at room temperature, the

mixtures were diluted with serum-free medium and added to each well. The final concentration of siRNAs in each well was 100 nM. After incubation of hASCs to serum-free medium containing siRNAs for 4 h, the cells were cultured in growth medium for 24 h, and the expression levels of Smad2 and GAPDH were then determined by western blot analysis.

Lentiviral small hairpin RNA (shRNA) transduction

pLKO.1-puro lentiviral vectors expressing TGF-β type I receptor shRNA (TRCN0000039774) or nontarget control shRNA (SHC002) were purchased from Sigma-Aldrich. The functional sequence for TGF-β type I receptor in the pLKO.1-puro shRNA lentiviral vector is CCGGGCG-AGAACTATTGTGTTACAACTCGAGTTGTAACACAATAGT TCTCGCTTTTTG. To generate lentiviral particles, HEK293FT cells were co-transfected with the shRNA lentiviral plasmid (pLKO.1-puro) and ViraPower Lentiviral packaging mix (pLP1, pLP2, pLP-VSV-G; Invitrogen) using Lipofectamine plus™ (Invitrogen) and the culture supernatants containing lentivirus were harvested at 48 h after transfection. For lentiviral transduction, hASCs were treated with the shRNA-expressing lentivirus in the presence of 5 μg/ml polybrene (Sigma-Aldrich) and stable cell lines expressing shRNA were generated by selection with puromycin (10 μg/ml). To ensure shRNA-mediated silencing of TGF-β type I receptor expression, the mRNA levels of TGF-β type I receptor and GAPDH were determined by RT-PCR.

ELISA

TGF-β1 level was determined by a commercially available human TGF-β1 ELISA kit (R&D Systems) according to the manufacturer's instructions. In brief, 100 μl of diluted capture antibody was added to each well of a 96-well microplate and then incubated overnight at room temperature for coating of the capture antibody. After blocking of each well with 100 μl of blocking solution, samples or standards were transferred to wells. After incubation for 2 h at room temperature, wells were washed with phosphate-buffered solution and 100 μl of horseradish peroxidase-conjugated detection antibody was dispensed into each well. Following 2 h incubation at room temperature, the plate was washed, and then 100 μl of substrate solution was added to each well. After incubation at room temperature for 20 min, the reaction was stopped by the addition of 2N H_2SO_4 to each well and the absorbance of each solution at 450 nm was determined by using a PowerWavex microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT). The concentration of TGF-β1 in the samples is determined by comparison to the standard curve.

Western blot analysis

hASCs were treated under appropriate conditions, washed with PBS, and then lysed in lysis buffer (20 mM Tris-HCL, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1% Triton X-100, pH 7.4). The lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto a nitrocellulose membrane and stained with 0.1% *Ponceau S* solution (Sigma-Aldrich). After blocking with 5% nonfat milk, the membranes were immunoblotted with primary antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence Western blotting system (Amersham Biosciences, Piscataway, NJ).

Depletion of TGF-β1 from RA-SF with anti-TGF-β1 antibody

TGF-β1 was immunodepleted from RA-SF by immunoprecipitation with anti-TGF-β1 antibody. In brief, aliquots (30 μl) of a suspension (50% slurry) of protein G-agarose beads (Sigma-Aldrich) in PBS were mixed with 0.2 μg of anti-TGF-β1 or normal mouse antibodies at 4°C for 1 h with intermittent shaking. After recovery by centrifugation, the beads were washed three times and used for immunodepletion of TGF-β1 from RA-SF. 1% RA-SF or 0.2 ng/ml TGF-β1 were incubated with the protein G-agarose beads immobilized with anti-TGF-β1 or normal mouse antibodies for 1 h at 4°C. The immune complexes absorbed to protein G-agarose beads were precipitated by centrifugation. The resultant supernatants were collected and immediately used for experiments.

Adenoviral infection

Recombinant adenoviruses expressing Smad7 or LacZ were kindly provided by Dr. Kohei Miyazono (Tokyo University, Japan) and were used individually at a multiplicity of infection of 50 as described previously (Fujii *et al*., 1999).

Statistical analysis

The results of multiple observations are presented as mean ± SD. For multivariate data analysis, group differences were assessed with two-way ANOVA, followed by post hoc comparisons tested with Scheffe's method.

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